Mohammad Anis · Naseem Ahmad
Editors

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Editors
Mohammad Anis
Plant Biotechnology Laboratory
Department of Botany
Aligarh Muslim University
Aligarh, Uttar Pradesh, India

Naseem Ahmad
Department of Botany
Aligarh Muslim University
Aligarh, Uttar Pradesh, India

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Preface

The extinction of plant species is progressively taking place due to their being trapped in the vicious circle of ever-increasing industrialization, deforestation, global warming, climate change, and also unscrupulous human activities. The situation warrants acceleration of efforts to develop methods for their germplasm preservation. In this context, the importance of in vitro morphogenesis cannot be overemphasized, as the interplay of morphogenic factors, which can precisely be managed in vitro – grown plant system cannot be done ex vitro. Furthermore, its application for germplasm preservation becomes imperative, particularly in case of hybrids which must be propagated vegetatively, where seeds are not produced, the plant is systemically infected, or the plant material is very limited. The application of micropropagation techniques has witnessed major advances and numerous benefits over the last few decades and is the only aspect of biotechnology that has been convincingly documented with regard to its feasibility for mass-scale propagation commercially.

Molecular biology and biotechnology have now become an integral part of tissue culture research. The tremendous impact generated by genetic engineering and consequently the generation of transgenics has helped in the manipulation of plant genomes at will. There is indeed rapid development in this area with commendable success in India. It has, therefore, become increasingly difficult to author a book on the subject. Hence, this edited volume would hopefully prove informative to readers. The book provides a source material to researchers intending to initiate work in these areas.

The editors acknowledge the unstinted support received from contributors who spared valuable time in writing chapters for this volume and also sincerely thank the publishers for their cooperation in making this book a reality. We are indebted to Dr. H. C. Chaturvedi, former emeritus scientist (CSIR), and Dr. A. K. Sharma, former head, Tissue Culture Lab, CSIR-National Botanical Research Institute (NBRI), Lucknow, who introduced us to plant tissue culture and to the intricacies of the technique.

While preparing this book, we have received unflinching support from colleague and research students in the Plant Biotechnology group. Prof. Altaf Ahmad extended full cooperation and read a number of manuscripts. Postdocs of the Plant Biotechnology Laboratory, Dr. Ankita Varshney, Dr. Nigar Fatima, Dr. Ruphi Naz, and Dr. Saad Bin Javed, and doctoral students, Ms. Afsheen Shahid, Ms. Mehrun

Nisha Khanam, Mr. Sheikh Altaf Husain, Mr. Anees Ahmad, and Mr. Naushad Alam, extended full cooperation and solicited timely help.

Once again, thanks to all those who helped in various ways.

Aligarh, Uttar Pradesh, India

Mohammad Anis
Naseem Ahmad

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Contributors

Jyothi Abraham Postgraduate and Research, Department of Botany, St. Thomas College, Kottayam, Kerala, India

Diwakar Aggarwal Department of Biotechnology, Multani Mal Modi College, Patiala, India

Naseem Ahmad Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Behzad Ahmadi Agricultural Biotechnology Research Institute of Iran (ABRII), Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

Mohammad Anis Plant Biotechnology Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

K. Arora Botany Department, National P.G. College, Lucknow, India

M.C. Aruna Plant Cell Biotechnology Department, CSIR-Central Food Technological Research Institute, Mysore, India

Suchitra Banerjee Plant Biotechnology Division, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, UP, India

Somika Bhatnagar Temasek Life Sciences Laboratory, Singapore, Singapore

Amita Bhattacharya Division of Biotechnology, CSIR-Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh, India

Mª José Cernadas Instituto de Investigaciones Agrobiológicas de Galicia, CSIC, Santiago de Compostela, Spain

Bhuwan Chandra G.B. Pant Institute of Himalayan Environment & Development, Almora, Uttarakhand, India

Near Anandi Academy, Bankhola, Mandalsera, Bageshwar, Uttarakhand, India

Smitha Chandrasekharan Temasek Life Sciences Laboratory, Singapore, Singapore

Elena Corredoira Instituto de Investigaciones Agrobiológicas de Galicia, CSIC, Santiago de Compostela, Spain

Nisha Dhiman Academy of Scientific and Innovative Research, New Delhi, India

Department of Biotechnology, CSIR-Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh, India

Mohamed A. El-Esawi Botany Department, Faculty of Science, Tanta University, Tanta, Egypt

L.A. Elkonin Department of Biotechnology, Agricultural Research Institute of South-East Region, Saratov, Russia

G. Gambhir Department of Biotechnology, Dr Yashwant Singh Parmar University of Horticulture and Forestry, Solan, Himachal Pradesh, India

A. Gaur Department of Biotechnology, Dr Yashwant Singh Parmar University of Horticulture and Forestry, Solan, Himachal Pradesh, India

Parvatam Giridhar Plant Cell Biotechnology Department, CSIR-Central Food Technological Research Institute, Mysore, India

T. Gouthaman Department of Plant Science, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

PG & Research Department of Botany, Government Arts College (M), Krishnagri, Tamil Nadu, India

Yan Hong School of Biological Sciences, Nanyang Technological University, Singapore, Singapore

M. Jain Tissue Culture Laboratory, CSIR-National Botanical Research Institute, Lucknow, UP, India

Laura V. Janeiro INLUDES, Diputación Provincial de Lugo, Lugo, Spain

K. Kalaiarasi Plant Genetic Engineering and Molecular Biology Lab, Department of Biotechnology, School of Biosciences, Periyar Palkalai Nagar, Periyar University, Salem, TN, India

Amanpreet Kaur Department of Biotechnology, Thapar University, Patiala, India

Norzulaani Khalid Centre for Research in Biotechnology for Agriculture, University of Malaya, Kuala Lumpur, Malaysia

Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia

Sana Khan Plant Biotechnology Division, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, UP, India

Anil Kumar Department of Biotechnology, Thapar University, Patiala, India

P. Kumar Department of Biotechnology, Dr Yashwant Singh Parmar University of Horticulture and Forestry, Solan, Himachal Pradesh, India

Sandopu Sravan Kumar Plant Cell Biotechnology Department, CSIR-Central Food Technological Research Institute, Mysore, India

V. Kumaresan Department of Plant Science, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

Department of Botany, Aringar Anna Govt. Arts College, Salem, Tamil Nadu, India

A.R. Lavanya Department of Plant Science, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

Department of Botany, Periyar E. V. R. Govt. Arts College, Tiruchirappalli, Tamil Nadu, India

Mª Teresa Martínez Instituto de Investigaciones Agrobiológicas de Galicia, CSIC, Santiago de Compostela, Spain

Pratibha Misra CSIR, National Botanical Research Institute, Lucknow, India

Remya Mohanraj Department of Biology, Houston Community College, Houston, TX, USA

Raquel Montenegro Instituto de Investigaciones Agrobiológicas de Galicia, CSIC, Santiago de Compostela, Spain

S. Muthukrishnan Department of Plant Science, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

M. Muthukumar Department of Plant Science, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

Mohammad Nadeem G.B. Pant Institute of Himalayan Environment & Development, Almora, Uttarakhand, India

Department of Botany & Microbiology, King Saud University, Riyadh, Saudi Arabia

Shyamal K. Nandi G.B. Pant Institute of Himalayan Environment & Development, Almora, Uttarakhand, India

Lok Man S. Palni G.B. Pant Institute of Himalayan Environment & Development, Almora, Uttarakhand, India

Biotechnology Department, Graphic Era (Deemed) University, Dehradun, Uttarakhand, India

Hemant Pandey G.B. Pant Institute of Himalayan Environment & Development, Almora, Uttarakhand, India

Agro Division, Merino Industries Ltd, Achheja, Hapur, Ghaziabad, Uttar Pradesh, India

Asra Parveen Plant Tissue Culture and Genetic Engineering Laboratory, Department of Botany, Gulbarga University, Kalburgi, Karnataka, India

Vanita Patial Academy of Scientific and Innovative Research, New Delhi, India

Department of Biotechnology, CSIR-Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh, India

D.K. Purshottam Tissue Culture Laboratory, CSIR-National Botanical Research Institute, Lucknow, UP, India

S.K. Rai Tissue Culture Laboratory, CSIR-National Botanical Research Institute, Lucknow, UP, India

Srinath Rao Plant Tissue Culture and Genetic Engineering Laboratory, Department of Botany, Gulbarga University, Kalburgi, Karnataka, India

A.S. Rao Department of Biotechnology, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

M.V. Rao Department of Plant Science, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

M. Sudhakara Reddy Department of Biotechnology, Thapar University, Patiala, India

Syed Saema CSIR, National Botanical Research Institute, Lucknow, India

Mª del Carmen San José Instituto de Investigaciones Agrobiológicas de Galicia, CSIC, Santiago de Compostela, Spain

H. Sandhya Plant Tissue Culture and Genetic Engineering Laboratory, Department of Botany, Gulbarga University, Kalburgi, Karnataka, India

T. Senthil Kumar Department of Industry University Collaboration, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

Mehran E. Shariatpanahi Agricultural Biotechnology, Research Institute of Iran (ABRII), Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

A.K. Sharma Tissue Culture Laboratory, CSIR-National Botanical Research Institute, Lucknow, UP, India

M. Sharma Tissue Culture Laboratory, CSIR-National Botanical Research Institute, Lucknow, India

S. Sharma Department of Biotechnology, Dr Yashwant Singh Parmar University of Horticulture and Forestry, Solan, Himachal Pradesh, India

D.K. Srivastava Department of Biotechnology, Dr Yashwant Singh Parmar University of Horticulture and Forestry, Solan, Himachal Pradesh, India

Boon Chin Tan Centre for Research in Biotechnology for Agriculture, University of Malaya, Kuala Lumpur, Malaysia

Robert Thangjam Department of Biotechnology, School of Life Sciences, Mizoram University, Aizawl, Mizoram, India

T. Dennis Thomas Postgraduate and Research, Department of Botany, St. Thomas College, Kottayam, Kerala, India

Department of Plant Science, School of Biological Sciences, Central University of Kerala, Kasaragod, Kerala, India

S.N. Timofeeva Botanical Garden, Saratov State University, Saratov, Russia

V.S. Tyrnov Department of Genetics, Saratov State University, Saratov, Russia

Laiq ur Rahman Plant Biotechnology Division, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, UP, India

Silvia Valladares Fundación Promiva, Madrid, Spain

P. Venkatachalam Plant Genetic Engineering and Molecular Biology Lab, Department of Biotechnology, School of Biosciences, Periyar Palkalai Nagar, Periyar University, Salem, TN, India

M. Vijaya Venkatesh Department of Plant Science, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

O.I. Yudakova Department of Genetics, Saratov State University, Saratov, Russia

About the Editors

Professor Mohammad Anis possesses about 31 years of teaching and research experience on Biotechnology and Cytogenetics. He has published over 185 research and review articles in Journals of International repute. He is recipient of National (1992) and Overseas (1994) Biotechnology Associateship of DBT, Govt. of India, INSA Visiting Scientist to Frankfurt University (1998) and Institute of Genetics and Plant Biotechnology, Nitra (2006), Slovak Republic, Eminent Scientist Award (2007) by National Environmental Science Academy, New Delhi, Vigyan Ratan Samman (2010) of Council of Science and Technology, U.P., Professor P. Maheshwari Medal (2013) and Mid-Career award of UGC (2015) for his significant contribution in Medicinal Plant Biotechnology. He has been Program Coordinator of several major projects like DST-FIST (I and II), UGC-DRS (I and II) and DBT-HRD.

Dr. Naseem Ahmad is Young Scientist SERB-DST, New Delhi in the Department of Botany, Aligarh Muslim University, Aligarh. He has more than 6 years of Postdoctoral experience in the area of Morphogenesis, Plant Tissue Culture and Molecular Biology. He has published about 37 research papers in Journals of International repute, 03 book chapter and 41 abstracts in various conferences. He is a member in the Editorial Board of various journals, holds Life Membership of many Learned/scientific societies and is a Fellow of Indian Botanical Society (FBS). He is recipient of "National Scholarship" of Slovak Republic (2013), Yuva Vaigiyanik Samman (2009), CST, UP; Young Scientist (2010) by NESA, New Delhi, Rashtriya Gaurav Award (2015) by India International Friendship Society, New Delhi and Young Scientist of the year (2015) by International Foundation for Environment and Ecology (IFEE), Kolkata. He has received Post Graduate Merit Scholarship in M.Sc. and Best oral presentation awarded at BBAU (Central University) Lucknow and has availed Senior Research Fellowship (SRF) of CSIR, New Delhi.

Part I

In Vitro Regeneration

Plant Tissue Culture: A Journey from Research to Commercialization

1

Mohammad Anis and Naseem Ahmad

Abstract

Tissue culture was a subject of academic interest for a long time. In recent years, it has become a useful tool for agriculture and medicine. It has therefore been a popular area of biological research. Considerable amount of literature has been generated, but it is not commensurate with the results obtained. The continuous and non-organized exploitation has resulted in many medicinal plants becoming rare, and a good number have even become extinct. Therefore, tissue culture has emerged as a science with a vast potential for human welfare ranging from large-scale plant production in horticulture and forestry, human health, plant protection as well as environmental protection. In vitro rejuvenation holds remarkable potentials for the production and superior plant-based medicine. There are mainly four approaches for in vitro germplasm preservation, which may lead to development of a tissue bank; cryopreservation, normally growing and multiplying shoot culture, slow-growth culture and regenerative long-term excised root culture. The main parameter for evaluating the worth of these approaches includes practicability, prolonged retention of regenerative potentiality and the least chances of genetic instability.

M. Anis (✉)
Plant Biotechnology Laboratory, Department of Botany, Aligarh Muslim University, Aligarh 202 002, Uttar Pradesh, India
e-mail: anism1@rediffmail.com

N. Ahmad
Department of Botany, Aligarh Muslim University, Aligarh 202 002, Uttar Pradesh, India

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Abbreviations

BA	6-benzyl adenine
2iP	2-isopentenyl adenine
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Kn	6-furfurylaminopurine
PGRs	Plant growth regulators
MS	Murashige and Skoog (1962) medium
NAA	α-Naphthalene acetic acid

1.1 Introduction

Biodiversity is nature's fabric of life. The economic prosperity of any country depends on this natural capital. Today we are in a global battle for the conservation of this natural wealth. Being driven primarily by climate disruption, habitat changes and over-exploitation, biodiversity loss is pushing earth towards the sixth mass extinction. Continuous and often indiscriminate collections of medicinal plants in bulk quantity from diverse ecosystem, coupled with destruction of natural habitats, are resulting in irreplaceable loss of valuable genetic diversity. Medicinal plants account for one-third of the species in the 'Red Data Book of India'. The country's rich biodiversity has been sadly and seriously affected with the increasing human population. The in vitro multiplications of protocols offer a potential technique of generating sufficient material for commercial planting. Its utilization in forestry, agriculture and horticulture is growing worldwide. Large numbers of plants have been recovered initiated from a sole entity in a comparatively short instance and space (Bhojwani and Razdan 1983). Micropropagation is fast, uses little quantity of shoots and succeeds when other methods fail (Fay 1992). The technique has been used globally for monitoring of secondary metabolite at various stages of growth and differentiation.

Tissue culture becomes a popular area of research with most laboratories jumping on the bandwagon, changing their names or opening a new section to include tissue culture. Conventionally these plants take a long time for multiplication and have a low rate of fruit/seed set and poor seed viability/germination, and often roots/rhizome of few years old plant contains the effective principle. Thus, in order to obtain active ingredients from storage organs, often whole plants are dug out which eliminate its chances of survival and perpetuation in nature.

Recent advancement in biotechnological methodology of culturing plant cell and tissues has provided new means of rapidly propagating and conserving the endangered and other vulnerable plant species.

As per directives of the University Grants Commission (UGC) in 2003, the curricula for both UG and PG were revised where greater emphasis was made on the courses related to Plant Biotechnology and Molecular Biology. Prof. M. Anis, the

group leader, established a laboratory and study programme in Plant Biotechnology in the Botany Department, AMU, Aligarh. On the basis of the research output made in this area during the last 15 years, he was instrumental in arranging huge grants from various government agencies. Based on the overall achievements and progress, the department was supported under the Special Assistance Programme DRS-I (2009–2014) and DRS-II (2016–2021), DST-FIST-I (2006–2010), DST-FIST-II (2011–2016) and DBT-HRD (2008–2013). This has paved the way for establishing a Plant Molecular Biology and Nanobiotechnology lab with an aim to expand academic operations by offering new courses and upgrading programmes to attract a wider spectrum of students and researchers.

A number of reproducible protocols originated from tissue culture studies using different morphogenic pathways on plants belonging to different categories have been established for the multiplication and conservation of phytodiversity. In addition, evaluation on the effect of different light intensities on photosynthesis and antioxidant enzymes during acclimatization of in vitro regenerated plants has been carried out. Since the possibility of occurrence of genetic variation (somaclonal variation) during in vitro process cannot be ruled out, we have been focusing on enhanced axillary shoot proliferation which is least prone to somaclonal variation.

The group of Plant Biotechnology in the University, Botany Department, has made significant contributions towards the development and progress of tissue culture technology in the country for mass propagation and morphogenic studies on large number of plants, including recalcitrant species that are difficult to be propagated from seeds. The research team has made pioneer and excellent contribution towards the mass propagation of several ornamental, medicinal, fruit and woody trees including endangered plants.

The present communication describes various approaches of in vitro manipulation for plant regeneration in selected plants, with medicinal/economic importance. The results are of great practical significance for their mass propagation with conservation and have been published in journals of international repute.

1.2 *Salix alba* L.

Salix alba (*Salicaceae*) is a large tree with olive-green, yellow or purple branches frequently cultivated in Western Himalaya up to an altitude of 2400 m. It is vegetatively propagated by cutting during February to March and used mostly in post and planks, house building, packing boxes, furniture, agricultural implements, etc. The regeneration potential of various explants was evaluated by manipulating various culture conditions. Among the various treatments of different cytokinins (BA, Kin, 2iP) singly on woody plant medium, BA was found superior in comparison with others in nodal explant. However, amalgamation of auxins (IAA, IBA or NAA) with optimum doses of BA was superior in the production of a maximum of 12.77 shoots with 1.83 cm average shoot length induced from nodal explants.

The presence of various additives, viz. silver nitrate, glutamine, ammonium nitrate or adenine sulphate, favours the production of good-quality shoots. Among

all, 2.0 mg/l of $AgNO_3$ was found to be the optimum for proper growth and development of shoots.

In vitro isolated shoots from the cluster were transferred to the media containing different auxins (IAA, IBA or NAA) at various doses for root induction. Among the various treatments tested, 0.5 μM IBA was found best for highest root induction. Plantlets with proper root and shoot systems were acclimatized using standard procedure. All the regenerants were lastly shifted to pots in the net house where they grew well lacking any noticeable morphological dissimilarity. No somaclonal variation among regenerants was observed as confirmed by PCR-based DNA markers (Khan 2014).

1.3 *Erythrina variegata* L.

Erythrina variegata L. (*Fabaceae*) is highly medicinal and is being used in traditional medicinal system in various parts of Asia, for liver disorder treatment to leprosy. Its extracts have been reported to show hypoglycaemic, antidiuretic, antihyperlipidaemic and sedative properties. It is a salt and drought tolerant and even grows in waterlogged conditions, giving it the ability to survive even near the seashores. It also possesses aesthetic value because of its beautiful inflorescence and is in high demand in the international market. Its vegetative propagation is, however, limited because of the requirement of large cutting which is difficult to transport. Therefore, it is a very good candidate for in vitro and physiological studies which are not possible using conventional methods. For developing an efficient regeneration system, nodal explants were incubated on MS culture medium fortified with different doses and amalgamations of plant growth regulators (PGRs). Combination of 5.0 μM 6-benzylaminopurine (BA) and 0.5 μM 1-naphthaleneacetic acid (NAA) was found to be most effective and induced maximum number of shoots (~13) per explant with average (4.8 cm) mean shoot length in 93.6 % cultures. Addition of cobalt (≤50 μM) to the medium significantly enhanced the growth parameters of the culture, increasing the number of shoots to more than 16 shoots/explant after 8 weeks of culture on the standardized medium. Rooting in in vitro obtained shoots was proficiently induced on full-strength MS medium fortified with 2.5 μM indole-3-butyric acid (IBA) which yielded more than three roots/shoots with mean root length of 3.2 cm. The cultures transferred from the media supplemented with optimized cobalt concentration showed better rhizogenic competence as compared to the one transferred from medium lacking cobalt. Cobalt exposure increased the percentage (83.5 %) of explants showing root induction on the similar medium as compared to unexposed cultures (74 %). Genetic characterization of the regenerants was also done using PCR-based DNA markers, to ensure that cobalt exposure or the use of plant growth regulators in the protocol has not compromised the genetic integrity of the progeny plantlets. Screening of 570 bands produced by DNA-based ten selected ISSR primers did not record any polymorphism among the regenerants, establishing their clonal nature. Thus, the developed regeneration protocol for *Erythrina variegata* can be used for its propagation and conservation and in other in vitro manipulations for plants' improvement (Javed and Anis 2015).

1.4 *Withania somnifera* L. (Dunal)

Withania somnifera (winter cherry) commonly called as ashwagandha belongs to family *Solanaceae*. It is also known as Indian ginseng and considered as highly representative of plant kingdom in the Indian system of medicine. It is an erect greyish shrub growing up to the height of 75 cm. It is composed of about 12 alkaloids, 40 withanolides and several sitoindosides. The major constituents are mainly present in the leaves. The roots are also composed of glucose, starch, dulcitol, reducing sugar and withanol, and currently an estimation of withaferin A and withanolides D was reported by HPLC analysis (Ganzera et al. 2003).

It is commonly used in the Ayurvedic system of medicine and chiefly claimed to possess potent aphrodisiac rejuvenative and life-prolonging properties. It is also used as a memory enhancer and in other gastric problems (Williamson 2002). It also helps various ailments like chronic fatigue, weakness, teeth loose, impotency, dehydration, weakness in the bones, premature ageing, muscle tension and emaciation. Leaves and fruits are also helpful in various problems like tumour, carbuncle, ulcers and tubercular glands.

Roots are also useful in rheumatism, general ability, nervous exhaustion, memory loss, spermatorrhoea and constipation. In Ayurveda, the roots are also prescribed for gynaec disorders, bronchitis, inflammation, skin diseases, fever rheumatism, etc. (Fatima 2013).

It propagates through seeds, but seeds possess very short viability with low germination rate which resists its propagation via seeds even after stratification. Due to indiscriminate use and ruthless collection from the wild, the species is now getting the category of endangered.

Therefore, an efficient propagation system is necessary for the propagation and commercial utilization of important medicinal plant. In order to reduce the pressure on natural population and also to provide an alternative method for the production of planting material, the present experiments were taken into consideration in order to develop an efficient in vitro method which can be utilized for mass propagation of selected medicinal plant.

Direct shoot bud emergence was noticed on the Murashige and Skoog (1962) culture medium with the supplementation of different doses of various cytokinins (BA, Kin, 2iP) either single or in combination with different auxins (IAA, IBA or IAA) in nodal and shoot tip explants. However, both explants failed to show any micropropagation response on control MS medium devoid of growth regulators.

Among various treatments tried, maximum shoot regeneration was achieved on MS medium supplemented with BA (2.5 μM) and NAA (0.5 μM) in nodal segments. One hundred percent rooting frequency was observed in cultured shootlets on rooting media composed of NAA (0.5 μM), on one-half MS medium. Histological examinations also confirm the induction of various shoot primordia in both nodal and shoot tip explants. The obtained complete plantlets with rooted shoots were acclimatized with green house and transferred to natural light with 95 % survival rate (Fatima and Anis 2012).

In order to improve the regeneration potential, the effect of phenyl urea derivative, thidiazuron (TDZ), was also studied using nodal explants. MS culture medium containing TDZ (0.0–10.0 μM) was successful in producing shoot and retaining high shoot development rate and subsequent elongation on hormone-free MS medium. TDZ at a dose of 0.5 μM was mainly effective in bud break which formed optimum multiplication frequency (98 %) and a number of shoots (23.8 ± 0.33) with shoot length (4.83 ± 0.66 cm), after 4 weeks of culture. These induced shoots when transferred onto the MS medium lacking TDZ showed the greatest shoot number (32.4 ± 0.24) with shoot height (7.66 ± 0.08 cm) latterly of the fourth subculture passage. Among the various doses of IBA (50–500 μM) experienced for ex vitro rooting, the maximum percentage of rooting was obtained in Soilrite™ when the lower end of the isolated shootlets was employed with 200 μM (IBA) for 15 min, which induced maximum roots (18.3 ± 0.16) with root length of (7.63 + 0.08 cm) per shoot. After proper hardening in the plant growth chamber, the regenerated plantlets were transported to the net house where they grow well, reach maturity and show normal flowering (Fatima and Anis 2011).

The morphogenetic response of copper sulphate and zinc sulphate on nodal segment was also studied. Inclusion of micronutrients $CuSO_4$ (25–200 μM) and $ZnSO_4$ (50–500 μM) in an already standardized MS medium showed better response in shoot bud formation and lengthening. $ZnSO_4$ gives a better response in comparison with $CuSO_4$; about 61 and 66 shoots per explant were obtained with 100 μM $CuSO_4$ and 300 μM $ZnSO_4$, respectively. Rooting in micropropagated shoots was achieved on one-half MS + NAA (0.5 μM). Chlorophyll a, chlorophyll b, total chlorophyll and carotenoid content in the micropropagated plants increase with the increasing copper and zinc concentration up to optimum dose of 100 and 300 μM of $CuSO_4$ and $ZnSO_4$, respectively, in the medium. Micropropagated plantlets were hardened by the following standard procedure with 95 % survival rate. All the regenerated plants were morphologically similar (Fatima et al. 2011).

Non-embryogenic, synthetic seeds were produced by encapsulating nodal segments (containing axillary buds) of *Withania somnifera* L. in calcium alginate hydrogel comprising MS culture medium. A 3% sodium alginate with 100 μM calcium chloride $CaCl_2$ was found to be the optimum concentration for the creation of consistent syn seeds. The effect of different treatments, i.e. MS medium containing different doses of cytokinins (0.5, 1.0, 2.5, 5.0 and 10.0 μM) along with optimum dose of auxins NAA (0.5) on in vitro regeneration response of synthetic seeds, was assessed. The optimum percentage (86.2 %) of the transformation of calcium alginate-coated nodal segments into plantlets was obtained on MS medium composed of BA (2.5 μM) and NAA (0.5 μM) after 4 weeks of incubation. Rooted plantlets were achieved on one-half MS supplemented with 0.5 μM NAA. Plantlets obtained from stored synthetic seeds were hardened accordingly. Significant enhancement in the pigment contents (chlorophyll, carotenoids) and net photosynthetic rates with an increase in acclimatization days may be due to the proper working of photosynthetic machinery. Activities of antioxidant enzymes, i.e. superoxide dismutase, catalase and peroxidase, were significantly increased which suggest their preventive role in membrane oxidation and damage to biological molecules.

Also, an enhanced level of lipid peroxidation, as indicated by MDA content, is a sensitive diagnostic index of oxidative injury, clearly indicating its positive determining role in combating oxidative stress. The generated RAPD and ISSR patterns from regenerated plantlets with the mother plant were similar which confirms the genetic stability among the clones. The synthetic seed technology could possibly pave the way for the conservation, short-term storage and germplasm exchange with potential storability and limited quarantine restrictions (Fatima et al. 2013).

1.5 *Cuphea procumbens* Orteg.

The stimulatory effect of different doses of three cytokinins, BA, Kin and 2iP, on in vitro shoot bud induction, proliferation and multiplication of a potential medicinal herb *Cuphea procumbens* was investigated. Young nodal explant excised from 15 days old using cotyledonary node explants excised from 15-day-old sterilized seedlings and multiple shoots were induced on MS medium augmented with different doses of cytokinins. Maximum shoot regeneration frequency (70 %), mean number (9.33) of shoots per explant and the highest shoot length (4.16 cm) were obtained on MS medium enriched with 2.5 μM BA along with 0.5 μM NAA after 4 weeks of incubation. The addition of 200 mg/l casein hydrolysate to the standardized medium increases regenerants' growth. Microshoots of 4 cm length were successfully rooted on one-half MS medium supplemented with different concentrations of IBA. The in vitro raised healthy plantlets with properly developed roots and shoots were acclimatized and maintained in the net house with 80 % survival. Random amplified polymorphic DNA (RAPD) marker analysis of ten randomly selected in vitro raised plantlets confirms their genetic fidelity with the mother plant. The results suggested that the culture environments used for explant multiplying are suitable for clonal propagation of the selected remedial plant as these do not seem to hinder with genetic integrity of regenerants. High multiplication rate associated with observed genetic stability clearly indicates the efficacy of the present in vitro clonal propagation protocol of their valuable plants of high commercial value (Fatima et al. 2012).

1.6 *Syzygium cumini* L.

Syzygium cumini (*Myrtaceae*) is a large evergreen tree, native to the Indian subcontinent and adjoining regions of Southeast Asia. It possesses antioxidant, antimicrobial, anti-inflammatory and antiamnesic activities and is used in various neurological disorders. The fruit has various promising therapeutic values (antidiabetic properties) with various phytoconstituents, such as tannins, alkaloids, steroids, flavonoids, terpenoids, fatty acid and vitamins. The plant is propagated with seeds and vegetative methods. Vegetative methods are less effective and seed propagation may result in genetic variation. The seeds have short dormancy period and lose viability after maturation. A study was conducted to evaluate the effect of metatopolin (an aromatic cytokinin) at different concentrations with IAA, IBA or NAA on MS medium.

Among the various concentrations (0.5–10.0 μM) tested, 5.0 μM was found to be the optimum single treatment. However, the mean number of shoots per explant increased considerably when the combination of optimum metatopolin with different auxins was tried. Among all the tested concentrations, metatopolin (5.0 μM) + NAA (2.0 μM) proved to be the best treatment for induction of maximum shoots (25.37) with shoot length (6.54 cm) per explant (Naaz et al. 2014).

For rooting, isolated shoots (4 cm) from clumps were excised and transferred to the rooting medium containing various concentrations of IBA and NAA on full- or half-strength MS medium. Of the different treatments evaluated, the best rooting response (85%) with average root number (6.33) and root length (7.13 cm) was observed on half-strength MS medium containing NAA (5.0 μM). Properly rooted plantlets with four to five fully expanded leaves were successfully hardened off in growth room and finally to the normal environmental conditions. No detectable variation among the potted plants in respect to morphological and growth characteristics was observed. The genetic integrity among regenerants was also confirmed by using PCR-based DNA markers (RAPD/ISSR) (Naaz 2015).

1.7 *Albizia lebbeck* L. (Benth.)

Albizia lebbeck (*Fabaceae*) is indigenous to tropical Southern Asia and found mainly in India, Australia, Bangladesh, etc. It is a deciduous, hermaphrodite woody tree, attaining a height of 30 m. It is used to treat boils, cough, eye flu, gingivitis, lung problem and abdominal tumours. In addition, antiprotozoal, hypoglycaemic, anticancer and analgesic properties have also been reported.

Conventionally, it is propagated through seeds or microcuttings. Propagation through seeds is not useful due to the long seed dormancy. Moreover, the progeny from seeds is not homogeneous. Therefore, tissue culture technique is applied for propagation which provides an alternative for mass production of plants with uniform characteristics.

The manipulations of various culture conductions were carried out for in vitro production of plantlets from different explants, viz. seedling-derived cotyledonary node, node, cotyledon, hypocotyls, root and mature nodal explants. Among the various experiments carried out, hypocotyl explants excised from 15-day-old aseptic seedling produced an optimal shoot regeneration frequency (81%) and number (22) of shoots on MS medium supplied with 7.5 μM BA after 4 weeks of incubation.

Further, excellent response in shoot multiplication was recorded when shoot clusters were subcultured to a medium augmented with 7.5 μM BA and 0.5 μM NAA, producing highest number of shoots (34) per hypocotyl explant with mean shoot length of 6.3 cm after 8 weeks of culture.

Adventitious root induction in in vitro isolated shoots was readily achieved with various auxins (IAA, IBA or NAA) at different concentrations. The maximum root regeneration frequency was achieved on MS medium supplemented with IBA (2.0 μM) which produced an average of 5.2 roots with mean root length (4.4 cm) per shootlet. The micropropagated plantlets were acclimatized in soil with 80%

survival rate. Various physiological parameters during hardening were also evaluated. The estimation of photosynthetic pigments and antioxidant enzyme analysis has been an important parameter in determining the ability of the plants to survive oxidative stress and played an important role for better adaptation of regenerated plantlets transferred from in vitro to ex vitro environment (Perveen 2013).

1.8 *Acacia gerrardii* (Benth.)

Acacia gerrardii (*Fabaceae*) is a small-size tree legume commonly available in arid river valleys. Its unusual papery bark, ample shade and spring flowers make an excellent tree to mix with traditional and landscape species. Generally, it is propagated through seeds but seeds are recalcitrant with short seed viability. Vegetative propagation method through cutting is rather slow, and a good number of cuttings died during transportation and plantation. Various treatments containing cytokinins (BA/Kin) at different concentrations either single or in augmentation with auxins (IAA, IBA or NAA) have been evaluated on MS medium in order to establish an in vitro method for its propagation from CN explants excised from aseptically raised seedlings. The explants failed to show any response on control MS medium devoid of plant growth regulators. However, the addition of cytokinin helped in shoot bud induction in CN explants. Among all the concentrations tested, 5.0 μM BA exhibited 5.5 shoot per explant in 90 % cultures. Kin (5.0 μM) was found to be least effective. A combination of auxin and cytokinin showed synergism in shoot bud induction and proliferation. The combined effect of BA (5.0 μM) and NAA (0.5 μM) resulted in an increase in shoot number (Varshney et al. 2013).

The regenerated shootlets were rooted well in vitro on a medium containing full-strength MS salts and IBA (2.0 μM). The in vitro regenerated plantlets were successfully acclimatized and established in normal garden soil under full sun with 70 % survival rate.

1.9 Current Status of Plant Tissue Culture Commercialization in India

The response of various explants from different genotypes to different plant growth regulators clearly shows where tissue culture is today and where it is heading as an equal partner with molecular biology, as a tool in basic plant biology and in various other areas of application. Knowledge of tissue culture has contributed greatly to our understanding of the factors responsible for growth, metabolism, differentiation and morphogenesis of plant cells. The techniques of plant tissue culture have been employed as an important aid to conventional methods of plant improvement. These have been used as a tool for the propagation of genetically manipulated superior clones and for ex situ conservation of valuable germplasm.

In recent years, there has been an explosion in the number of commercial plant tissue culture units in India. Till date, 95 commercial tissue culture production units

have been recognized by the Department of Biotechnology, Government of India under the National Certification System for tissue culture-raised plants (NCS-TCP 2016). The potential for the domestic market is enormous, and by conservative estimates, it is around Rs. 200 crores with an annual growth rate of 20 %. The production capacity of commercial tissue culture units ranges between 0.5 million and 10 million plants per annum with an aggregate production capacity of about 200 million plantlets per year.

Micropropagation industry in India is providing major support to the Indian agriculture in four crop groups: fruits, ornamentals, spices and forestry/plantation crop. Banana is the largest selling tissue culture food crop. TC papaya plants are now marketed for extraction and processing of papain. TC anthuriums, orchids and gerberas have attained commercial importance. TC rose plants are used as pot plants. Nearly 500 ha are under tissue culture cardamom cultivation in Southern India recording 20–30 % increase in yield. Vanilla cultivation is expected to increase from the existing 50 ha to more than 400 ha in the coming years using TC plants. Sugar companies have in-house units of micropropagation of sugarcane. 'Jain Tissue Culture', working since 1995 for propagation and supply of 'Tissue Culture Planting Material' in the country, is the biggest laboratory for banana, pomegranate and strawberry in the world. There is a growing demand for bamboo and eucalyptus for selective reforestation. Thus, from few research laboratories several years ago, tissue culture is rapidly becoming a commercial industry in the country. Today, micropropagation and in vitro conservation have been standardized for various plant species. It no longer remains an empirical science and is now being employed in studying intricate pathways of plant metabolites and molecular genomics of plants.

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2 Selection of Elites and In Vitro Propagation of Selected High-Value Himalayan Medicinal Herbs for Sustainable Utilization and Conservation

Shyamal K. Nandi, Lok Man S. Palni, Hemant Pandey, Bhuwan Chandra, and Mohammad Nadeem

Abstract

Reduction in the forest cover from the Indian Himalayan region (IHR), due to overexploitation, has resulted in decreased availability of non-timber forest products, including medicinal plants of high economic value. With the ever-increasing human population and growing demand for plants and plant-derived products,

S.K. Nandi (✉)
G.B. Pant Institute of Himalayan Environment & Development,
Kosi-Katarmal, Almora 263643, Uttarakhand, India
e-mail: shyamal_nandi@rediffmail.com

L.M.S. Palni
G.B. Pant Institute of Himalayan Environment & Development,
Kosi-Katarmal, Almora 263643, Uttarakhand, India

Biotechnology Department, Graphic Era (Deemed) University,
Clement Town, Dehradun 248002, Uttarakhand, India

H. Pandey
G.B. Pant Institute of Himalayan Environment & Development,
Kosi-Katarmal, Almora 263643, Uttarakhand, India

Agro Division, Merino Industries Ltd, Achheja,
Hapur, Ghaziabad 245101, Uttar Pradesh, India

B. Chandra
G.B. Pant Institute of Himalayan Environment & Development,
Kosi-Katarmal, Almora 263643, Uttarakhand, India

Near Anandi Academy, Bankhola, Mandalsera, Bageshwar 263642, Uttarakhand, India

M. Nadeem
G.B. Pant Institute of Himalayan Environment & Development,
Kosi-Katarmal, Almora 263643, Uttarakhand, India

Department of Botany & Microbiology, King Saud University, Riyadh 11451, Saudi Arabia

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there has been tremendous anthropogenic pressure on these primary producers. Many plant species are a source of high-value drugs; due to increasing global demand for the 'naturals', they are being subjected to reckless, often illegal harvesting, well beyond the natural regeneration capacity. This has led to many species being listed in the Red Data Book or in various IUCN threat categories. Improper harvesting (season and/or age of the plant/plant parts) not only results in uneconomical yields due to low content of active ingredients but also adversely affects the process of natural regeneration. There is, therefore, an urgent need for commercially important species to be subjected to improved management practices and regulated harvesting to generate better economic benefits on one hand and to encourage cultivation for sustained utilization as well as economic development of the region on the other. This twin strategy would also help to improve the conservation status of such species.

In order to meet such challenges, in vitro propagation (tissue culture) techniques have provided a well-recognized potential for rapid multiplication of elite clones for the supply of much needed good-quality planting material for cultivation and also to achieve conservation objectives. Keeping these goals in mind, studies were taken up to assess the active ingredient content of plants/plant parts collected from natural populations growing in different locations/altitudes in the wild and to develop in vitro propagation methods for selected high-value alpine medicinal herbs (*Aconitum balfourii*, *A. heterophyllum*, *Picrorhiza kurrooa* and *Podophyllum hexandrum*). Using elite plant material, attempts have been made to establish tissue culture protocols that involved the induction of multiple shoots, improved rooting and subsequent development of suitable methods for hardening and field transfer. In a few cases, the survival and growth of tissue culture-raised (TCR) plants was also monitored to evaluate their field performance.

Abbreviations

BAP	6-Benzylaminopurine
IBA	Indole-3-butyric acid
IAA	Indole-3-acetic acid
IHR	Indian Himalayan region
GA_3	Gibberellic acid
Kn	Kinetin
MS	Murashige and Skoog
NAA	α-Naphthalene acetic acid
PGS	Plant growth substance
SR	Seed raised
TCR	Tissue culture raised
TDZ	(Thidiazuron): 1-phenyl-3 (1,2,3-thiadiazol-5-yl) urea

2.1 Introduction

The forest cover from the Indian Himalayan region (IHR) has been substantially reduced over the years, and it varies from 10.14% (Jammu and Kashmir) to 90.38% (Mizoram) across IHR states (Anonymous 2013). The recommended cover of 67% and above is not present in many of the Himalayan states. This has adversely affected the availability of non-timber forest products, including medicinal plants of high commercial and therapeutic value. The increasing human population and the growing demand for plants and plant-based products have collectively placed very high anthropogenic pressure on these primary producers. Many plant species are known sources of high-value drugs, and due to the increasing global demand for the 'naturals', they are being subjected to reckless, often illegal harvesting, well beyond their natural regenerative capacity. This has led to many species being listed in the Red Data Book and/or in various threat categories (Nandi et al. 2002; Anonymous 2003; Ved et al. 2003).

The life and economy of the hill people, to a large extent, depend on the plants, and thus any reduction in the forest cover does have a great negative effect on natural resources including their living conditions. Moreover, improper harvesting (season and/or age of the plant/plant parts) results in uneconomical yields due to the suboptimal content of active ingredients and also adversely affects the process of natural regeneration. There is, therefore, an urgent need for all such commercially important species to be subjected to improved management practices and regulated harvesting to generate improved long-term economic benefits on one hand and to encourage their cultivation for sustained utilization as well as economic development of the region on the other. Keeping these goals in mind, studies were taken up to assess the active ingredient content of plants/plant parts collected from natural populations of selected medicinal plants growing in different locations/altitudes in the wild and to develop in vitro propagation (tissue culture based) methods for these high-value alpine medicinal herbs (*Aconitum balfourii*, *A. heterophyllum*, *Picrorhiza kurrooa* and *Podophyllum hexandrum*). Using elite (in terms of high active principle content) plant material, attempts have been made to establish their in vitro (tissue) cultures, induce multiple shoots, improve rooting of shoots and subsequently develop suitable methods for hardening before field transfer. In a few cases, the survival and growth of in vitro-raised (IVR) plants was also monitored to evaluate field performance.

A brief description of all four species selected (Fig. 2.1) for in vitro propagation has been provided below.

Aconitum balfourii Stapf. [=*A. atrox* (Brhul) Muk.; family, Ranunculaceae; English name, aconite; local names, 'Meetha' and 'Bish'] is a highly valued medicinal herb endemic to the alpine and subalpine belts of the IHR and grows above 3200 m altitude (Samant et al. 1998). Its tuberous roots are used by various ethnic communities for curing different ailments (rheumatism, fever, etc.) and are important source of ingredients used in the preparation of Indian Ayurvedic medicines (Chopra et al. 1984; Anonymous 1988). The medicinal properties have been attributed to several diterpenoid alkaloids, mainly aconitine, balfourine, bikhaconitine and pseudaconitine, the latter being highly toxic and biologically 2.5 times more active than aconitine (Chopra et al. 1984; Khetwal et al. 1992).

Fig. 2.1 Different medicinal plants used in the study growing under the natural habitat. (**a**) *Aconitum balfourii*, (**b**) *A. heterophyllum*, (**c**) *Picrorhiza kurrooa* and (**d**) *Podophyllum hexandrum*

Aconitum heterophyllum Wall. (family, Ranunculaceae; common name, aconite; local name, 'Atish') is an erect herbaceous rosette distributed in the subalpine and alpine regions of IHR at 3000 m and above. The tubers and roots are being used as tonic and for curing different ailments like fever, diarrhoea and dyspepsia. The tubers and roots are known to contain alkaloids like atisine, heteratisine and aconitine (Pelletier et al. 1968; Pandey et al. 2008). Both *A. balfourii* and *A. heterophyllum* pass through long juvenile phase and their propagation, mainly through seeds, is quite poor under natural conditions. Flowering and fruiting are erratic due to harsh climatic conditions, and only limited or no viable seeds are produced (Pandey et al. 2000 and references therein). At present *A. balfourii* and *A. heterophyllum* are under 'vulnerable' and 'critically endangered' category, respectively (Ved et al. 2003).

Picrorhiza kurrooa Royle ex Benth. (family: Scrophulariaceae; local name, 'Kutki') is endemic to Himalayan alpines and distributed between 3300 and 4800 m

altitude. The extracts of runners and roots have been used in several 'Ayurvedic' preparations, prescribed for hepatic disorders. The plant is also used in traditional as well as modern system of medicine as a stomachic, purgative, antiperiodic and brain tonic and in dyspepsia and fever. It contains picroside I, II and III and kutkoside as major bioactive compounds (Weinges et al. 1972; Jia et al. 1999). Indiscriminate collection of this plant from the wilds and lack of organized cultivation have led to considerable depletion of its natural populations, and it is presently categorized as 'critically endangered' and listed in CITES Appendix II (Anonymous 2003; Ved et al. 2003).

Podophyllum hexandrum (family, Podophyllaceae; English name, mayapple; Hindi name, 'Bankakri'; local names, 'Banwaigan' and 'Papri') has gained considerable importance because the rhizomes of this species (and that of some other *Podophyllum* species) contain several lignans, out of which one of the non-alkaloid compounds, podophyllotoxin, is extremely important (Van Uden et al. 1989; Canel et al. 2000; Tabassum et al. 2014). It is a potent antiviral agent (Beutner and von Krogh 1990) as well as the starting compound for the preparation of semisynthetic compounds, namely, etoposide, Etopophos and teniposide, which are effective antitumour agents used in the treatment of lung cancer, variety of leukaemia and other solid tumours (Van Uden et al. 1989; Canel et al. 2000; Lee and Xiao 2005). Two species, namely, *P. hexandrum* Royle (occurring in Central Himalaya) and *P. sikkimensis* R. Chatterji and Mukerji (found in Eastern Himalaya) have been reported from India. *P. hexandrum* (generally growing above 2800 m) contains three times more podophyllotoxin as compared to the American species, *P. peltatum*, and hence its ever-increasing demand. Its uncontrolled collection from the natural populations is inflicting tremendous damage; *P. hexandrum* is currently placed under 'endangered' category and listed in CITES Appendix II (Anonymous 2003; Ved et al. 2003). Details of these three species can be found in a recent article (Paul et al. 2013).

2.2 Analysis of Active Ingredients

2.2.1 Aconitine and Pseudaconitine

Quantification of diterpenoid alkaloids, namely, aconitine and pseudaconitine, was carried out in tubers of *Aconitum heterophyllum* and *A. balfourii* collected from higher altitudes of Indian Central Himalaya [seven locations (3000–3600 m) in Garhwal and four locations (3250–3430 m) in Kumaun region of Uttarakhand] following column, thin-layer and high-performance liquid chromatography (HPLC) with the aim of identifying elites (Pandey et al. 2008). The aconitine levels in different populations of *A. heterophyllum* varied from 0.13 to 0.75 % (dry weight basis); maximum and minimum levels were detected in tubers from Phurkia (3260 m) and Kafni (3400 m), respectively. In *A. balfourii* the amount of aconitine and pseudaconitine also varied and was found to range from 0.13–0.83 % to 0.06–0.62 %, respectively. The highest level of pseudaconitine (0.62 %) was recorded in samples collected from Phurkia Bugyal (3430 m), while the lowest and about tenfold less value (0.06 %) was recorded in tubers from Kafni population (3400 m);

highest level of aconitine (0.83 %) was also recorded in tubers collected from Phurkia Bugyal (3430 m), while the lowest values were found in samples from Kedarnath population (3600 m). The active principle content could not be correlated with altitude (Pandey et al. 2008). However, in another study, the amount of pseudaconitine and aconitine content in *A. balfourii* and *A. heterophyllum* was found to generally increase with altitude (Bahuguna et al. 2000). The amount of pseudaconitine and aconitine reported by Pandey et al. (2008) was higher than the values reported for these species by Bahuguna et al. (2000). The existing natural populations are likely to be of seed origin and thus the observed variation in active ingredient content could be attributed to genotypic differences. Ecological factors like habitat, temperature and soil characteristics are likely to affect qualitative and quantitative changes in aconitine analogues; reported variation in the levels of active principles in different studies could also result from the methods used for the extraction, purification and estimation.

A number of important chemical compounds identified from various *Aconitum* species along with their biological activities have been summarized in recent reviews (Srivastava et al. 2010; Sharma and Gaur 2012).

2.2.2 Picrosides

The runners/stolons of *P. kurrooa* are known to contain different medicinal and bioactive compounds which act as hepatoprotective agents and been identified as picroside I, picroside II, kutkoside, apocynin, androsin, cucurbitacin glycosides, catechol, etc. (Weinges et al. 1972; Jia et al. 1999). *P. scrophulariiflora* is also a source of iridoid glycosides such as picroside I, picroside II and kutkoside; however, it contains an additional phenylethanoid glycoside and plantamajoside which are absent in *P. kurrooa* (Li et al. 1998), and hence *P. scrophulariiflora* is a better substitute for *P. kurrooa*. Kutkoside was thought to be a single compound; however a recent report indicates that kutkoside is a mixture of several iridoid glycosides, namely, picroside II, picroside IV and 6-ferulloylcatalpol (Bhandari et al. 2010). Extracts of both *P. kurrooa* and *P. scrophulariiflora* have been reported to contain high antioxidant activity (Ray et al. 2002; Bhandari et al. 2010; Tiwari et al. 2012).

There are limited reports on active ingredient content of runner/stolon of *P. kurrooa* collected from different populations. Purohit et al. (2008) analyzed five high-altitude populations (narrow and broadleaf plants, collected from Tungnath, Kilpur, Valley of Flowers, Kuwari Pass and Panwali Kantha at 2700–3800 m altitudes) from Garhwal Himalaya in Uttarakhand and reported that picrotin and picrotoxin content ranged from 1.00 to 6.05 mg/g. In general, the broadleaf plants from all the populations showed higher content than the narrow ones. The minimum (1.0 mg/g) picrotin and picrotoxin content was found in narrow leaf plant samples from Kuwari Pass (2800–3800 m) population, while the maximum amount (6.05 mg/g) was reported from broadleaf samples from Valley of Flowers (2700–3600 m) population (Purohit et al. 2008). Sharma et al. (2012b) analyzed runner samples from seven accessions of *P. kurrooa* collected from high-altitude regions of Himachal Pradesh, India (e.g.

Chamba, Manikaran, Manali, Khoksar, Marhi, Keylong and Rohtang from 996 to 3978 m altitude) by reversed-phase HPLC and reported that the highest level of the major bioactive compounds, picroside I (3.5 %) and picroside II (2.0 %), was found in Rohtang population (3978 m). The variability of these major constituents within the same species at different altitudinal ranges would thus help in identifying superior clones (elites) for multiplication and conservation.

In another study (Tiwari et al. 2012) picroside content and antioxidant activity were determined in rhizomes of *P. scrophulariiflora* and *P. kurrooa* collected from Munsyari region of Uttarakhand, India. Separation and quantification was achieved by HPTLC and subsequently by densitometrically. The study revealed that picroside I and picroside II content was higher in *P. scrophulariiflora* than in *P. kurrooa*. Picroside I content was found to be 1.26 and 1.61 %, and picroside II was 0.48 and 0.61 % in *P. kurrooa* and *P. scrophulariiflora*, respectively. The antioxidant potential of these two species using DPPH assay was found to be quite high, i.e. at a concentration of 0.1 mg/ml, the scavenging activities of *P. kurrooa* and *P. scrophulariiflora* were found to be 37.70 and 34.30 %, respectively (Tiwari et al. 2012). In another investigation, high levels of picrosides (picroside I and picroside II, up to 7.33 %) were reported in rhizomes collected from one of the three different populations of *P. scrophulariiflora* from the Eastern Himalayan region (Bantawa et al. 2010). These workers also reported a micropropagation protocol for rapid multiplication of this high picroside containing population which can be used for cultivation and conservation.

2.2.3 Podophyllotoxin

Podophyllotoxin levels were determined in rhizome and root samples of *P. hexandrum* plants (with known leaf morphological variants, i.e. 1 L, 2 L and 3 L; 4 L samples could not be obtained) collected from 17 different populations (2800–3600 m altitudes) along an altitudinal gradient spread across Uttarakhand state (Pandey 2002; Pandey et al. 2015). Extraction, purification and subsequent analysis (by HPLC) were carried out by published methods (Van Uden et al. 1989; Nadeem et al. 2007), and the results indicated a wide variation in the podophyllotoxin content, ranging from 0.012 to 5.80 % (on dry weight basis); among these morphological variants, 2 L plants of Kedarnath area (highest altitude, 3600 m) exhibited maximum content, both in rhizomes and roots (Pandey 2002; Pandey et al. 2015). In another investigation, analyses of rhizomes collected from eight populations along an altitudinal gradient (2740–3350 m, i.e. Dhungiadhaung, 2740 m; Dwali, 2790 m; Juharpani, 2900 m; Khamia, 3125 m; Khatia, 3240 m; Kathlia I, 3250 m; Phurkia, 3260 m; and Kathlia II. 3350 m) of Kumaun region in Indian Central Himalaya indicated variation in podophyllotoxin levels, ranging from 0.36 % to 1.08 % (Nadeem et al. 2007). It was observed that the minimum podophyllotoxin content (0.36 %) was recorded in samples from Dwali (2790 m), while the maximum (1.08 %) was detected in samples from Kathlia II (3260 m) populations; the regression analysis revealed a positive correlation between podophyllotoxin content and increase in the altitude (Nadeem et al. 2007).

Podophyllotoxin levels in rhizomes have been reported to vary considerably, and values up to 8.26 % were found in samples collected from the states of Uttarakhand and Himachal Pradesh in IHR (Purohit et al. 1998, 1999; Sharma et al. 2000). Furthermore, levels ranging from 3.02 to 9.53 % were reported from 28 populations occurring at various altitudes (1570–4300 m) in Himachal Pradesh (Naik et al. 2010). Similar levels were also estimated from the rhizome buds of various populations collected from Zanskar valley of Jammu and Kashmir state in IHR (Kitchlu et al. 2011). Sharma (2013) has also reported podophyllotoxin levels ranging from 3.44 to 5.87 % in rhizomes collected from Himachal Pradesh, but no relationship between the active principle content and altitude was observed.

Besides rhizomes, leaves of *P. peltatum* have also been reported to be a rich source of podophyllotoxin (Bastos et al. 1996; Canel et al. 2001; Moraes et al. 2000, 2002). However, the occurrence of podophyllotoxin in leaves of *P. hexandrum* from wild has been reported only recently (Pandey et al. 2013; Sharma 2013). In a detailed study, podophyllotoxin content in leaf and stem samples of *P. hexandrum* plants (with leaf morphological variants, i.e. 1 L, 2 L and 3 L) collected from seven different populations (2800–3600 m) of Uttarakhand was analyzed by established methods, and the content was found to range from 0.001 to 0.60 %; among these morphological variants, 3 L plants of Dodital area (altitude, 3100 m) exhibited maximum content, both in leaf and stem samples (Pandey et al. 2013). However, these estimates were found to be lower as compared to values up to 5.80 % reported from rhizomes/roots collected from the same region (Pandey 2002, Pandey et al. 2015). Sharma (2013) also determined the podophyllotoxin content of *P. hexandrum* leaf samples from four high-altitude populations (2730–3978 m, Himachal Pradesh), and the maximum amount (0.30 %) was found in Marhi (3300 m) population. The podophyllotoxin content of leaves was nearly two-fold higher (0.60 %) in a study conducted by Pandey et al. (2013) as compared to the report by Sharma (2013). On the other hand, levels of up to 5.2 % have been reported, both in leaves and rhizomes of *P. peltatum* (Bastos et al. 1996; Canel et al. 2001; Moraes et al. 2000, 2002, 2005; Cushman et al. 2005; Zheljazkov et al. 2011). Thus leaves can be an alternate and readily renewable source of the compound which is routinely in high demand by pharmaceutical industries.

It is important to mention that in all above cited studies, a wide variation in podophyllotoxin content was observed. Thus, the observed differences in content in various populations of *P. peltatum* and *P. hexandrum* can be ascribed to genotypic differences (Bastos et al. 1996; Moraes et al. 2000; Nadeem et al. 2007; Naik et al. 2010; Pandey et al. 2013). Further, the age of the plant also influences active ingredient content in rhizomes of *P. hexandrum* (Pandey et al. 2007; Sharma et al. 2000). In this investigation analyses were carried out on samples from wild populations; thus, the age of sampled plants could not be ascertained. It is possible that variations arise due to the presence of different chemo types in natural populations as also on the method of extraction (Bastos et al. 1996; Canel et al. 2001). Both biotic and abiotic factors, including soil conditions, are known to affect the lignan yield in *P. peltatum* (Moraes et al. 2005).

Table 2.1 Podophyllotoxin content in different parts of *P. hexandrum* plants (1- to 5-year-old, seed raised) grown at a lower altitude (Kosi, 1150 m)

	Podophyllotoxin content (% of dry wt)		
Plant age (year)	Rhizome	Roots	Leaf
1	0.016 ± 0.001	0.022 ± 0.008	0.005 ± 0.001
2	0.017 ± 0.002	0.039 ± 0.003	0.009 ± 0.003
3	0.040 ± 0.001	0.056 ± 0.000	ND
4	0.080 ± 0.021	0.159 ± 0.012	ND
5	0.108 ± 0.026	0.011 ± 0.005	0.0003 ± 0.0001
LSD ($P = 0.005$)	0.056	0.003	0.007

Source: Pandey et al. (2007)
LSD Least significant difference, ± = Standard error of mean; Values are an average of three determinations. *ND* Not detected

An attempt was also made to grow plants of *P. hexandrum* at a lower altitude (1150 m altitude) in order to examine if there are changes in podophyllotoxin content in plants of known age; such study should also help in suggesting a suitable strategy for promoting cultivation at alternate locations to reduce the pressure on natural populations and for developing cultivation packages. The podophyllotoxin content in various plant parts (rhizomes, roots and leaves) of *P. hexandrum* (expressed as % of dry wt) is summarized (Table 2.1, Pandey et al. 2007). The content in rhizomes was low and similar for 1- and 2-year-old plants and increased gradually reaching a maximum value (0.108 %) in 5-year-old plants. In general, it was higher in roots than in the rhizomes of 1- to 4-year-old plants. The content in the roots increased up to 4 years when highest level (0.159 %) of podophyllotoxin was recorded; this level was higher than that present in rhizomes of 5-year-old plants. However, the levels in the roots declined drastically in 5-year-old plants. The podophyllotoxin content of leaves was considerably lower than that of rhizomes and roots of the same plants and was found in the range of 0.0003–0.009 %; the levels were below detectable limit in 3- and 4-year-old plants. It must be added that various growth parameters (leaf area, leaf length and above- and below-ground biomass) recorded a concomitant increase with plant age (Pandey et al. 2007).

The podophyllotoxin content of rhizomes of young plants (1-, 2- and 4-year-old) was found to increase with age in population growing at 2400 m (Purohit et al. 1999). The reported values for plants of any given age were higher than that found in the present study (Table 2.1). However, prior to this study, no information was available on the podophyllotoxin content in rhizomes, roots and leaves of plants of a known age series along with the growth parameters (including dry matter production) of seed-raised plants grown at a relatively lower altitude. Growing of alpine plants in accessible locations at near-natural conditions or at lower altitudes can be an overall strategy to promote their cultivation at relatively convenient locations, with a view to reduce pressure on the wild populations.

The podophyllotoxin content in rhizomes of another Himalayan species, i.e. *P. sikkimensis*, reported for the first time from our laboratory, ranged from 0.06 % to

0.73 % in plants collected from Thangu area of Sikkim in Eastern Himalaya (Paul et al. 2013). Though the levels are well below those reported in *P. hexandrum* or *P. peltatum*, estimations from other populations are required to be carried out in order to provide a clearer picture on its active ingredients.

2.3 Tissue Culture (In Vitro) Studies

2.3.1 *Aconitum balfourii*

Tubers (at the time of senescence of aerial parts) from an identified population of *A. balfourii* were collected from Kedarnath area (District Rudraprayag, Uttarakhand; 3300 m; 30°43′ to 30°45′ N and 79°3′ to 79°4′ E) and grown in the Institute Nursery at Kosi-Katarmal (District Almora, Uttarakhand; 1150 m; 29°38′15″ and 79°38′10′ E). Apical portions of fully grown plants were de-topped to encourage the growth of axillary buds. These buds (along with a small portion of the stem) were excised and used as explants which were disinfected and cultured as described earlier (Pandey et al. 2004). The explants were transplanted in test tubes containing 20 ml of Murashige and Skoog's medium (Murashige and Skoog 1962, MS) supplemented with sucrose (3 %, w/v), agar (0.8 %, w/v) and BAP (4.5, 13.5 or 22.5 μM); these explants were subcultured at least four to five times at 3 days interval to obtain contamination-free cultures. The axillary buds sprouted to form shoots and young leaves from these shoots were used for callus induction.

Callus formation occurred along the leaf margins within 5 weeks on the MS medium supplemented with various combinations of BAP (0.5–4.5 μM) and NAA (5.4–26.9 μM); maximum (75 %) explants with calli were obtained on MS medium supplemented with 4.5 μM BAP and 26.9 μM NAA. Hence, healthy and proliferating callus growing on this medium was transferred to the fresh medium containing different concentrations of BAP (4.5–22.2 μM) and NAA (0. 5–5.4 μM) for shoot induction. It was found that relatively lower levels of NAA (0.5–5.4 μM) and BAP (4.5 μM) resulted in excellent shoot regeneration. The adventitious shoot formation was maximum (100 %) on the medium supplemented with 4.5 μM BAP and 1.4 μM NAA, resulting in nearly six shoots/callus lump (Fig. 2.2a). Therefore, this medium was routinely used for shoot multiplication. Subsequently, single shoots were separated and subcultured on the MS medium containing BAP alone (0.5–44.4 μM) for shoot multiplication and elongation.

BAP at 1.1 μM concentration produced maximum number of shoots (24.7 per flask, 4.2 per cultured shoot/subculture) as well as resulted in shoot elongation (about 3.5 cm) within 4 weeks (Fig. 2.2b). The excised shoots developed on the MS medium supplemented with 1.1 μM BAP were placed on the medium supplemented with various concentrations of IBA for rooting. Root formation was initiated within 15–18 days in cultures supplemented with IBA (4.9, 12.3 or 24.5 μM), and maximum rooting (89 %) was observed on the medium containing 12.3 μM IBA (Fig. 2.2c). Well-rooted microshoots were first transferred to Erlenmeyer flasks containing sterilized soil rite, moistened with one-half MS salts and hardened for 2 weeks

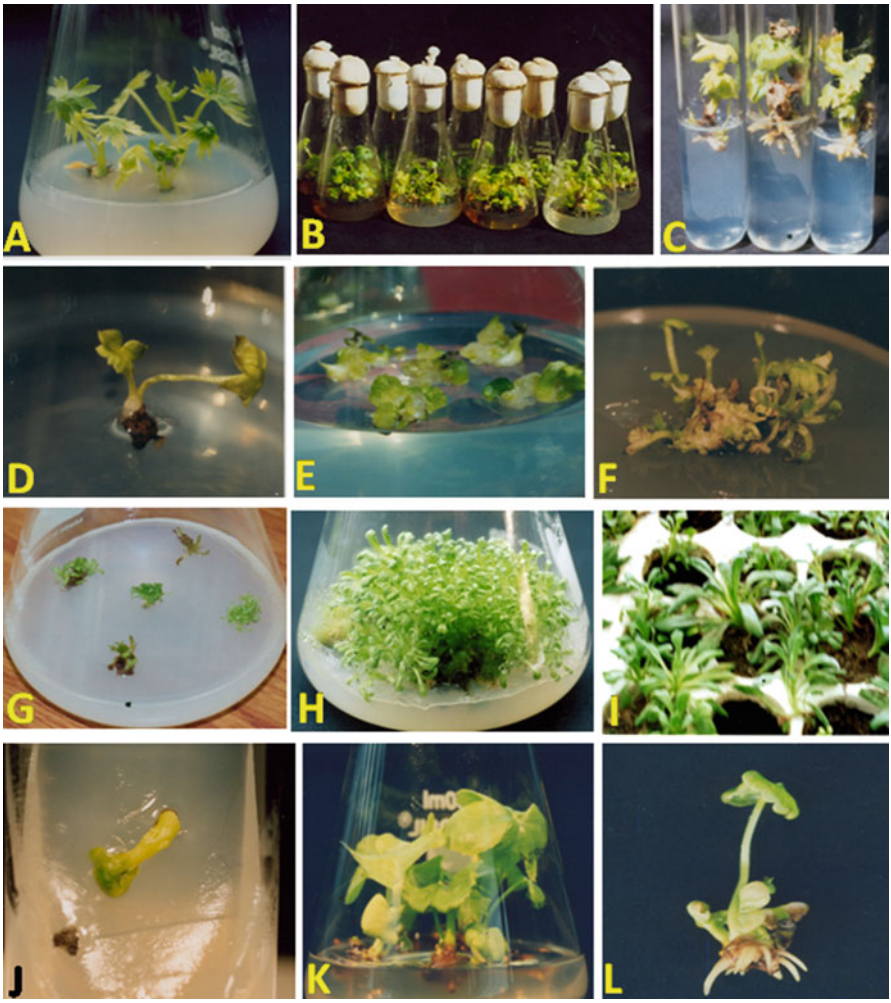

Fig. 2.2 Different stages during in vitro propagation of selected medicinal herbs: *Aconitum balfourii* (**a**–**c**), *A. heterophyllum* (**d**–**f**), *Picrorhiza kurrooa* (**g**–**i**) and *Podophyllum hexandrum* (**j**–**l**). (**a**) In vitro shoot formation on the MS medium supplemented with 4.5 μM BAP and 1.4 μM NAA; (**b**) shoot multiplication on the MS medium containing 1.1 μM BAP only; (**c**) rooting of shoots on the MS medium supplemented with 12.3 μM IBA after 4 weeks of culture; (**d**) germinated seed after 6 weeks of inoculation on the MS medium; (**e**) appearance of shoot budlike structures in cultures on the MS medium supplemented with 5.0 μM BAP and 1.0 μM NAA; (**f**) shoot multiplication from seedling explants (without radicle) on the MS medium containing 1.0 μM BAP; (**g**) initiation of shoot multiplication from cotyledonary node explants after 4 weeks of inoculation on the MS medium containing 1.0 μM BAP; (**h**) multiplication and profuse shoot formation on the MS medium containing 1.0 μM BAP; (**i**) hardened plantlets under greenhouse conditions, 4 months after ex vitro growth; (**j**) culture of excised embryos on the MS medium supplemented with BAP and IAA; (**k**) multiple shoot induction on the MS medium supplemented with BAP and IAA (1.0 μM each) and (**l**) induction of roots on shoots after transfer to the MS medium containing 0.5 μM IAA

under aseptic conditions in the culture room. These flasks were then shifted to the greenhouse (25 °C, RH 65 %) at Kosi-Katarmal and the plantlets transferred to plastic cups containing soil rite. Following 2 months of hardening (regular watering with one-half MS salts), the plants were transferred to a mixture of soil and FYM (1:1) and allowed to grow for another 2 months before transportation to their natural habitat. This substantially improved the ex vitro survival during hardening, and the roots were also found to proliferate (increase in number as well as in length) during this period of hardening. The survival of these plants was found to be 50 %, and 20 % of such plants formed normal tuberous roots within a year (Pandey et al. 2004).

Using the above-mentioned protocol, a callus lump (approx. size 9 × 8 × 5 mm, l × w × h) derived originally through a leaf explant (size 5 × 5 mm, l × w) of *A. balfourii* can produce up to six shoots directly through organogenesis, and each shoot can in turn provide four additional shoots per subculture, thus resulting in a total of 24 shoots within 9 weeks. Although an earlier study on this species (Singh et al. 1998) reported formation of embryos, adventitious shoots and roots, quantitative data on these aspects were not reported.

Quantitative analysis of diterpenoids by thin-layer chromatography followed by HPLC, in tubers of *A. balfourii* derived from seedlings and tissue culture-raised (TCR) plants showed that both aconitine and pseudaconitine (% of dry wt) were almost similar; aconitine content was 0.01 % in both types of plants, while pseudaconitine was 0.40 % in seedlings and 0.41 % in the TCR plants (Pandey et al. 2004). The results of the above study indicate that TCR plants compared favourably with the seed-raised plants of *A. balfourii*. More recently in vitro multiplication of this species was reported using both leaf and root explants taken from plant and passing through a callus phase; following shoot multiplication, root induction and hardening about 95 % of plantlet survival was observed in pots placed under greenhouse conditions (Bist et al. 2011; Sharma et al. 2012a). It would be important to add that the above-mentioned studies must be substantiated with DNA-based markers like RAPD, ISSR, etc. to confirm the genetic fidelity of these TCR plants.

In vitro studies in other species of *Aconitum* have also been carried out. As observed by Pandey et al. (2004), BAP has also been found to be effective in axillary bud proliferation and shoot multiplication in *A. noveboracense* and *A. napellus* (Cervelli 1987; Watad et al. 1995). Application of higher concentration of NAA with lower concentration of BAP has been reported to result in callus induction in *A. heterophyllum* (Giri et al. 1993). In *A. carmichaeli*, 22.1 μM BAP was extremely effective in stimulating the growth of shoot tip explants leading to formation of multiple shoots (Hatano et al. 1988), whereas a lower concentration (4.5 μM) of BAP produced greatest number of usable rosettes from the nodal explants in *A. napellus* and *A. noveboracense* (Cervelli 1987). Herbaceous species generally demand greater care, and therefore, during the initial phase of hardening, gradual removal of sucrose and salts helps the TCR plants to shift to autotrophic mode of nutrition (Bhojwani and Razdan 1996).

It should be mentioned that the electrophoretic pattern of storage proteins of TCR microtubers of potato was also found to be identical to that of field-produced tubers (Dodds et al. 1992). Similarly, it has been demonstrated that micropropagated

plants of *A. carmichaeli* showed less variation in alkaloids content when compared to those of field-grown plants (Hatano et al. 1988; Shiping et al. 1998).

The potential of using plant callus, cell and suspension cultures for long-term and sustainable production of active principles has been realized (Petersen and Alfermann 2001; Collin 2001, and references therein). Cell cultures have also been used extensively for biotransformation of various molecules to produce a variety of compounds of interest (Giri et al. 2001b). Recently, higher aconitine accumulation was reported in the presence of precursor acetyl-CoA (compared to control) when roots of *A. balfourii* were cultured in vitro (Sharma et al. 2014). Higher aconitine content (0.024 %) was also reported in roots of TCR plants when grown in hydroponics compared to TCR plants (0.012 %, Sharma and Gaur 2012). Besides, hairy root cultures induced by *Agrobacterium rhizogenes* have the ability of continued growth on plant growth substance-free medium and are known to produce elevated levels of active ingredients in several medicinal species (Giri et al. 1997, 2001a; Baiza et al. 1998). Studies on cell and hairy root culture in *A. balfourii* have not been reported so far.

2.3.2 *Aconitum heterophyllum*

Seeds of *A. heterophyllum* were collected from Kedarnath area (3300 m; District Rudraprayag, Uttarakhand) and stored at 4 °C (dark, 2 months). These were washed, surface disinfected, and inoculated (25 seeds/petri dish) onto the MS basal medium containing agar (0.8 %, w/v) and sucrose (2.0 %, w/v). Seeds were found to germinate within 5–6 weeks, and after another 3 weeks, the cotyledons were found to fully emerge (Fig. 2.2d). To initiate cultures, the cotyledons were separated from the seedlings and divided into two groups: the middle portion (where cotyledons unite in a sheathing base) and the outer portion (surrounding the middle region, Pandey 2002). Excised explants (middle/outer portions) were placed on the MS medium supplemented with NAA (5.0–25.0 μM) and BAP (5.0 μM) for callus induction. Pale-green calli were formed after 6 weeks of culture; in general, both types of cotyledon segments (middle and outer portions) exhibited similar performance. Maximum proliferation, i.e. 73 and 67 %, was observed in middle and outer portions, respectively, on the MS medium supplemented with 25.0 μM NAA and 5.0 μM BAP. Such callus maintained on this medium was later transferred to the medium containing 5.0 μM BAP and very low amounts of NAA (0.1–1.0 μM) for shoot initiation. The MS medium supplemented with 1.0 μM NAA and 5.0 μM BAP resulted in the formation of shoot budlike structures (Fig. 2.2e). These shoots did not last long and dried up within 3–4 weeks (Pandey 2002).

Germinated seedlings (radicle portion excised) containing cotyledons, hypocotyls, and hypocotyl sheath with apical meristem at the base were cultured on the MS medium containing BAP or thidiazuron (TDZ) for shoot proliferation. When the seedlings (without radicle) were cultured on the medium containing BAP (0.01, 0.1 and 1.0 μM), best response (80 %) was obtained on a medium containing 1.0 μM BAP with maximum shoot formation (six shoots/explant) after 8 weeks (Fig. 2.2f).

These shoots were excised from the clumps, and individual shoots were then cultured on the medium containing 1.0 μM BAP for multiplication. On the other hand when the seedlings (without radicle) were cultured on the medium containing TDZ (0.1, 1.0, and 10.0 μM), it resulted in early emergence of leaves. The use of 0.1 μM TDZ resulted in the emergence of the first leaf after 9 days of incubation in comparison to 32 days in control; the second leaf was found to emerge within 16 days in comparison to 42 days in control. However, the shoots thus formed could not survive for long and died within 2–3 weeks (Pandey 2002).

Although attempts were made in this investigation to obtain microshoots for further growth and multiplication, success could not be achieved. Further attempts are underway to obtain improved growth of such microshoots. The cytokinin, BAP, has been found to be quite useful for shoot bud proliferation and shoot multiplication in several species of *Aconitum*, including *A. heterophyllum* and *A. balfourii* (as mentioned above). It is worth mentioning that in vitro propagation of *A. heterophyllum* has been reported (Giri et al. 1993; Jabeen et al. 2006), and these workers have also used BAP to obtain shoot formation; mass scale propagation of plants has, however, not been reported.

The establishment of hairy root cultures in *A. heterophyllum* and the production of active ingredients were successfully demonstrated (Giri et al. 1997). They reported that the total aconitine content of transformed roots was 3.75-fold higher than that of non-transformed roots. However, further reports on the production of active compounds either by hairy roots or through cell culture have not appeared. Recently, the protocol for in vitro plant regeneration in *A. violaceum*, another Himalayan medicinal herb, has been demonstrated (Mishra-Rawat et al. 2013 a, b).

2.3.3 *Picrorhiza kurrooa*

Seeds of *P. kurrooa* were collected from Pindari area (3400 m; District Bageshwar, Uttarakhand; 30°6′ to 39°15′ N and 70°55′ to 80°5′ E); these were removed from the spikes, air dried, and stored at 4 °C (dark, 3 months). Seeds were then washed, surface disinfected and inoculated (25 seeds/petri dish) on the MS basal medium containing agar (0.8 %, w/v) as described earlier (Chandra et al. 2004, 2006). Germinated seeds were used for obtaining 'cotyledonary node' or 'shoot tip' explants.

Two weeks following seed germination under in vitro conditions, the radicle, cotyledons and a part of the hypocotyl (3.0–5.0 mm below the cotyledonary node) were removed from the seedlings, and the remaining portions of the explants containing a small part of the hypocotyl and the cotyledonary node (cotyledonary node explant) were cultured on the MS medium supplemented with BAP or kinetin (Kn, 1.0–10.0 μM). The other set of germinated seeds were allowed to germinate in vitro and grown (avg. height of seedlings 2.5 cm) till the first leaf emerged. 'Shoot tip' portions (0.5 cm) were carefully excised from seedlings, under aseptic conditions, and further subcultured on the MS medium supplemented with BAP or Kn (0.1–10 μM).

The response of cotyledonary node on the medium supplemented with various levels of cytokinins (BAP or Kn) resulted in multiple shoot formation within 3–4 weeks on the medium containing 1.0 μM BAP or 2.5 μM Kn (Fig. 2.2g). The maximum (66.7 %) proportion of explants were found to form healthy shoots (4.8 shoots per explant) on the MS medium containing 1.0 μM BAP, while the maximum number of shoots (5.5 per explant) was observed on the medium with 1.0 μM Kn. Subculturing was carried out at 4–6 weeks interval on the medium containing 1.0 μM BAP in order to obtain good and steady supply of shoots, devoid of any callus formation at the base of the shoots.

Shoot tip explants also proliferated and formed multiple shoots (within 4 weeks) on the MS medium irrespective of the concentration of BAP or Kn (up to 2.5 μM) used; higher concentrations (5.0 and 10.0 μM) of these cytokinins were, however, ineffective. All the explants (100 %) cultured on the medium supplemented with 1.0 μM BAP developed multiple shoots, while 53.3 % explants were found to form multiple shoots using the same concentrations of Kn. The maximum number of shoots (ca. 12 per explant) was obtained on the medium containing 1.0 μM BAP (Fig. 2.2h). The average length of shoots was also higher (4.0 cm) on this medium. The minimum shoot formation (average <1.0) was found when 0.1 μM Kn was used (Chandra et al. 2004).

Transfer of individual shoots (3.0–5.0 cm height) to PGR-free medium resulted in over 60 % rooting (without callus formation) after 11 days of inoculation. However, the rooting efficiency could be improved (up to 100 %) by addition of any of the three auxins, namely, NAA, IBA or IAA (0.1, 0.5, 2.5 μM) added to the MS medium. The mean number of roots formed per shoot was highest (8.3) when 0.5 μM NAA was used. The rooted plants were transferred to thermocole trays or cups containing a mixture of soil and sand (2:1, v/v) and placed for hardening in a greenhouse (25 °C; RH, 90 %) at Kosi-Katarmal (1150 m). The plantlets (Fig. 2.2i) were found to grow normally after 4 months, under greenhouse conditions; these were then transferred to high-altitude experimental site in village Khaljhuni (2450 m), hardened for a week in a polyhouse and field transplanted in plots for assessing growth and performance (Chandra et al. 2004).

In this study multiplication of *P. kurrooa* has been achieved using both cotyledonary nodes and shoot tips as explants taken from in vitro-raised seedlings. Cotyledonary node explants from in vitro-grown seedlings, like in the present study, have exhibited organogenic competence in several herbaceous species (Mallick and Rashid 1989; Jackson and Hobbs 1990). Cytokinins like BAP or Kn, at lower concentrations, proved to be extremely effective for induction of multiple shoots and subsequent shoot multiplication in both the types of explants used. In previous reports on in vitro propagation of *P. kurrooa*, these two cytokinins were also found to induce shoot multiplication in explants taken from a mature plant (Lal et al. 1988; Upadhyay et al. 1989). In the present study, shoot tips were found to be superior for obtaining multiple shoots; the maximum number of shoots was found on the medium containing BAP. Moreover, the higher concentration of BAP resulted in hyperhydric (vitrified) shoots during subsequent subcultures. Lowering the cytokinin concentration in the medium resulted in regenerating normal shoots from the

base of vitrified shoots. Vitrification of the shoot in cytokinin-supplemented medium has also been reported earlier in *P. kurrooa* (Upadhyay et al. 1989).

Rooting of microshoots has been reported in *P. kurrooa* by incorporation of various auxins, namely, NAA, IBA and IAA into the rooting medium (Lal et al. 1988; Upadhyay et al. 1989). While the report by Lal et al. (1988) did not indicate rooting percentage, 89 % rooting was achieved in a subsequent study using the MS medium supplemented with 1.0 μM NAA, with root initiation taking place after 20 days (Upadhyay et al. 1989). In the present investigation, auxins were found to induce cent per cent rooting in microshoots. Furthermore, the time required for root initiation was also reduced (8 days) when a lower concentration (0.1 μM) of NAA or IBA was used; this also resulted in minimizing or avoiding callus formation at the base of the shoots.

It is important to mention that following in vitro multiplication, microshoots of *P. kurrooa* could also be encapsulated; assessment of genetic fidelity of proliferating microshoots indicated true-to-type nature and compared well with the mother plant (Mishra et al. 2011a). This technology will be useful for storage and transport of microshoots for plantation at a later period when the climatic conditions become congenial at higher altitudes. In another study, Sood and Chauhan (2009) reported high-frequency callus induction and subsequent plant regeneration from various explants of *P. kurrooa*.

A rapid and efficient tissue culture protocol was developed (Bantawa et al. 2010) for an elite clone of another important Himalayan species of *Picrorhiza*, i.e. *P. scrophulariiflora* using explants taken from rhizome explants on woody plant medium supplemented with BAP and Kn. About 90 % of the regenerated microshoots could be rooted using NAA, without basal callus formation. Subsequently more than 1000 plants were hardened and field planted under natural conditions (Bantawa et al. 2010).

In addition to the above studies, it has been successfully demonstrated that hairy root lines of *P. kurrooa* produced elevated levels of active ingredients (Verma et al. 2007; Anonymous 2010; Mishra et al. 2011b). Such studies would offer possibilities for in vitro commercial production of important metabolites and assist in the conservation of important and/or endangered Himalayan medicinal plants.

2.3.4 *Podophyllum hexandrum*

Seeds of *P. hexandrum* Royle were collected from the Pindari region (3260 m; District Bageshwar, Uttarakhand) and stored at 4 °C (2 months). These were then washed with water, surface disinfected and allowed to imbibe in sterile distilled water (overnight) as described earlier (Nadeem et al. 2000). The embryos were carefully excised and transferred onto the MS medium containing 3.0 % (w/v) sucrose and 0.8 % (w/v) agar and supplemented with various concentrations of PGSs [BAP (0.5–5.0 μM), IAA (1.0–4.0 μM) or NAA (0.5–5.0 μM)] and activated charcoal (0.4–1.0 %, w/v). Excised embryos germinated within 7 days of inoculation on the basal medium or on the medium supplemented with BAP (0.5–4.0 μM);

a prominent cotyledonary tube with cotyledonary leaves and a distinct radicular portion were observed (Fig. 2.2j).

When the excised embryos were placed on the medium supplemented with 1.0–4.0 μM IAA and 1.0 μM BAP, multiple shoots were formed. The highest shoot multiplication rate (5.0 shoots/embryo) was observed on the medium containing both IAA and BAP (1.0 μM each, Fig. 2.2k). The base of the cotyledonary leaf in the embryos swelled to give rise to multiple shoots in about 4–5 weeks. These shoots were then separated and cultured individually for root induction. While the basal medium did not induce rooting, reducing its strength by half resulted in rooting of 16.6% shoots. However, rooting increased only slightly (25%) when the medium was supplemented with 0.5 μM IAA (Fig. 2.2l, Nadeem et al. 2000).

The well-rooted microshoots were transferred into the 250 ml flasks containing sterilized vermiculite, moistened with one-half MS salts, and allowed to harden for 15 days under aseptic conditions in the culture room. The plantlets were then transferred to polybags containing vermiculite and kept in a polyhouse (25–30 °C, 50% shading with green/black net) for 30–35 days; the polybags were covered with polythene from the top to maintain high humidity (>90% RH). Subsequently the plants were transferred to polybags containing soil and kept in the same polyhouse for another 3 months; during this period all the hardened plants showed new shoot emergence and behaved like normal field-grown plants (Nadeem et al. 2000).

The radicular, cotyledonary tube and cotyledonary leaves of germinated embryos were dissected and also cultured separately on the MS medium containing varying combinations and concentrations of NAA, BAP and GA_3. Callusing was induced from the basal end of embryos in most combinations. The calli so obtained remained greenish to creamish white up to the time of the first subculture; some calli, however, turned yellow and friable following the second subculture. Subsequently, somatic embryogenesis was recorded when the callus was subcultured on the medium supplemented with 5.0 μM NAA and 0.5 μM BAP. For maturation of somatic embryos, calli bearing different stages of embryos were transferred to various media combinations (MS, one-half MS, MS + charcoal and MS + 0.5 μM NAA). Irrespective of various combinations tried, nearly all somatic embryos were found to mature. Although somatic embryos of different sizes were obtained, the smaller ones turned green after a week on the MS medium supplemented with 0.5 μM NAA. The radicular portions elongated on the medium containing activated charcoal in 2–3 weeks, and these could be made into 'synthetic seeds' by encapsulation in sodium alginate beads. Subsequently, these could be germinated to form complete plants on a medium containing 1.0 μM BAP and 2.5 μM GA_3 (Nadeem et al. 2000). The plants were then hardened as mentioned above.

Tissue culture studies on *P. hexandrum* have been reported earlier (Arumugam and Bhojwani 1990); while multiple shoot formation from zygotic embryos was demonstrated, rooting of these shoots was not reported by these authors. The study by Nadeem et al. (2000) outlined above seems to be the first report of in vitro propagation of this species via multiple shoot formation and subsequent rooting. Addition of 0.5 μM IAA to the MS medium enhanced the rooting process. Somatic embryogenesis followed by subsequent germination is also beneficial for propagation,

because it eliminates the additional step of root induction that is required in propagation through multiple shoot formation (also see Nadeem et al. 2000). The TCR plants have been successfully grown and maintained in pots; these plants exhibited winter dormancy behaviour similar to that of field-raised plants and remained healthy. In a later study also using rhizome explants, in vitro plantlet regeneration in *P. hexandrum* was achieved via direct organogenesis (Chakraborty et al. 2010). These workers used the MS medium supplemented with NAA and BAP for shoot regeneration, and after multiple shoot formation in a medium containing IAA, the microshoots were rooted on one-half MS liquid medium containing IBA (Chakraborty et al. 2010). However, subsequent field transfer of these TCR plantlets was not reported. In spite of the efforts on the development of TC protocols for *P. hexandrum* (above-mentioned studies) and *P. peltatum* (Sadowska et al. 1997; Moraes-Cerdeira et al. 1998; Kim et al. 2007), large-scale propagation and cultivation trials of such plants have not been reported. Efforts must, therefore, be made to undertake large-scale multiplication of elite clones as well as conduct cultivation trials of these two species.

Production of podophyllotoxin by callus, root, suspension and hairy root cultures has also been demonstrated. Kadkade (1981, 1982) reported podophyllotoxin production (up to 0.65 %) from callus cultures of *P. peltatum*. Moreover, cell lines of the same species with the capacity to produce podophyllotoxin were also reported (Kutney et al. 1991). Subsequently embryogenic cell and adventitious root culture systems in *P. peltatum* were reported to produce podophyllotoxin, the later producing higher levels (Anbazhagan et al. 2008). Callus and suspension cultures derived from *P. hexandrum* were shown to produce podophyllotoxin (Van Uden et al. 1989, 1990; Woerdenberg et al. 1990; Chattopadhyay et al. 2001, 2002). Later Li et al. (2009) demonstrated an improved and effective method of podophyllotoxin production by root cultures of *P. hexandrum*. Hairy roots of *P. hexandrum* were also demonstrated to produce podophyllotoxin (Giri and Narsu 2000; Giri et al. 2001a); however, the levels reported were low. This alternate and continuous method has the potential for commercial production of high-value podophyllotoxin.

Another alternative approach for the production of podophyllotoxin through endophytic fungi is being vigorously perused nowadays. Ever since reports on taxol production by endophytic fungi isolated from yew species (Stierle et al. 1993; Strobel et al. 1996), there has been a growing interest in the biosynthetic capabilities of endophytes which are considered to be rich and diverse source of natural products. An endophyte isolated from *Nothapodytes foetida* was reported to produce camptothecin (Puri et al. 2005). Later the same group isolated an endophytic fungus *Trametes hirsuta* from *P. hexandrum*, as a novel alternative source of podophyllotoxin and related aryl tetralin lignans (Puri et al. 2006). Further, two strains (PPE5 and PPE7) of the fungus *Phialocephala fortinii* Wang and Wilox capable of podophyllotoxin production were isolated from *P. peltatum* (Eyberger et al. 2006). More recently, isolation of an endophytic fungus, *Fusarium solani*, from *P. hexandrum* collected from Kumaun region of Indian Himalaya, and podophyllotoxin production were reported (Nadeem et al. 2012). It is interesting to mention that isolation and identification of an endophytic strain of *Fusarium oxysporum* producing

podophyllotoxin were reported from *Juniperus recurva* (Kour et al. 2008). In view of the low amount of podophyllotoxin production by endophytes, their role needs further investigation. Moreover, optimization of conditions and isolation of high-yielding strains are essentially necessary to improve production.

2.4 Field Plantations and Performance

Field plantation and further assessment of plant performance (raised by tissue culture as well as conventional methods) were carried out at a high-altitude experimental site in village Khaljhuni (District Bageshwar, Uttarakhand; 2450 m; 30°6′12″N, 79°58′29″ E; Fig. 2.3a–c).

2.4.1 Soil and Climate Condition

Soil in the plantation site was found to be acidic (pH 5.7) with average soil moisture content of 23 % (April to June). Nitrogen, phosphorus and carbon contents were recorded as 0.39, 0.006 and 3.0 % (dry weight basis), respectively. The mean maximum temperature during winter months (October 2003 to March 2004) was 14.3 ± 2.8 °C and the minimum was 7.2 ± 1.2 °C, whereas during summer months (April 2003 to September 2003), these were 25.9 ± 0.4 °C and 16.0 ± 1.5 °C, respectively.

2.4.2 Field Preparation and Plantation

Field plantation to assess growth and biomass production was conducted in the cultivated land of a local farmer at village Khaljhuni (2450 m). Keeping in mind the land topography and small size of plots, separate plots were used for each species. The land was ploughed, levelled and raised and plain beds were prepared (Pandey 2002; Chandra 2002).

2.4.3 Plantation in the Field

2.4.3.1 *Aconitum balfourii*

Plants [TCR and seed raised (SR); grown in Kosi-Katarmal, Almora, transported and hardened at Khaljhuni] were planted in raised beds of 5 × 4 m (length x width). The spacing between rows as well as between plants was 15 cm. The growth of TCR plants was slow with 50 % survival in the first 3–4 months; subsequently the remaining plants also failed to survive. The growth and survival rate of seed-raised (SR) plants was 80 % till the end of the growing season (Fig. 2.3e, Pandey 2002).

In addition, tubers from field-grown plants were cut transversally into three segments of equal length, i.e. top, middle and basal, and planted in field plots of

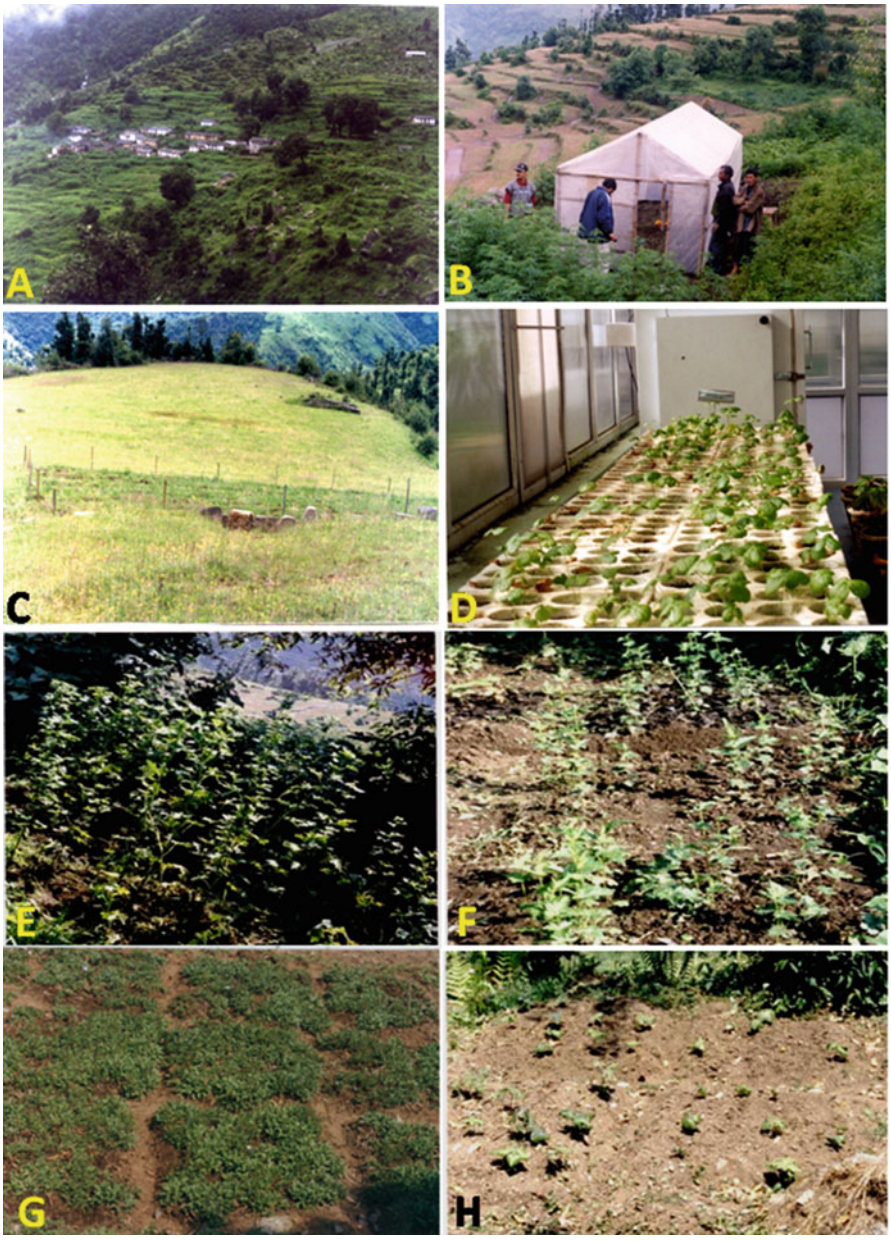

Fig. 2.3 Field plantation of selected medicinal herbs in a high-altitude field station located at Khaljhuni (2450 m). (**a**) A view of village Khaljhuni; (**b**) view of a polyhouse in the nearby area used for raising and hardening of plants; (**c**) fenced area of demonstration plots; (**d**) seedlings of *P. hexandrum* raised in the greenhouse at Kosi-Katarmal (1150 m); (**e**–**h**) demonstration plots of different herbs; (**e**) *Aconitum balfourii* (seed-raised plants); (**f**) *A. heterophyllum* (seed-raised plants); (**g**) *Picrorhiza kurrooa* (in vitro-raised plants) and (**h**) *Podophyllum hexandrum* (seed-raised plants)

15 × 7 m (length × width). The spacing between rows was 30 cm whereas between plants was 15 cm. The plants exhibited satisfactory growth during the season; however, tuber formation was hampered at the end of the season and the tubers could not be located during the next year (April, Pandey 2002).

2.4.3.2 *Aconitum heterophyllum*

Since TCR plants of *A. heterophyllum* were not available, SR plants (raised at Kosi-Katarmal and hardened at Khaljhuni as in *A. balfourii*) and tuber segments (taken from natural population) were planted in plots of 5 × 4 m (length × width). Tubers were cut into uniform-sized segments and planted with spacing as in the case of *A. balfourii* seedlings and tubers. Like *A. balfourii*, the plants exhibited 80 % survival during the first 3–4 months followed by satisfactory growth till the end of the growing season (Fig. 2.3f); however, tuber formation was hampered at the end of the season and the tubers could not be located during the next year (April, Pandey 2002).

2.4.3.3 *Picrorhiza kurrooa*

Plants (TCR and SR) of *P. kurrooa* (raised in Kosi-Katarmal and then hardened in Khaljhuni) were planted in plain and raised beds of 6 m length and 4 m width. The spacing between rows as well as between plants was 15 cm. Nine-month-old (4 months in the greenhouse and 5 months in the experimental field) SR and TCR plants were compared for growth in terms of plant height, number of leaves, runner biomass, etc. (Chandra 2002). It was observed that the average plant height was higher (7.1 cm) in TCR plants (Fig. 2.3g) in comparison to SR plants (5.5 cm), but the runner diameter was higher in SR plants (2.0 mm as compared to 1.7 mm in TCR plants). The total biomass (dry weight of runners) per plant was also higher for SR plants (3.5 g as compared to 2.4 g in TCR plants). The leaf parameters were also recorded, and thicker leaves were found in TCR plants. Higher leaf area and specific leaf mass were recorded in SR plants (3.7 cm^2 and 3.5 mg/cm^2, respectively, as compared to 2.2 cm^2 and 2.9 mg/cm^2 for TCR plants). Among other parameters studied, chlorophyll 'a' content was found to be higher for seedlings (0.82 mg/g FW as compared to 0.69 mg/g FW in TCR plants), while chlorophyll 'b' content was slightly higher in TCR plants. Per cent relative water content (RWC) of leaves was essentially similar for both sets of plants (Chandra et al. 2004).

In addition, runner segments obtained vegetatively from the field-grown plants (containing three nodes, 5.0–6.0 cm long) were planted in plain and raised beds of 30 m length and 16 m width. Similarly, seedlings that were raised in trays or in beds under polyhouse conditions were transplanted to field beds containing farm yard manure during the month of March in plain and raised beds. The spacing during planting was kept as mentioned above. Runner cuttings were more successful for multiplication as well as for higher biomass production within a short period than cultivation through seeds. Both types of plants showed satisfactory growth and multiplication in the field. In the following year (April), new shoots developed from these runners and plants were found to grow well during the second year (Chandra 2002).

2.4.3.4 *Podophyllum hexandrum*

Plants (TCR and SR) grown in Kosi-Katarmal (Fig. 2.3d) and then hardened at high altitude (Khaljhuni) were planted in plots of 7×4 m (length × width). The spacing between rows as well as between plants was 15 cm. It was observed that the growth of TCR plants was slow with only 30% survival up to the first 3 months; subsequently there was no survival. The survival rate of SR plants (Fig. 2.3h) was also very low (20%) till the end of the growing season, and rhizome formation was not observed in the next growing season (Pandey 2002).

Rhizome segments of (4.0–5.0 cm long transverse segments containing axillary buds in each segment) were planted in nursery beds of 5 m length and 8 m width. The spacing between rows was 20 cm, whereas the spacing between plants was 15 cm. One-year-old seed-raised plants were also planted in plots of 4 m length and 2 m width with similar spacing as for the rhizome segments. The growth and survival of these segments/plants was unsatisfactory and rhizome formation was not observed during the next growing season (Pandey 2002).

2.4.4 Weeding, Irrigation and Fungicide Treatment

Manual weeding was carried out at regular intervals, following weed growth. Irrigation, particularly in peak summer months (May), was done as and when required according to the species and the status of soil moisture content (Chandra 2002). In general, no infection occurred, but in plots of *P. kurrooa*, infection with powdery mildew was observed at the time of pre-senescence (July–August). These plots were treated with a systemic fungicide, Bavistin (1%, w/v), by manual spraying at 48 h interval (thrice).

2.4.5 Harvesting

After completion of the reproductive phase, *P. kurrooa* plants were found to be mature for harvesting (during September). The TCR as well as the SR plants did not flower in the first season. Thinning of runners was done in the field itself; the roots along with runners were dug and excised with a sharp knife. The excised runners were planted in separate plots to get another crop (Chandra 2002).

Efforts were made to grow TCR plants along with SR plants of the target species on the farmer's field located at a higher altitude and assess their growth and performance. This is possibly the first report wherein attempt has been made to assess the field performance of TCR plants. Although TCR plants were subjected to sufficient hardening in the greenhouse and then further acclimatized at the high-altitude field station prior to plantation, among the four species tried, performance of only *P. kurrooa* was satisfactory; survival of the other three species was poor. It must be mentioned that during hardening under the greenhouse, TCR plants of *P. kurrooa* were inoculated with some bacterial isolates (e.g. *Bacillus subtilis*) having antagonistic properties against pathogenic fungi (Chandra et al. 2004). This step is important as

maximum mortality is generally caused by fungal infection during early weeks of transfer of rooted plants to the soil. The survival rate of plants (both TCR as well as SR) in the field can perhaps be improved if the time taken during transport of plants from the greenhouse to the field planting is reduced. Since the field is located in a distant and difficult-to-reach place in the subalpine Himalayan zone, a major climatic shift, in addition to transport shock, may have affected the final survival of plants in the field.

It needs to be mentioned that due to shortage of TCR plants, further field trials could not be carried out in the next season (second year). While in vitro (tissue culture) technology has potential for large-scale production for subsequent commercial cultivation, and based on the success recorded with *P. kurrooa*, concerted efforts are required for the remaining three species. The four target species are listed under 'vulnerable', 'endangered' or 'critically endangered' category and their export is regulated. All these four species are currently in the 'List of medicinal plants prohibited' of the Ministry of Commerce, Government of India (Lakhanpal 1998). In spite of this, only limited attempts have been made to develop agrotechnologies to promote their cultivation. Agrotechnologies for some medicinal herbs, including *A. heterophyllum*, *A. balfourii* and *P. kurrooa*, have been reported using seed, tubers and runners as propagules (Purohit 1997; Maikhuri et al. 1998). Thus, tissue culture propagation protocols reported in this communication, along with the conventional methods, would be useful in initiating programmes on systematic cultivation with proper coordination and linkages between the government, state forest departments, State Biodiversity Boards, state medicinal plant boards, researchers and farmers; the efforts should be directed towards ensuring long-term benefits to the local community, as well as providing protection to wild populations of such important plants.

2.5 Conclusions

Medicinal and aromatic plants form an integral and essential part of the lives of hill communities, and the inhabitants depend on these plants for use in healthcare. These plants are well-known source of active principles in Ayurvedic, Unani and other traditional systems of medicines. At present these plants are major sources of many high-value drugs. Concomitant with the ever-increasing global demand for the 'naturals', these species are being subjected to reckless, often illegal harvesting, well beyond their natural regeneration capacity. The medicinal/pharmaceutical properties are mainly attributed to secondary metabolites. Various investigations have indicated a wide variation in active ingredient content in plant parts among the populations of the same species, and hence exact knowledge of its content is extremely important. Our findings clearly support this for all the species described above. Besides the known source of ingredients, i.e. underground parts, other plant parts (i.e. stem and leaf) and alternative means (callus, cell and suspension culture and hairy roots) of obtaining these compounds must be exploited. The above investigations suggest that besides rhizomes/roots, leaves of *P. hexandrum* can be harvested and thus the plants can be a renewable source of podophyllotoxin. It is

well known that in vitro (tissue culture) techniques are an alternate and effective means of propagation. These are being used not only in forestry, horticulture, land rehabilitation and other afforestation programmes but can facilitate the recovery of rare and endangered plant species (Bajaj 1989; Palni et al. 1998; Nadeem et al. 2000; Nandi et al. 2002; Pandey et al. 2002; Chandra et al. 2006; Purohit et al. 2015). However, it must be emphasized that tissue culture-raised plants need to be carefully hardened and acclimatized. The overall success would depend upon efficient field transfer, following proper hardening (using both physical and biological means), continued monitoring of growth, regeneration ability of rhizomes and periodic estimation of active ingredients. Since in vitro techniques are known to induce variability, the plants so raised should be screened for useful somaclonal variants, which may be exploited for obtaining plants or cultures with high secondary metabolite content. Moreover, this technique is used for rapid supply of large number of desired quality planting material (clone) which is phenotypically uniform and genetically akin to the mother plants. Thus determination of genetic fidelity of TCR plants is extremely important for validating true-to-type or variant nature of clones. Molecular markers have been routinely applied to detect genetic integrity among TCR plants and have been successfully carried out in tea (Devarumath et al. 2002; Mondal and Chand 2002; Bag et al. 2008), bamboo (Das and Pal 2005; Agnihotri et al. 2009), medicinal plants of IHR (Mishra et al. 2011a; Giri et al. 2011; Purohit et al. 2015) and several other species.

As most alpine medicinal plants require several years to reach optimal harvestable size, in vitro techniques can be effectively applied for rapid and clonal propagation and thus pave the way for conservation along with economic exploitation. Data on morphological and some physiological parameters of field-planted TCR *P. kurrooa* plants showed comparable results like those of seedlings/mother plants (this study). Similar results have also been reported for a temperate bamboo, *Thamnocalamus spathiflorus* (Bag et al. 2000) and 'maggar' bamboo, *Dendrocalamus hamiltonii* (Agnihotri et al. 2008, 2009; Bag et al. 2012). Moreover, reports of occurrence of podophyllotoxin in leaves and stems of *P. hexandrum* from various populations of Indian Central Himalaya in our laboratory (Pandey 2002; Pandey et al. 2013) are significant as leaves can be a renewable source of podophyllotoxin and can be exploited instead of harvesting the entire plant. The outcome of the above-mentioned studies is likely to result in the development of technology packages for mass scale propagation and cultivation of these species following proper field trials. This will not only help in restoration of degraded lands but also result in deriving economic benefits by the local communities.

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3 In Vitro Approaches for Conservation and Sustainable Utilization of *Podophyllum hexandrum* and *Picrorhiza kurroa*: Endangered Medicinal Herbs of Western Himalaya

Nisha Dhiman*, Vanita Patial*, and Amita Bhattacharya

Abstract

The western Himalaya is a rich repository of unique plants that are valued for their medicinal properties. Many of these plants are extensively utilized in pharmaceutical industries, and there is a huge global demand for them. Since most of these plants have become either rare, threatened, or endangered, there is an urgent need to conserve them. *Podophyllum hexandrum* and *Picrorhiza kurroa* are two endangered medicinal herbs that are being ruthlessly uprooted for their active principles, i.e., podophyllotoxins and picrosides, respectively. Hence, different plant tissue culture approaches have been employed for their conservation and sustainable utilization. The successes achieved till date in these approaches have been reviewed in the present article.

Keywords

Cell suspension • Genetic transformation • Micropropagation • *Podophyllum hexandrum* • *Picrorhiza kurroa* • Secondary metabolite production

* Both the authors are contributed equally.

N. Dhiman • V. Patial
Academy of Scientific and Innovative Research, New Delhi, India

Division of Biotechnology, CSIR-Institute of Himalayan Bioresource Technology, Palampur 176061, Himachal Pradesh, India

A. Bhattacharya (✉)
Division of Biotechnology, CSIR-Institute of Himalayan Bioresource Technology, Palampur 176061, Himachal Pradesh, India
e-mail: amitabhatta@ihbt.res.in

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3.1 Introduction

The entire stretch of mountainous terrains extending from southwest Afghanistan, northern Pakistan, northwestern India, and western parts of Nepal constitutes the "Western Himalayas." In India, the region spans through the states of Jammu and Kashmir, Himachal Pradesh, and Uttarakhand. The region is characterized by remarkable altitudinal gradients and distinct climatic zones that range from tropical at the base of the mountains to permanently snow clad alpine regions beyond the tree line. The life forms that inhabit each of these altitudinal gradients differ markedly and contribute toward the amazing natural wealth of the western Himalayan biodiversity. The region being comparatively drier than the eastern parts of Himalayas supports various flora and fauna that are distinctively different from their eastern counterparts. These serve as rich repositories of medicinal bioresources. Many of these medicinally important plants are specifically confined to unique eco-geographical niches and suffer from the risks of habitat degradation and climate change. As a result, several valuable plant species have been depleted from nature. With increasing popularity of herbal medicines in developed countries (British Medical Association 1993; Najar and Agnihotri 2012), the plants are also suffering from tremendous overexploitation. Thus, many plants of western Himalaya are becoming rare, threatened, endangered or critically endangered and figure prominently in the IUCN Red List (Verma et al. 2012). *Podophyllum hexandrum* Royle (Indian mayapple or Bankakdi) of family Berberidaceae (Fujii 1991; Giri and Narasu 2000; Kushwaha et al. 2007) and *Picrorhiza kurroa* Royle ex Benth. (Kutki) of family Scrophulariaceae are two such endangered medicinal herbs of western Himalaya (Nayar and Sastry 1990; Kushwaha et al. 2007; CITES 2007).

Of the two plants, *P. hexandrum* is acclaimed for podophyllotoxins or aryltetralin lignans present in the roots and rhizomes of the plant (Jackson and Dewick 1984; Liu et al. 2015). The compounds are known for their anticancer, antifungal, and immunomodulatory properties (Kamil and Dewick 1986; Broomhead and Dewick 1990; Canel et al. 2000; Lerndal and Svensson 2000; Ganie et al. 2010; Kushwaha et al. 2010). Podophyllotoxins and their derivatives are extensively used as precursors for the synthesis of commercial anticancer drugs such as VP-16-213 (etoposide Vumon®), VM-26 (teniposide) and etopophos®, Pod-Ben-25, Condofil, Verrusol, Warticon, GL331, Top 53, NK 611, and CPH 82 (Uden et al. 1989; Huang et al. 1996; Pagani et al. 1996; Gordaliza et al. 2004; Liu et al. 2004; Kharkwal et al. 2008; Nagar et al. 2011; Bhattacharyya et al. 2012; Kumar et al. 2015). These are approved drugs for treating lung and testicular cancers, leukemia, psoriasis, and rheumatoid arthritis (Staheliin and Von Warburg 1991; Liu et al. 2004; Chen et al. 2007). Podophyllotoxins are also used for the treatment of venereal warts (Yanofsky et al. 2012). These usages have led to ruthless uprooting and overharvesting of the underground parts of the plant to the extent that it has become endangered (Gupta and Sethi 1983; Nayar and Sastry 1990; Airi et al. 1997). Hence, the plant now figures prominently in the IUCN Red List and demands immediate and urgent conservation.

On the other hand, *P. kurroa* is known for its hepatoprotective (Chander et al. 1990; Sinha et al. 2011), anticancer, antidiabetic, cardioprotective, anticholestatic, antiulcerogenic, antiasthmatic, anti-inflammatory, and antidepressant properties (Singh et al. 1993; Joy et al. 2000; Ram 2001; Chauhan et al. 2008; Husain et al. 2009; Patani et al. 2012; Navade et al. 2013). These properties are primarily due to "kutkin," the main active constituent that accumulates predominantly in the roots and rhizomes of the plant. Kutkin is a mixture of two major C-9-iridoid glycosides, picroside I (6-O-trans cinnamoyl catalpol) and picroside II (6-vanilloyl catalpol) (Jia et al. 1999; Mondal et al. 2013). The detailed chemical structures of both picroside I and picroside II were elucidated by Kitagawa et al. (1971) and Weinges et al. (1972). *P. kurroa* is highly sought by Indian herbal industries wherein 5000 tonnes of the plant constitute the annual demand. This is in sharp contrast to the annual supply of *P. kurroa*, which is less than 100 tonnes (Kumar 2006). Thus, the plant is suffering from ruthless and illegal overharvesting from its natural habitats. Natural populations of the plant is also rapidly dwindling due to habitat specificity, restricted distribution, overgrazing, landslides, poor natural regeneration, and anthropogenic activities (Chandra et al. 2006; Bantawa et al. 2009). Presently, the plant is categorized as "endangered" in the Appendix II of the "Convention on International Trade in Endangered Species of Wild Fauna and Flora" (CITES 2000) and the "International Union for Conservation of Nature and Natural Resources" (Leaman 2007). Hence, there is an urgent need to conserve the plant.

While there are various methods of plant conservation, tissue culture is popularly used in case of valuable but rare, endangered, and threatened (RET) plants. The method has been extensively used as an alternative route for rapid clonal multiplication of valuable germplasm and their reintroduction in nature (Basavaraju 2005; Narula et al. 2007; Srivastava et al. 2010; Pant 2013). Tissue culture has also been used to produce secondary metabolites in root, callus, and cell suspension cultures. However, these approaches require extensive optimization, and the success rates are often limited. In this regard, the efforts made towards the conservation and sustainable utilization of *Podophyllum hexandrum* and *Picrorhiza kurroa* are reviewed in the present article.

3.2 *Podophyllum hexandrum*

The major focus of *P. hexandrum* tissue culture has been the development of in vitro systems for podophyllotoxins production. However, mass propagation of the plant through shoot multiplication, somatic embryogenesis, and caulogenesis has also been attempted by a few workers (Table 3.1).

3.2.1 Micropropagation

Till date, only seedlings generated from zygotic embryos were used for shoot multiplication (Nadeem et al. 2000). Germination of excised embryos on Murashige

Table 3.1 Efforts made for in vitro regeneration and podophyllotoxin production in *Podophyllum hexandrum*

Sr. no.	Explant	Medium	Response	References
1	Roots	B5 + 2% coconut water, 4% sucrose, and 4 mg l^{-1} NAA	Callus and cell suspension	Uden et al. (1989)
2	Rhizome (callus derived from rhizome)	B5 + 2% coconut water, 4% sucrose, 4 mg l^{-1} NAA and 2.5 mM each of phenylalanine, coniferin, tyrosine, cinnamic acid, caffeic acid, coumaric acid, and ferulic acid independently	Podophyllotoxins increased by 12.8-fold in cell suspension developed on coniferin supplemented medium	Uden et al. (1990)
3	Roots	B5 + 4% sucrose, 4 mg l^{-1} NAA and 3 mM of β-cyclodextrin-complexed coniferyl alcohol or noncomplexed coniferyl alcohol/coniferin	Higher accumulation of podophyllotoxins in cell suspension fed with 3 mM of β-cyclodextrin-complexed coniferyl alcohol and water-soluble coniferin	Woerdenbag et al. (1990)
4	Roots	B5 + 0.1–1.2 mg l^{-1} 2,4-D, 0.2–2.0 mg l^{-1} GA_3 and 0.05–1.0 mg l^{-1} BA	Callus culture	Heyenga et al. (1990)
			Podophyllotoxin, 4′-demethylpodophyllotoxin and podophyllotoxin-4-O-glucoside at levels similar to the original explants	
5	Zygotic embryos	(i) MS + 2.0 μM BA and 0.5 μM IAA	(i) Callusing and somatic embryogenesis	Arumugam and Bhojwani (1990)
		(ii) MS medium + 6% sucrose or 2.5 μM NAA	(ii) Further development and maturation	
		(iii) MS basal	(iii) Somatic embryo germination	
6	Zygotic embryos	(i) MS + either 0.5 μM or 10 μM 2,4-D and 1 μM BA	(i) Callus maintenance	Arumugam and Bhojwani (1994)
		(ii) MS + 2.5 μM NAA	(ii) Cotyledonary embryo development	
		(iii) MS basal	(iii) Embryo germination	

7	Cell suspension	Culture medium + cyclodextrin/cyclodextrin-complexed deoxypodophyllotoxin/deoxypodophyllotoxin	Maximum podophyllotoxin and β-D-glucoside of podophyllotoxin accumulation (2.87 % on a dry weight basis) in deoxypodophyllotoxin-fed cultures after 9 days	Uden et al. (1995)
8	Zygotic embryos	(i) MS basal	(i) Germination within 1 week	Nadeem et al. (2000)
		(ii) MS medium + 1 μM IAA and 1 μM BA	(ii) Multiple shoots in 4–5 weeks	
		(iii) MS medium + 2–4 μM IAA and 1 μM BA	(iii) Rooting of micro-shoots	
		(iv) MS 5mM NAA and 0.5 mM BA	(iv) Somatic embryos from callus after 16 weeks of culture	
9	Roots	(i) ½ MS + 1 mg l^{-1} 2,4-D, 0.1 mg l^{-1} BAP, and 1 mg l^{-1} GA_3	(i) Callusing	Giri et al. (2001)
		(ii) MS + 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} BAP	(ii) Suspension culture	
		Agrobacterium rhizogenes strains A4, K599, and 15834		
10	Roots	(i) MS + 2 mg l^{-1} IAA and 5 g l^{-1} activated charcoal	(i) Friable callus culture	Chattopadhyay et al. (2001)
		(ii) MS + 2 mg l^{-1} IAA, 10 g l^{-1} PVP and 1.5 mg l^{-1} pectinase	(ii) Suspension culture	
11	Roots	(i) MS + 2 mg l^{-1} IAA and 5 g l^{-1} activated charcoal	(i) Friable callus culture	Chattopadhyay et al. (2002a)
		(ii) MS + 2 mg l^{-1} IAA, 10 g l^{-1} PVP, and 0.005 mg l^{-1} pectinase	(ii) Suspension culture	
12	Roots	(i) MS + 11.4 μM IAA, 10 g l^{-1} PVP, 1.5 mg l^{-1} pectinase, and 30 g l^{-1} glucose	(i) Suspension culture initiation	Chattopadhyay et al. (2002b)
		(ii) MS + 5 g l^{-1} PVP and 30 g l^{-1} glucose	(ii) Suspension culture maintenance	
13	Roots	(i) MS +2 mg l^{-1} IAA and 5 g l^{-1} activated charcoal	(i) Callus culture	Chattopadhyay et al. (2003a)
		(ii) MS + 2 mg l^{-1} IAA, 10 g l^{-1} PVP, and 1.5 mg l^{-1} pectinase	(ii) Suspension culture	

(continued)

Table 3.1 (continued)

Sr. no.	Explant	Medium	Response	References
14	Roots	Medium same as Chattopadhyay et al. (2001)	Podophyllotoxin accumulation in batch mode was 21.4 g l^{-1} and 13.8 mg l^{-1} after 24 and 26 days	Chattopadhyay et al. (2003b)
		Culturing in a 3 L stirred-tank bioreactor under low-shear condition in batch and fed batch modes of operation		
15	Cell cultures	Cytotoxicity of podophyllotoxins produced in cell culture tested using human breast cancer cell line (MCF-7)	50 % inhibition by 1 nM podophyllotoxin, provided it was applied during the beginning of cell growth	Chattopadhyay et al. (2003c)
16	Cell cultures	Bioreacter scale culture in cell retention cultivation mode	Podophyllotoxin accumulation in batch mode was 21.4 g l^{-1} and 13.8 mg l^{-1} after 24 and 26 days, respectively	Chattopadhyay et al. (2004)
17	Excised embryo culture	B5 + GA_3	Germination and en masse plant production	Kharkwal et al. (2004)
18	Zygotic embryos	LS (in dual shake flasks and dual bioreactors)	Suspension culture	Lin et al. (2003a, b)
19	Zygotic embryos	B5 and MS	Root cultures	Sagar and Zafar (2005)
20	Root segments	(i) Half strength B5 + 0.5–1.5 mg l^{-1} 2, 4-D, and 0.2–1.0 mg l^{-1} BA	(i) Callus initiation	Sultan et al. (2006)
		(ii) MS + 0.5 mg l^{-1} BA and 1 mg l^{-1} IAA	(ii) Shoot proliferation	
		(iii) MS + 0.5–1.0 % activated charcoal and 0.5–2.0 mg l^{-1} NAA	(iii) High rooting efficiency	
		(iv) Jiffy pots containing sand, soil, and vermiculite in 1:1:1 ratio	(iv) Hardening under polyhouse conditions	
21	Zygotic embryos	(i) Solid nutrient agar slab	(i) Embryo germination	Ahmad et al. (2007)
		(ii) MS + 0.5–2 μM NAA, 1–2.5 μM BAP, and 2.5 μM GA_3	(ii) Callusing	

22	Excised embryos	MS + 1 mg l^{-1} IAA, 0.5 mg l^{-1} BA, and 1 % activated charcoal	Maximum plantlet growth	Sultan et al. (2009)
23	Juvenile and mature explants from in vitro-grown seedlings	B5 and MS + different concentrations of 2,4-D, GA_3, and 6-BA	Callus and cell suspension	Majumder and Jha (2007; 2009)
24	Zygotic embryo and rhizomes	MS + different concentrations of IBA, GA_3, hydroquinone, and activated charcoal	Root culture	Li et al. (2009)
25	Rhizome explants	(i) MS + 11.42 μM IAA	(i) Multiple shoot formation	Chakraborty et al. (2010)
		(ii) MS + 2.68 μM NAA and 11.1 μM BAP	(ii) Multiple shoot formation	
		(iii) Half strength liquid MS + 100 μM IBA	(iii) Root formation	
26	Shoots derived from zygotic embryo	WPM + 1.5 mg l^{-1} IAA and 0.5 mg l^{-1} NAA	In vitro rooting	Guo et al. (2012)
27	Callus developed by Majumder and Jha (2009)	Half strength liquid B5 + 1 % (w/v) sucrose, 0.1 mg l^{-1} BAP, 1 mg l^{-1} each of 2,4-D, and GA_3	Cell suspension	Majumder (2012)
28	Mature leaves	(i) MS + 2.68 μM NAA and 8.88 μM BAP	(i) Callus culture	Bhattacharyya et al. (2012)
		(ii) MS + 60 mM total nitrogen, 1.25 mM potassium dihydrogen phosphate, 6 % glucose, and 11.41 μM IAA	(i) Suspension culture	
29	Excised zygotic embryos	MS + 1.5 mg l^{-1} 2,4-D	Callus establishment and transformation	Rajesh et al. (2013)
		A. tumefaciens strains LBA4404, EHA101, and EHA105		
30	Zygotic embryos	(i) Three fourth strength MS + 3 g l^{-1} PVP and 4 % sucrose	(i) Callusing and somatic embryogenesis	Rajesh et al. (2014a)
		(ii) Three fourth strength MS + 1 mg/L ABA, 3 g l^{-1} PVP, and 4 % sucrose	(ii) Maturation of somatic embryos	

(continued)

Table 3.1 (continued)

Sr. no.	Explant	Medium	Response	References
31	Zygotic embryos	(i) MS + 0.6 mg l^{-1} GA_3	(i) Germination of zygotic embryos	Rajesh et al. (2014b)
		(ii) MS + 5 mg l^{-1} GA_3	(ii) Direct somatic embryogenesis	
		(iii) MS + 1.5 mg l^{-1} 2,4-D	(iii) Callusing	
		(iv) MS + 1.5 mg l^{-1} each of 2,4-D, NAA, 0.5–2.5 mg l^{-1} picloram, or 0.5–2.5 mg l^{-1} ABA	(iv) Indirect somatic embryogenesis	
		(v) MS + 5 mg l^{-1} GA_3	(v) Germination of somatic embryos	
32	Shoot, leaf and root segments	(i) Half strength MS + 3 mg l^{-1} IBA and 2% sucrose	(i) Adventitious root development by root explant only	Rajesh et al. (2014c)
		(ii) Half strength MS basal + 6% sucrose	(ii) Maximum biomass and podophyllotoxin accumulation	

and Skoog (MS) medium, supplemented with 1 μM each of IAA (Indole-3- acetic acid) and BA (6-Benzyl adenine), and the use of seedlings for multiple shoot formation was reported by Nadeem et al. (2000). Although different concentrations of IAA were combined with 1 μM BA for rooting of the micro-shoots, only MS medium supplemented with 0.5 uM IAA evoked the best response. The rooted plantlets were finally transferred to pots containing vermiculite and hardened successfully. Another group reported the employment of "excised embryo culture" for extraction and germination of zygotic embryos on B5 medium (Gamborg et al. 1968) supplemented with GA_3 (gibberellic acid). The workers achieved 89.14 % germination from both mature as well as immature zygotic embryos but these were not utilized for in vitro shoot multiplication. Rather, seedling-raised plants were generated en masse and transferred to soil under green house conditions (Kharkwal et al. 2004).

On the other hand, somatic embryogenesis has been the focus of different groups working on *P. hexandrum* tissue culture. In this regard, Arumugam and Bhojwani (1990) were the first to report indirect somatic embryogenesis from excised zygotic embryos. The workers achieved both callusing followed by somatic embryogenesis on half strength MS medium supplemented with 2 μM BA and 0.5 μM IAA. The differentiated globular somatic embryos were multiplied on the same medium but these failed to mature further. Therefore, 6 % sucrose or 2.5 μM NAA (naphthalene acetic acid) was used in MS medium to support the normal development and maturation of zygotic embryos. However, basal MS medium was required for somatic embryo germination. Optimal somatic embryogenesis at 25 °C but their suppression under light and higher temperatures was also reported. In a separate study, the researchers maintained the embryogenic calli for 3 years on basal MS medium containing 10 μM 2,4-D (2,4-dichlorophenoxy acetic acid) and 1 μM BA or 0.5 μM 2,4-D (Arumugam and Bhojwani 1994). However, the calli had to be subcultured on MS medium containing 2.5 μM NAA for further development into somatic embryos and their germination on basal MS medium. Another group used radicals and cotyledonary leaves of germinated somatic embryos for callus initiation on basal MS medium supplemented with different concentrations of NAA, BA, and GA_3. Somatic embryogenesis was initiated only when the calli were transferred to MS medium containing 5.0 μM each of NAA and BA (Nadeem et al. 2000). After a long gap of 14 years, Rajesh et al. (2014a) achieved both direct and indirect somatic embryogenesis, wherein the indirect or callus-mediated pathway led to induction of high frequency of somatic embryos on basal MS medium. However, supplementation of 5.0 mg l^{-1} GA_3 was required for 79 % normal somatic embryo germination. When direct somatic embryos were further cultured on MS medium supplemented with 1.5 mg l^{-1} 2, 4-D in dark, callus development occurred and 1.8 mg g^{-1} dry weight podophyllotoxins were produced per 1.2 g of callus. The amount recorded was higher than that of field grown plants. In a parallel study, Rajesh et al. (2014b) developed an efficient method of indirect somatic embryogenesis and plantlet regeneration from zygotic embryos cultured on three fourth strength MS medium supplemented with 3.0 g l^{-1} polyvinylpyrrolidone (PVP) and 4 % sucrose in dark. The somatic embryos matured in the presence of 1.0 mg l^{-1} ABA (abscisic acid), 3.0

g l^{-1} PVP, and 4 % sucrose but highest germination (91.1 %) occurred when 1.0 mg l^{-1} GA_3 was present. Accumulation of podophyllotoxins (2.8 mg l^{-1}) in the somatic embryos was highest when sucrose was increased to 8 %. The plantlets were also hardened in growth chamber.

Besides somatic embryogenesis, caulogenesis was attempted by a few workers. In this regard, Sultan et al. (2006) employed root segments of in vitro-grown seedlings for initiation and proliferation of callus on B5 medium. Supplementation of MS medium with 0.5–5.0 mg l^{-1} BAP and 0.5–3.0 mg l^{-1} IAA resulted in indirect shoot regeneration. The shoots developed further on MS medium containing 0.5 mg l^{-1} BAP (6-benzylaminopurine) and 1.0 mg l^{-1} IAA, but high percentage of rooting was achieved only when 0.5 mg l^{-1} NAA and 1.0 mg l^{-1} IAA were used along with activated charcoal. The rooted plantlets showed healthy growth and survival upon transfer to jiffy pots containing sand, soil, and vermiculite in 1:1:1 ratio under poly house conditions. However, the exact survival percentage of plants was not mentioned. Later, the same group cultured germinated excised embryos on MS medium supplemented with 0.5–3.0 mg l^{-1} IAA, 0.5–2.0 mg l^{-1} NAA, 1.0–2.0 mg l^{-1} 2,4-D, 0.5–4.0 mg l^{-1} BAP, and 0.5–1.0 mg l^{-1} kinetin (Kn). The workers achieved maximum plantlet growth on MS medium supplemented with 1.0 mg l^{-1} IAA, 0.5 mg l^{-1} BA, and 1 % activated charcoal (Sultan et al. 2009). In the following year, Chakraborty et al. (2010) claimed the development of an efficient protocol for direct regeneration of *P. hexandrum* plants from rhizome explants. They reported the use of different PGR combinations, but the highest rate of multiple shoot formation was recorded on MS medium supplemented with 11.42 μM IAA and activated charcoal within 3 months from culture initiation. The next best combination was reported to be 2.68 μM NAA and 11.1 μM BAP. Rooting of in vitro shoots was reported only on half strength liquid MS medium containing 100 μM IBA (indole-3-butyric acid). However, when leaf explants were used, only callus formation but no shoot regeneration was reported. Guo et al. (2012) was the only group who used WPM or woody plant medium (Lloyd and McCown 1980) for shoot cultures followed by their rooting on medium containing 1.5 mg l^{-1} IAA and 0.5 mg l^{-1} NAA. When these shoots were transferred to jiffy pots containing turfy soil and perlite (2:1), 98.1 % survival was recorded. The plants were also reinstated in nature under open shady places at an altitude of 1700 m.

3.2.2 Root Cultures of *Podophyllum hexandrum*

Large-scale cultivation of adventitious roots offers a great opportunity for in vitro podophyllotoxins production. Roots derived from 2 months old in vitro raised seedlings were cultured on both B5 and MS media but higher growth with enhanced production of podophyllotoxins was achieved only on the former (Sagar and Zafar 2005). Similarly, Li et al. (2009) used mature embryos and rhizomes to establish root cultures on MS medium supplemented with different concentrations of IBA, GA_3, hydroquinone, and activated charcoal. Of these, only hydroquinone promoted increased length of rhizomes after 40 days, whereas activated charcoal hastened

optimal root biomass and podophyllotoxins accumulation. Another group, Rajesh et al. (2014c) developed adventitious roots from root segments and explored the possibilities of producing podophyllotoxins in them. The workers tested the effects of different parameters such as carbon sources, media strength, pH of initial medium, and the ratios of ammonium, nitrate, and phosphate in the culture medium. Finally, half strength MS medium containing 3.0 mg l^{-1} IBA with initial pH of 6.0 was optimized. The study also revealed that 2% sucrose was most effective in maximizing the biomass, whereas 6% sucrose was required for maximum podophyllotoxins accumulation. In addition, ammonium at 10 mM, nitrate at 20 mM, and phosphate at 2.25 mM were optimized. The optimized conditions led to a maximum podophyllotoxin accumulation up to 6.4 mg g^{-1}. However, 1.25 mM phosphate supported the highest growth.

3.2.3 Callus and Cell Cultures of *Podophyllum hexandrum* for the Production of Podophyllotoxins

Several studies have shown that secondary metabolites produced in tissue cultures are often higher than that in the parent plants. Hence, the technique became an attractive route for commercial-scale production of plant metabolites. Of the different techniques used in in vitro secondary metabolite production, suspension cultures being fast growing is considered to be the most effective. Moreover, it can also be manipulated easily.

In this regard, Uden et al. (1989) was the first person to report the production of podophyllotoxins in the cell cultures derived from root explants of in vitro raised plantlets. Suspension cultures were raised from callus developed on B5 medium supplemented with 2% coconut water, 4% sucrose, and 4.0 mg l^{-1} NAA. When these cultures were maintained under dark or light conditions at 26 °C, higher amounts of podophyllotoxins accumulation up to 0.3% on dry weight basis were recorded only in dark. In the following year, Uden et al. (1990) fed cell suspension cultures obtained from rhizomes with seven types of precursors such as phenylalanine, coniferin, tyrosine, cinnamic acid, caffeic acid, coumaric acid, and ferulic acid based on the phenylpropanoid pathway for enhanced production of podophyllotoxins. They also used a related compound, i.e., methylenedioxy cinnamic acid at 2.5 mM. Of these, only coniferin affected a 12.8-fold increase in podophyllotoxins. In a parallel work, Woerdenbag et al. (1990) used the same system for producing podophyllotoxins in cell suspension cultures obtained from root calli. However, when the cultures were fed with 3 mM of β-cyclodextrin-complexed coniferyl alcohol, higher accumulation of podophyllotoxins was recorded as in case of water-soluble coniferin (β-D-glucoside of coniferyl alcohol) fed cultures as compared to the poorly water-soluble noncomplexed coniferyl alcohol. In the same year, Heyenga et al. (1990) reported the production of tumor-inhibitory lignans in the callus cultures derived from root explants of in vitro raised seedlings. Callus was initiated and multiplied on B5 medium supplemented with 0.1–1.2 mg l^{-1} 2,4-D, 0.2–2.0 mg l^{-1} GA_3, and 0.05–1.0 mg l^{-1} BA. The cultures produced anticancerous lignans,

podophyllotoxin, 4′-demethylpodophyllotoxin, and podophyllotoxin-4-O-glucoside at levels similar to the original explants used for callus initiation. High levels of podophyllotoxins were recorded, particularly, when there was tissue differentiation. Moreover, the presence of plant growth regulators in the medium affected the relative proportions of podophyllotoxin and 4′-demethylpodophyllotoxin significantly.

In another study, Uden et al. (1995) developed cell cultures of *Linum flavum* and *P. hexandrum* for the bioconversion of cyclodextrin-complexed deoxypodophyllotoxin into podophyllotoxin and its 5-methoxy derivative. After developing callus cultures as per their earlier report of 1989, Uden et al. further studied the effect of cyclodextrin and its deoxypodophyllotoxin complex on the growth of cell suspension cultures. They found that the growth of the cultures was not affected by the presence of either of the compounds. Rather, maximum accumulation of podophyllotoxin and β-D-glucoside of podophyllotoxin (2.87 % on a dry weight basis) was recorded in deoxypodophyllotoxin-fed cultures after 9 days. This was the time when the highest bioconversion (33.2 %) occurred and corresponded with 192 mg l^{-1} of suspension cultures.

Chattopadhyay et al. (2001) developed suspension cultures on MS medium supplemented with 2.0 mg l^{-1} IAA, 10 g l^{-1} PVP, and 1.5 mg l^{-1} pectinase. PVP at 10 g l^{-1} was useful in eliminating problems such as browning of culture medium, clumping of cells, and reduction of pH in the medium. It also supported higher cell viability, biomass, and podophyllotoxin yield. Similarly, MS medium supplemented with 2.0 mg l^{-1} IAA and 5.0 g l^{-1} activated charcoal supported friable callus development. In the following year, the group used the same medium to develop calli from root explants after 3 weeks of culture at 20 °C (Chattopadhyay et al. 2002a). The workers also modulated the major media components such as carbon source, NH_4^+ to NO_3^- ratio, PO_4^-, and IAA for optimization of podophyllotoxin yield in the suspension cultures. They observed that glucose, inoculum, IAA, and pH were culture parameters that affected the podophyllotoxin yield significantly. However, production of podophyllotoxins was optimal only after the specific replacement of sucrose by glucose. Furthermore, the workers attempted submerged culturing in a 3 L stirred-tank bioreactor fitted with a low-shear, steric impeller for upscaling of podophyllotoxins (Chattopadhyay et al. 2002b). Podophyllotoxin accumulation was higher when the cells were gown in shake cultures at 100 rpm in dark. Upon screening B5, Eriksson, MS, Nitsch, Street, and White media, comparatively better growth and podophyllotoxin accumulation was supported on MS medium (Chattopadhyay et al. 2003a). Successful culturing in a 3 L stirred-tank bioreactor under low-shear condition in batch and fed batch modes of operation was also reported (Chattopadhyay et al. 2003b). Podophyllotoxin accumulation in batch mode was 21.4 g l^{-1} and 13.8 mg l^{-1} after 24 and 26 days, respectively. The nutrient feeding rate of 150 ml d^{-1} and substrate uptake rate of 105 g l^{-1} from incoming feed at non-limiting and non-inhibitory glucose concentrations were selected for cell retention bioreactors. After 60 days, there was an overall enhancement in the biomass (48.0 g l^{-1} dry cell weight) as well as podophyllotoxins (43.2 mg l^{-1}). However, when the bioreactor was optimized in the cell retention cultivated mode, the biomass and intracellular podophyllotoxin accumulation were 53.0 g l^{-1} and 48.8 mg l^{-1}, respectively

(Chattopadhyay et al. 2003c). When the podophyllotoxins produced in the cell cultures were studied for their cytotoxicity using human breast cancer cell line (MCF-7), there was 50 % inhibition by 1 nM podophyllotoxin, provided it was applied during the beginning of cell growth (Chattopadhyay et al. 2004).

Although other workers such as Ahmad et al. (2007) established callus cultures from excised embryo segments, no attempts were made by them to develop cell suspensions from these cultures. Majumder and Jha (2007, 2009) also established callus cultures from roots of in vitro raised seedlings but developed suspension cultures of only selected cell lines in B5 medium. When the cell lines were characterized using RAPD markers and podophyllotoxins, the podophyllotoxins content became stable after the fourth year of culture initiation.

Majumder (2012) also studied the effect of 100, 250, and 500 mg l^{-1} tryptophan, an indirect precursor of lignan biosynthesis on podophyllotoxins production in cell suspension cultures maintained in half strength liquid B5 medium containing 1 % (w/v) sucrose, 1.0 mg l^{-1} 2,4-D, 1.0 mg l^{-1} GA_3, and 0.1 mg l^{-1} BAP. Podophyllotoxin accumulation up to 2.7 times with no effect on biomass was observed as compared to control. In the same year, Bhattacharyya et al. (2012) initiated callus cultures from leaves in MS medium supplemented with 2.68 μM NAA and 8.88 μM BAP. However, cell suspension cultures developed in MS medium containing 60 mM nitrogen, 1.25 mM potassium dihydrogen phosphate, 6 % glucose, and 11.41 μM IAA. A seven to eight fold increase in podophyllotoxin accumulation was observed provided they were elicited with 100 μM methyl jasmonate after 9 days. MALDI TOF/TOF MS/MS analysis of the suspension cultures were also done to identify regulatory proteins.

In the present laboratory also, attempts were made to initiate callus cultures from leaves of plants growing in different locales of western Himalaya. MS medium containing 2 % sucrose, 5 μM IBA, and 10 μM BAP at pH 5.7 supported callus induction in leaves collected from Kukumseri region. After 1 month of callus induction, the medium had to be supplemented with 200 mg l^{-1} ascorbic acid to avoid polyphenol oxidation and browning of tissues. The calli proliferated further on the same medium (Fig. 3.1a–c). In contrast, the leaf explants collected from Parashar Lake at Mandi failed to respond. Hence, the response was considered to be region specific.

3.2.4 Secondary Metabolite Production Through *Agrobacterium*-Mediated Genetic Transformation of *Podophyllum hexandrum*

For the first time, Giri et al. (2001) attempted to enhance the production of podophyllotoxins through genetic transformation of *P. hexandrum*. The workers used the *Agrobacterium rhizogenes* strains A4, K599, and 15834 for transformation of callus derived from root explants. The transformed calli growing on half strength MS medium supplemented with 1.0 mg l^{-1} 2,4-D, 0.1 mg l^{-1} BAP, and 1.0 mg l^{-1} GA_3 showed a threefold increase in podophyllotoxins as compared to untransformed controls. Suspension cultures of the transformed calli were also initiated and

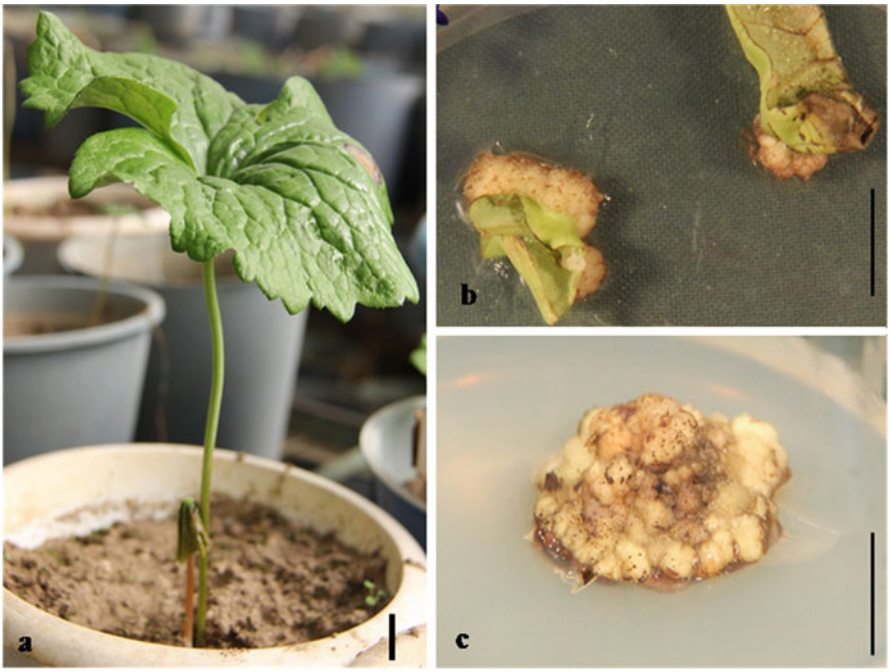

Fig. 3.1 Callusing in *P. hexandrum* plant collected from Kukumseri and maintained under polyhouse conditions, (**a**) 2-year-old plant, (**b**) initiation of callus on leaf segments, (**c**) callus proliferation. Bars = 1 cm

established on MS medium containing 1.0 mg l^{-1} 2,4-D and 0.1 mg l^{-1} BAP. In a separate strategy, Lin et al. (2003a, b) used the *A. rhizogenes* strains LBA9402 and TR105 for production of hairy roots from leaf disks of *Linum flavum*. Significant variation in coniferin accumulation was observed between hairy root lines originating from different *L. flavum* seedlings and/or *A. rhizogenes* strains. When roots were cultured on Linsmaier and Skoog (LS) medium with 2,4-D and NAA, coniferin accumulation was 58 mg g^{-1} dry weight. These hairy roots being a natural source of coniferin were further cocultured with *P. hexandrum* cell suspensions and maintained in dual shake flasks containing Linsmaier and Skoog (LS) medium in dual bioreactors. Increase in podophyllotoxins content was recorded. Earlier, availability and stability of coniferin in the medium were considered to be the key factors for podophyllotoxin synthesis during coculture. Therefore, podophyllotoxins in *P. hexandrum* cell suspensions was effectively increased through coniferin feeding (Lin et al. 2003a, b). Much later in 2013, Rajesh et al. used callus derived from zygotic embryos of *P. hexandrum* for *A. tumefaciens*-mediated genetic transformation. Different parameters governing genetic transformation such as acetosyringone concentration, time of cocultivation, and different strains of *A. tumefaciens* were tested. It was found that EHA105 harboring pCAMBIA2301 with *npt*II and *gus*A as selection marker and reporter genes, respectively, was the most effective as

compared to LBA4404 and EHA101 strains. While there was no effect of acetosyringone, the optimum time for cocultivation was 3 days on MS medium containing 1.5 mg l^{-1} 2,4-D. While complete elimination of residual *Agrobacterium* required the use of 200 mg l^{-1} timentin, kanamycin was used for the selection of the transformed somatic embryos. The putatively transformed plantlets were finally raised on basal MS medium and confirmed using GUS histochemical assay, PCR, and southern blotting. A transformation efficiency of 29.64 % was achieved.

3.3 *Picrorhiza kurroa*

3.3.1 In Vitro Regeneration

Clonal propagation of *P. kurroa* was first attempted by Lal et al. (1988) who used shoot tips for rapid proliferation of multiple shoots in basal MS medium supplemented with 3.0–5.0 mg l^{-1} Kn (kinetin). The workers found that medium containing 5.0 mg l^{-1} Kn evoked the best response of 50.6 ± 1.24 shoots per explant but concentrations beyond 7.0 mg l^{-1} promoted vitrification. Addition of 1.0 mg l^{-1} IAA improved shoot growth, leaf size, and stem thickness. Further, when the shoots were rooted and hardened in sterilized sand, soil, and manure at 1:1:1 ratio, survival of 87.7 % plants was recorded. Thereafter, several workers attempted micropropagation of *P. kurroa* and different explants were used by them (Mondal et al. 2013). Upadhyay et al. (1989) cultured terminal and single nodes on MS medium containing 0.11–2.25 mg l^{-1} BAP and 0.02–0.2 mg l^{-1} IAA or 0.03–0.35 mg l^{-1} GA_3. Of these, only 0.2 mg l^{-1} BAP was most effective. Again in 1996, Lal and Ahuja induced callusing on leaf explants by using MS medium containing 2.0 mg l^{-1} 2,4-D but supplementation of 4.0 mg l^{-1} NAA and 1.0 mg l^{-1} Kn was required for further growth and proliferation. Induction of shoot bud primordia occurred only when the calli were transferred to MS medium containing 0.25 mg l^{-1} BAP. Shoot growth and multiplication was however supported when nitrogen in MS medium was reduced to half strength and 0.12 mg l^{-1} BAP was added. Another group used axillary shoot buds and cotyledonary nodes of seedlings for shoot multiplication on MS medium containing 1.0 μM BAP or Kn (Chandra et al. 2004, 2006). For hardening the plants, the rooted shoots were transferred to a mixture of soil and sand at 2:1 ratio and also fortified with *Bacillus subtilis* and *Pseudomonas corrugate*. As a result, they achieved 92.5 and 85.0 % plantlet survival, respectively, after 2 months. However, when the same group hardened the plantlets in soil and sand (1:1) under high relative humidity (80.0 ± 5.0 %), only 65 % survival was recorded after 6 months (Chandra et al. 2006). Later in 2009a, Sood and Chauhan cultured axillary shoot tips on medium containing both Kn and IBA for shoot multiplication. The workers also developed a low-cost medium by replacing sucrose with table sugar and omitting agar-agar completely. In an attempt to develop a protocol for large-scale propagation of *P. kurroa*, liquid MS medium supplemented with 2.0 mg l^{-1} IBA, 3.0 mg l^{-1} Kn, and 3 % table sugar was tested (Sood and Chauhan 2009a). However, the number of shoots per explant was reduced to 27 only. The workers also cultured leaf

disks, nodal, and root segments on MS medium supplemented with 2.0 mg l^{-1} 2,4-D and 0.5 mg l^{-1} IBA and achieved callusing in 56.3, 38.8, and 70 % explants, respectively. Multiple shoot formation on these calli was also recorded on MS medium containing 0.5–2.0 mg l^{-1} BAP, 1.0–3.0 mg l^{-1} Kn, and 0.5–2.0 mg l^{-1} IBA. Of these, 2.0 mg l^{-1} BAP and 3.0 mg l^{-1} Kn was reported to be the best for shoot multiplication (Sood and Chauhan 2009b). The workers also hardened the rooted shoots in sterile mixture of sand, soil, and vermiculite (1:1:1). In the following year, Jan et al. (2010) optimized the process of indirect shoot regeneration from nodal explants. Although different concentrations of 2,4-D and BAP were used in MS medium, only 0.25 mg l^{-1} each of 2,4-D and BAP supported callus induction in 20 % explants. Indirect shoot regeneration followed by their elongation and multiplication was however evoked only when 0.2–1.0 mg l^{-1} NAA was used along with 0.1–0.5 mg l^{-1} each of IAA and IBA. As a result, there was a maximum of 18.5 ± 1.10 shoots per explant on 0.2 mg l^{-1} NAA as compared to 18.3 ± 0.57 shoots per explant in 0.6 mg l^{-1} NAA in 80 and 95 % of explants, respectively. As in case of earlier report, the workers used autoclaved mixture of sand, soil, and vermiculite at 1:1:1 ratio for hardening of rooted shoots and achieved 81.5 % plantlet survival. In the same year, Sharma et al. (2010) employed 2.0 mg l^{-1} Kn for shoot proliferation from leaf explants, axillary buds, and nodal explants. Maximum shoot regeneration was achieved by these workers on medium containing 2.0 mg l^{-1} Kn alone as well as in combination with 0.50 mg l^{-1} IBA. The workers also considered "synthetic seeds" as an effective system for ex situ conservation of *P. kurroa* and their large-scale plantations in degraded habitats (Sharma et al. 2010). However, the usefulness of the "synthetic seeds technique" was actually demonstrated by Mishra et al. (2011a). Hence, the workers developed alginate beads of somatic embryos and in vitro-grown shoots of *P. kurroa*. They also evaluated their revival potential after different durations of storage. The encapsulated micro-shoots showed 89.33 % revival after 3 months of storage at 4 °C with 42.66 % multiple shoot formation as well as 21.43 % shoot and root formation. However, transfer to half strength MS medium containing 0.2 mg l^{-1} NAA was necessary for healthy root formation. When these plantlets were transferred to greenhouse conditions, their survival was 95 %. Another group, i.e., Patial and coworkers, showed the benefits of 15-day pulse treatment of TDZ. The researchers used leaf explants from in vitro raised plants (derived from the leaves of field grown plants). They found that when the abaxial surfaces of leaf segments touched the culture medium (i.e., MS containing 2.32 μM Kn), there was maximum regeneration of indirect shoots (42.0) from the middle portion of about 94 % explants. Direct regeneration of shoot buds on plant growth regulator-free MS medium was also recorded. Shoots obtained either via direct or indirect method were further multiplied on MS medium containing 2.32 μM Kn (Patial et al. 2012) (Fig. 3.2a–g). Later, Sharma et al. (2015) attempted to culture 0.5–1.0 cm long shoot apices on MS medium supplemented with seaweed extracts. After 1 month, several fold enhancements in total biomass, length, and number of shoots as well as roots was recorded as compared to control. The plants also showed 80 % survival under greenhouse conditions.

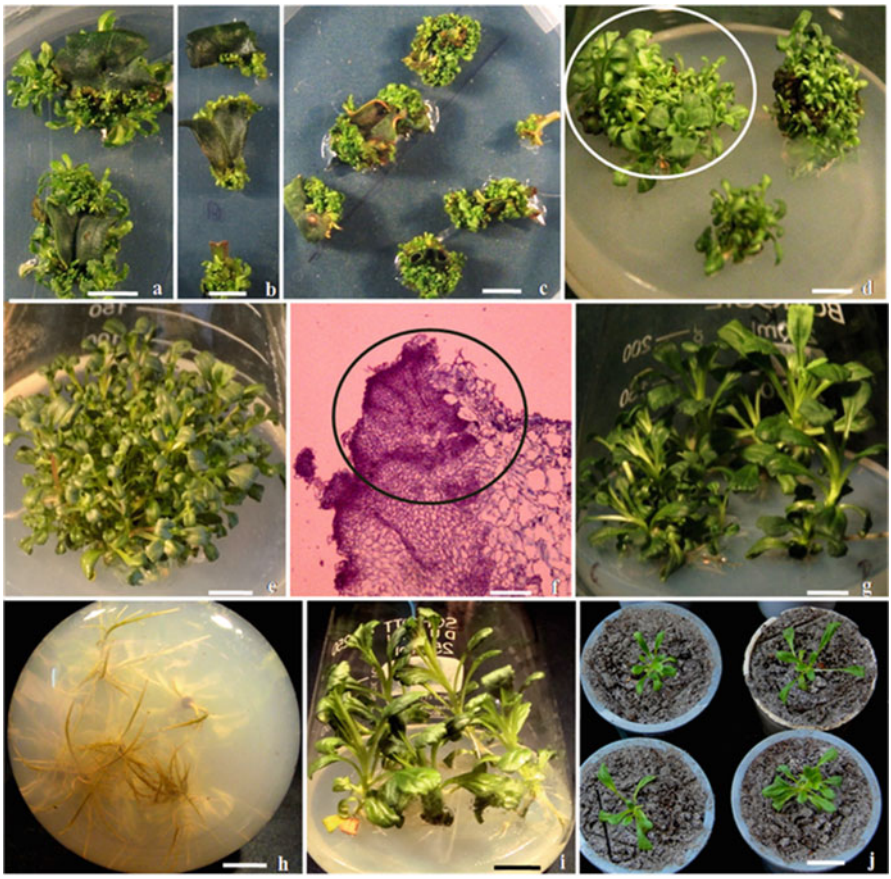

Fig. 3.2 Shoot regeneration from leaf explants of *P. kurroa* (**a**) shoot bud regeneration from ex vitro leaves, (**b**) direct shoot bud initiation from leaves of in vitro raised plant, (**c**) regeneration response of abaxial (on *left side*) and adaxial (on *right side*) surfaces of leaf explant, (**d**–**e**) maximum regeneration from middle portion of leaf explant (*encircled*), (**f**) histological section of leaf callus showing emergence of shoot bud (*encircled*), (**g**) shoot multiplication on MS medium containing Kn, (**h**) root formation on MS basal medium, (**i**) shoots at 15 °C, (**j**) hardened plants in polyhouse. Bars = 1 cm

Overall, various explants such as shoot tips, nodal segments, roots, and even leaf explants were used by researchers working on in vitro regeneration of *P. kurroa*. The percentage usage of these explants was found to range between 12 and 23 % (Fig. 3.3).

The step involving acclimatization of tissue culture-raised plants is crucial in governing the success of plant conservation and improvement. In this regard, hardening of tissue culture-raised *P. kurroa* plants has been a serious problem because of high percentage of plant mortality even after months of active and healthy growth. This is because of their high susceptibility to fungal diseases that cause rotting of

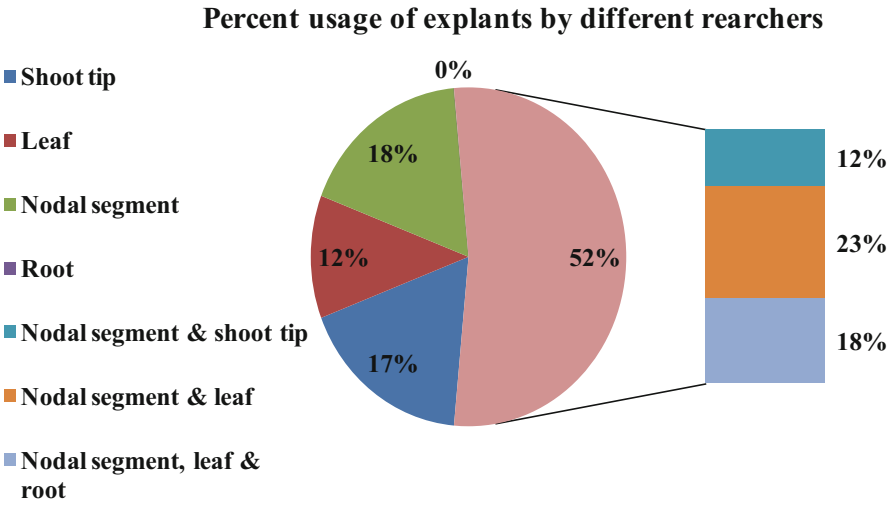

Fig. 3.3 Percent usage of explants by different researchers for initiation of aseptic cultures of *P. kurroa*

the aerial and underground parts. Therefore, different workers attempted biological hardening of tissue culture-raised plants of *P. kurroa*. Trivedi and Pandey (2007) used *Bacillus megaterium*, *B. subtilis*, and *Pseudomonas corrugate* for hardening in vitro raised shoots after rooting on MS medium supplemented with 0.22 mg l^{-1} IBA. The plants maintained for 8 weeks under greenhouse conditions at >80 % humidity showed 94 % survival as compared to control (38.5 %) upon transfer to larger pots. Patial et al. (2012), on the other hand, cultured rooted shoots (derived indirectly from leaf explants) at 15 °C for 10 days and achieved higher survival of the plants (80 %) as compared to controls (50.0 %) kept at 25 °C (Fig. 3.2g–j). The treated plants were found to have healthier leaves with thick cuticles and well-differentiated palisade and spongy parenchyma. These attributes were considered responsible for helping the plants to cope with stress imposed by the hardening process. In the following year, Thakur et al. (2013) attempted to optimize the root yield of *P. kurroa* by treating the plants with biofertilizers singly or in combination with farm yard manure and/or vermicompost. Farm yard manure or vermicompost in combination with *Azotobacter*, a phosphate-solubilizing bacteria (strain B or F), and vascular-arbuscular mycorrhizae resulted in 947 kg ha^{-1} root yield in 3-year-old plants. The fungal endophyte *Piriformospora indica* was also used for biological hardening (Das et al. 2015) and a 1.3-fold increase in the survival of tissue culture-raised plants was recorded under greenhouse conditions. Recently, Helena et al. (2015) developed an indirect system of regeneration from leaf and stem segments. MS medium containing 0.5 mg l^{-1} TDZ (thidiazuron) in combination with 0.3 and 0.5 mg l^{-1} IBA evoked callus induction from leaves and stem segments, respectively. However, shoot regeneration of about 89 % was evoked on MS medium containing 1.0 mg l^{-1} BA and 0.75 mg l^{-1} Kn in case of leaves, whereas 1.0 mg l^{-1} BA and

1.0 mg l^{-1} Kn for stem segments. When these shoots were transferred to half strength MS medium containing 0.5 mg l^{-1} 2–4, D and 0.4 mg l^{-1}NAA, there was 100 % root induction.

3.3.2 Genetic Transformation

Hairy root cultures of *P. kurroa* from leaf and stem explants was first reported by Verma et al. (2007). The researchers obtained 66.7 % relative transformation frequency after 3 weeks of transformation with *Agrobacterium rhizogenes* strain, LBA9402. They also evaluated nine independent opine and TL-positive hairy root somaclones or rhizoclones for their ability to produce kutkoside and picroside I during different phases of growth. Based on the inter-clonal variations in the contents of these compounds in the rhizoclones, the hairy root rhizoclone 14-P was selected for the highest biomass and kutkoside and picroside I contents. Four years later, Mishra et al. (2011b) used the *A. rhizogenes* strains, A4, and PAT405 for induction of hairy root cultures from leaf, internodal segments, and shoot tips. The hairy roots were evaluated after 8 weeks. The A4 strain was found to yield higher contents of both picrotin and picrotoxinin (8.8 and 47.1 μg l^{-1} on dry weight basis, respectively). In contrast, the PAT405 strain yielded 4.45 μg l^{-1} picrotin on dry weight basis as compared untransformed control (0.64 μg l^{-1} picrotin on dry weight basis). In the following year, Praveena and Rao (2012) employed *Agrobacterium rhizogenes*-mediated transformation as well as physical (UV radiation) and chemical (acridine dyes) mutagenesis approach for enhanced production of picrosides and kutkosides in in vitro roots. The kutkin content in the transformed roots was the highest (0.62 μg ml^{-1}) as compared to control (0.53 μg ml^{-1}) or roots subjected to UV radiation (0.58 μg ml^{-1}) and acridine dyes (0.24 μg ml^{-1}). In the same year, Bhat et al. (2012) attempted to transform the leaf explants of *P. kurroa* with the binary vector pCAMBIA1302 harboring the hygromycin phosphotransferase and green fluorescent protein (*gfp*) encoding genes in the strain GV3101 of *A. tumefaciens*. Their study revealed that in vitro-grown explants pre-cultured for 2 days on regeneration medium prior to cocultivation in presence of 200 μM acetosyringone was the most effective. Finally, putative transformants selected on 15 mg l^{-1} hygromycin were found to test positive in PCR (56 %) and also showed *gfp* expression in fluorescence microscopy.

3.4 Conclusions

In conclusion, the review is a compiled information on various in vitro approaches employed for the conservation and secondary metabolites production in *Podophyllum hexandrum* and *Picrorhiza kurroa*. The article offers a base line information from which various gaps can be identified and addressed for future research in these endangered medicinal herbs of western Himalaya.

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Effect of Plant Growth Regulators and Additives on Indirect Organogenesis of *Simarouba glauca* DC

4

A.R. Lavanya, M. Muthukumar, S. Muthukrishnan, V. Kumaresan, T. Senthil Kumar, M. Vijaya Venkatesh, and M.V. Rao

Abstract

A protocol of in vitro propagation of *Simarouba glauca* DC (Simaroubaceae) by indirect organogenesis under the influence of different combinations of plant growth regulators (PGRs) was standardized. Indole-3-butyric acid 1.0 mg/l produced the highest callusing response: 100 % in cotyledon and 95 % in internode explant. One hundred percent of shoot bud induction response with shoot number 18.0 for cotyledon and 85 % response of shoot bud induction with shoot number 12.5 for internode were observed in 6-benzyl adenine (BA) 3.0 mg/l, 6-furfuryl amino purine 0.5 mg/l, and $GA_3$1.0 mg/l. Organic additive coconut water that resulted in 95 % response with average 25.0 shoot number for cotyledon and 80 % response with average shoot number of 18.2 for internode was observed

A.R. Lavanya
Department of Plant Science, Bharathidasan University, Tiruchirappalli 620 024, Tamil Nadu, India

Department of Botany, Periyar E. V. R. Govt. Arts College, Tiruchirappalli 620 023, Tamil Nadu, India

M. Muthukumar • S. Muthukrishnan • M. Vijaya Venkatesh • M.V. Rao (✉)
Department of Plant Science, Bharathidasan University, Tiruchirappalli 620 024, Tamil Nadu, India
e-mail: mvrao_456@yahoo.co.in

V. Kumaresan
Department of Plant Science, Bharathidasan University, Tiruchirappalli 620 024, Tamil Nadu, India

Department of Botany, Aringar Anna Govt. Arts College, Attur, Salem 636 121, Tamil Nadu, India

T. Senthil Kumar
Department of Industry University Collaboration, Bharathidasan University, Tiruchirappalli 620 024, Tamil Nadu, India

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in the medium containing CW (15 %). GA_3 promoted only shoot multiplication with no shoot elongation. High concentration of GA_3 directly involved in shoot elongation rather multiplication, but it caused some irreversible root inhibition. α-Naphthalene acetic acid 2.0 mg/l showed 95 % and 80 % of rooting response with root numbers 8.5 and 7.2 in cotyledon and internode explants, respectively. The microshoots with the lengthy roots were transferred to paper cups for acclimatization. After a period of 12 weeks, acclimatized plants were successfully transferred to the field. The 56.8 % plants survived in the field.

Keywords
Paradise tree • *Simarouba glauca* DC • Organogenesis • Microshoots • GA_3 • Coconut water

4.1 Introduction

Simarouba glauca DC (Simaroubaceae), commonly known as paradise tree, is a fast-growing, multipurpose, medium-sized tree that grows up to 20 m high, with a trunk 50–80 cm in diameter. It is indigenous to the Amazon rain forest and other tropical areas in Mexico, Cuba, Haiti, Jamaica, and Central America (Cronquist 1944; Armour 1959). It can grow at elevations from sea level to 1000 m. It was first introduced to India by National Bureau of Plant Genetic Resources in the research station at Amravati, Maharashtra, in 1966 (Rath 1987; Bhagmal 1994).

It bears edible oil seeds containing 65 % oil (Armour 1959). It can also be used for industrial purposes in the manufacture of biofuels, soaps, detergents, lubricants, varnishes, cosmetics, pharmaceuticals, etc. (Govindarajuet al. 2009). The oil cake is a good organic manure and fruit pulp, leaf litter, and waste wood can be used to generate biogas. The shell and waste wood are used in thermal power station. The shells can be used in the manufacture of particle board and activated charcoal or as a fuel. Fruit pulp is used in beverage preparations (Rath et al. 1987). The pulp along with leaf litter can be used in the manufacture of vermicompost. The wood is generally insect resistant and used in the preparation of quality furniture, toys, and match industry and also as pulp in paper industry (Joshi and Joshi 2002).

The leaves and bark of *S. glauca* are used in the treatment of fevers, malaria, dysentery, diarrhea, astringent, digestive, anthelmintic, emmenagogue, colitis, intestinal parasites, dyspepsia, anemia, haemostatic, antiprotozoal, antiviral, anticancerous, skin depigmentation, snake bite, and a tonic (Grenand et al. 1987; Roig 1988; Rutter 1990; Girón et al. 1991; Cruz 1995; Bonte et al. 1996; Franssen et al. 1997; Joshi and Joshi 2002; Patil and Gaikwad 2011). The main active group of chemicals in *Simarouba* is called quassinoids which belong to the triterpene chemical family (Joshi and Joshi 2002). The various plant secondary substances in *Simarouba* include ailanthinone, dehydroglaucarubinone, glaucarubine, glaucarubolone, glaucarubinone, holacanthone, benzoquinone, canthin, sitosterol, melianone, tirucalla, simaroubidin, simarolide, simarubin, and simarubolide (Assendift et al. 1956; Kupchan et al. 1976; Polonsky et al. 1978; Franssen et al. 1997; Valeriote et al. 1998).

To overcome demand for supply of edible oil, *S. glauca* can be cultivated with advanced tool like plant tissue culture. Recent biotechnological approaches tend to help improving the in vitro raised plants' quality as well as yield. Regeneration of plants from callus, individual cells, and protoplasts has involved organogenic or embryogenic differentiation. These techniques have been useful in providing both spontaneous (Scowcroft et al. 1987) and mutagen-induced genetic variation. This could be used as an adjunct to traditional breeding methods for the modification and improvement of plants. Many of the changes observed in in vitro regenerated plants have potential agricultural and horticultural significance (Ahmed et al. 2001). In fact indirect organogenesis techniques have been quite useful for producing different tree cultivars of high quality and valuable genotypes. Indirect organogenesis is one such an important approach in producing variable cultivars and mass production, through which we can select such a good yielding plants. Variability might be an induced cause by exogenously applied plant growth regulators that trigger morphogenesis via cell-cycle disturbances (Peschke and Phillips 1992).

Previous reports had recorded only 5.83 shoots as their maximum yield per explant by micropropagation (Rout and Das 1995). One of the driving forces behind this growing interest is the challenging attempt to increase the number of plants produced while decreasing the interval of plant production time. The aim of this study was to establish a protocol for inducing indirect organogenesis of *S. glauca* under the influence of different combinations of plant growth regulators (PGRs).

4.2 Materials and Methods

4.2.1 Plant Material Collection

S. glauca seeds were procured from the Institute of Forest Genetics and Tree Breeding, Coimbatore, Tamil Nadu, India. Seeds were treated with con. H_2SO_4 for 1 h. Then the seed coat was softened by scrubbing the surface and washed thoroughly in running tap water. The seeds were rinsed with sterilized distilled water and placed in rotary shaker for uniform imbibitions using sterile distilled water for 3 days. On the fourth day, seeds were thoroughly washed with sterile distilled water, then sowed in the plastic bags containing soil and sand (1:1). Later, raised seedlings were transferred to earthen pots with the same soil and sand and maintained in the garden. The explants were collected from 2-month-old seedlings.

4.2.2 Selection of Explants and Sterilization

Cotyledon and internode explants (2–3 cm) were initially washed with few drops of liquid detergent (Teepol) for 5 min followed by rinsing in tap water for 10–15 min. The washed explants were treated with 70 % alcohol for 1 min followed by sterile distilled water, then with 0.1 % (w/v) mercuric chloride for 3 min. Finally the explants were rinsed 4–5 times with sterile distilled water and cut into small pieces each 0.5–1.0 cm long.

4.2.3 Callus Induction, Shoot Regeneration, and Rooting

The sterilized explants were cultured on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with different concentrations of auxins [indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and α-naphthalene acetic acid (NAA) (0.5–4.0 mg/l)]. The callus derived from all explants was cultured on shoot regeneration medium containing 6-benzyl adenine (BA), 6-furfuryl amino purine (KN) (0.05–4.0 mg/l), and gibberellic acid (GA_3) (0.1–2.0 mg/l) individually and in combination for shoot regeneration. Callus was subcultured on shoot regeneration (SR) medium (BA 3.0 mg/l, KN 0.5 mg/l, $GA_3$1.0 mg/l.) along with various concentrations of sodium citrate, glutamine (5–20 mg/l), and coconut water (CW) (5–20 %) to enhance the shoot quality. The regenerated multiple microshoots were excised and subcultured on MS medium containing IAA, IBA, and NAA (0.1–3.0 mg/l) for root induction.

4.2.4 Acclimatization and Hardening

Four-week-old in vitro raised plantlets with well-developed roots were removed from the culture medium, and roots were washed thoroughly under tap water. Plantlets were transformed to paper cups and earthen pots containing mixture of sand and soil (1:2) maintained inside a culture room at 25 ± 2 °C and 35μΣ $m^{-2}S^{-1}$ light intensity provided by cool white fluorescent tubes, and 70–80 % relative humidity was maintained by covering the plant with polythene bags to avoid contamination. Plantlets were watered with one fourth strength MS basal solution devoid of sucrose and mesoinositol at 3-day intervals for a period of 4 weeks. The acclimatized plantlets were then transferred to earthen pots containing soil and kept under shade for another 8 weeks before transferring to the field.

4.2.5 Culture Conditions

All media were supplemented with 30 g/l sucrose (Hi-Media) and solidified with 8 g/l agar-agar type II (Hi-Media). The pH of all media was adjusted to 5.6–5.8 before autoclaving. The cultures were incubated in culture room maintained at 25 ± 2 °C, under a 16 h photoperiod with a light intensity of 35μΣ $m^{-2}S^{-1}$ from Philips cool white fluorescent tubes with 55–60 % relative humidity.

4.2.6 Statistical Analysis

A minimum of 25 replicates were taken for each treatment and all the experiments were repeated thrice. The cultures were examined periodically and the morphological changes were recorded on the basis of visual observations. The experimental design was random and factorial. The data pertaining to shoot proliferation frequencies and shoot number, shoot elongation, and rooting were subjected to mean and

mean separation analysis by using Duncan's multiple range test (DMRT). All the above calculations and analysis were carried out using IBM SPSS Statistics V.20 for Windows (Software Package from SPSS Inc., 1989–2012; www.spss.com).

4.3 Results

4.3.1 Callus Induction

The callus was developed at the cut surfaces in all concentrations of auxins within 20–25 days of inoculation, and subsequently callus covered the entire surface of the explants within 15 days of culture. Green compact nodular callus was developed in all types of regulators but the percentage of response only varied. IBA 1.0 mg/l produced the highest callusing response 100 % in cotyledon (Figs. 4.1 and 4.3a) and 95 % in internode (Figs. 4.2 and 4.3b).

4.3.2 Shoot Regeneration

Green nodular callus was transferred to the MS medium supplemented with cytokinins (BA and KN (0.5–4.0 mg/l)) individually and in combination with fixed concentration of BA (3.0 mg/l) and varying concentrations of KN (0.05–1.0 mg/l) for shoot bud induction. BA (3.0 mg/l) induced the maximum shoot buds 80 % in cotyledon and 75 % in internode followed by KN (0.5 mg/l) that responds 70 % in cotyledon and 60 % in internode (Table 4.1).

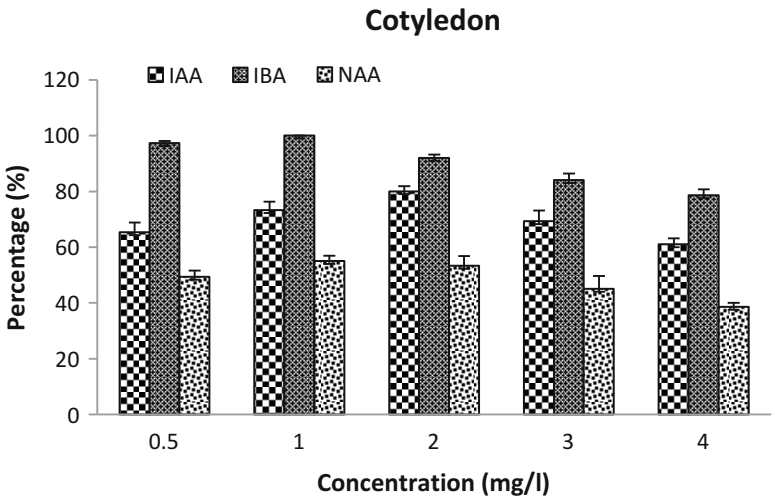

Fig. 4.1 Callus induction from cotyledon explants, after 35 days

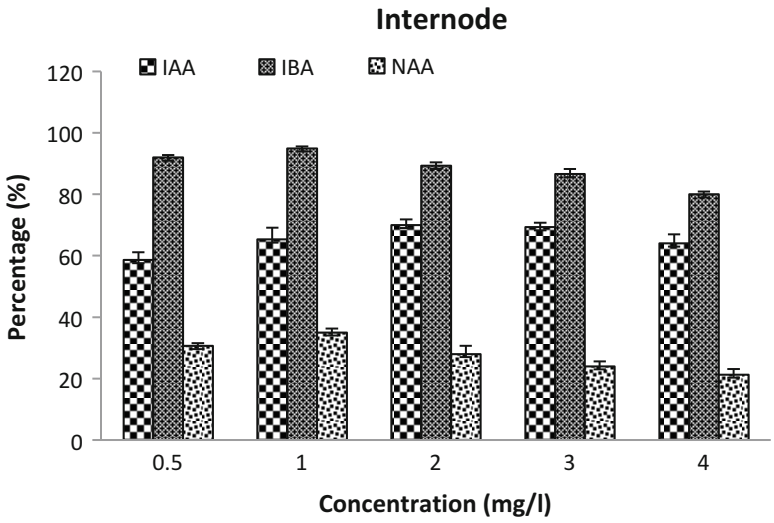

Fig. 4.2 Callus induction from internode explants, after 35 days

The synergistic effect of the two cytokinins BA (3.0 mg/l) and KN (0.5 mg/l) showed the highest response 95% and 80% of shoot bud induction and the shoot numbers 15 and 9.3 in cotyledon and internode explants, respectively (Table 4.1 and Fig. 4.3c).

For further regeneration of shoots, the callus was transferred to BA (3.0 mg/l) and KN (0.5 mg/l) with various concentrations of GA_3 (0.1–2.0 mg/l). One hundred percent of shoot bud induction response with shoot number 18.0 for cotyledon (Fig. 4.3d) and 85% response of shoot bud induction with shoot number 12.5 for internode were observed in GA_3 (1.0 mg/l) within 25 days (Table 4.1). All the treatments except GA_3 (2.0 mg/l) induced only the microshoots. At GA_3 (2.0 mg/l), the shoot length was increased with the reduction in multiplication rate (Fig. 4.3f).

4.3.3 Effect of Additives on Shoot Regeneration

To increase the further regeneration ability of the callus, the callus was transferred to the SR medium along with the additives like sodium citrate, glutamine, and CW. The ninety-five percent of response of the shoot bud induction with shoot number 25 (Fig. 4.3e) for cotyledon and 80% of the shoot bud induction response with shoot number of 18.2 for internode were observed in the medium containing CW (15%) followed by sodium citrate (10 mg/l), recording significant results compared to SR medium. The additives increased both the shoot number and the quality of the regenerated shoots (Table 4.2).

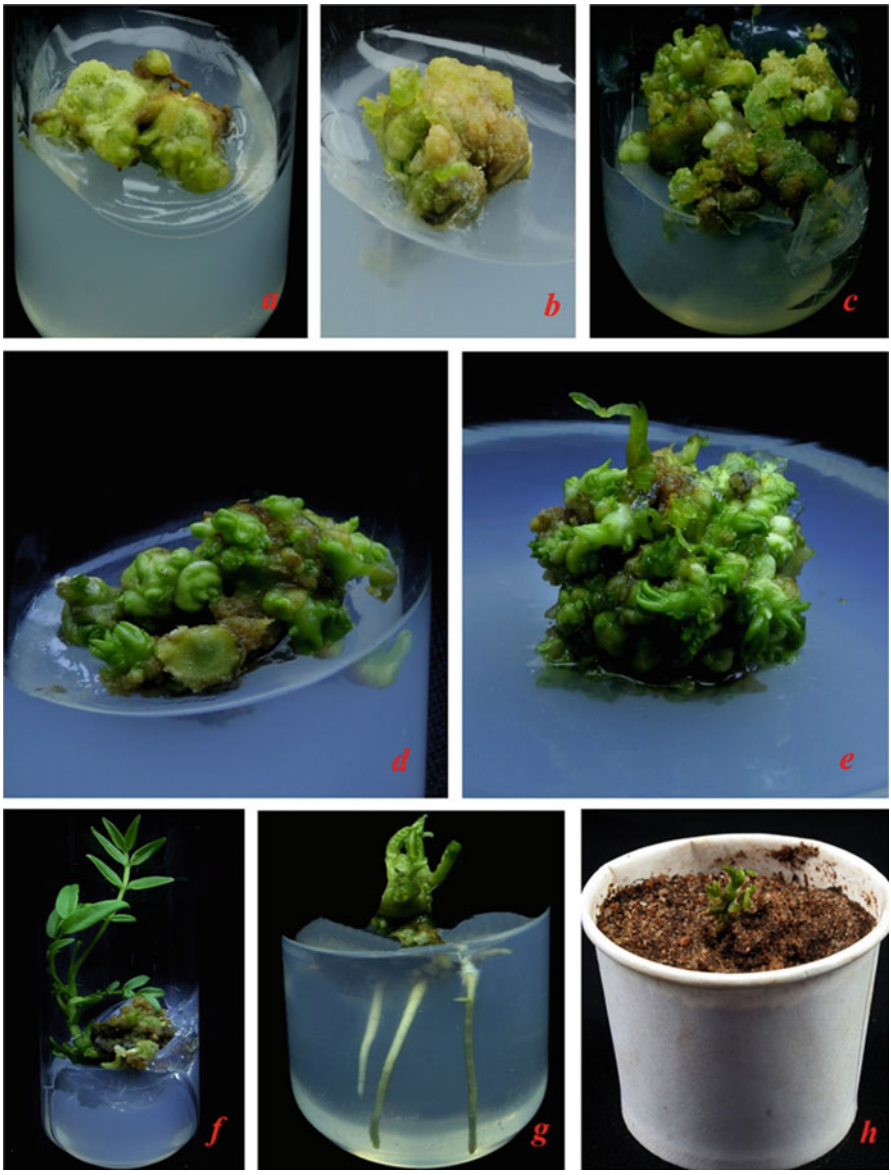

Fig. 4.3 Steps involved in indirect organogenesis of *Simarouba glauca*. (**a**, **b**) Callus induction and proliferation from cotyledon explant (MS + IBA 1.0 mg/l) (2.0×). (**c**) Green compact callus with shoot initiation on MS + BA (3.0 mg/l) + KN (0.5 mg/l) (2.0×). (**d**) Multiple shoots of cotyledon explant in SR medium, after 6 weeks (2.5×). (**e**) Multiple shoots on SR medium + CW 15 %, after 6 weeks (1.5×). (**f**) Elongated shoots (MS + BA 3.0 mg/l + KN 0.5 mg/l + GA_3 2.0 mg/l), after 45 days (1.0×). (**g**) Microshoots with well-developed roots (2.0×). (**h**) Acclimatized plantlet survived on paper cup, after 12 weeks in glass house (1.0×)

Table 4.1 Shoot regeneration response of callus from *Simarouba glauca* grown on MS medium supplemented with BA, KN, and GA_3, after 25 days

Plant growth regulators (mg/l)			Cotyledon		Internode	
			Shoot bud induction (%)	Shoot number*	Shoot bud induction (%)	Shoot number*
BA	**KN**	**GA_3**				
0.5			64.0^{lm}	5.8 ± 0.34^{hi}	58.6^{hi}	3.5 ± 0.27^{hi}
1.0			69.3^{jk}	7.0 ± 0.21^{h}	64.0^{g}	4.1 ± 0.26^{ghi}
2.0			77.0^{h}	8.5 ± 0.36^{f}	72.0^{e}	4.9 ± 0.50^{fgh}
3.0			80.0^{g}	10.4 ± 0.34^{d}	75.0^{d}	6.0 ± 0.65^{ef}
4.0			74.6^{hi}	9.3 ± 0.18^{de}	69.3^{f}	5.3 ± 0.42^{fg}
	0.5		70.0^{j}	6.0 ± 0.21^{hi}	60.0^{h}	4.0 ± 0.57^{hi}
	1.0		65.3^{l}	5.0 ± 0.30^{ij}	57.3^{i}	3.5 ± 0.29^{hi}
	2.0		62.6^{m}	3.8 ± 0.26^{jk}	49.3^{j}	2.9 ± 0.26^{ij}
	3.0		57.3^{n}	3.1 ± 0.55^{k}	42.6^{k}	2.3 ± 0.18^{j}
	4.0		50.0^{o}	2.8 ± 0.50^{k}	38.0^{l}	1.9 ± 0.14^{j}
3.0	0.05		84.0^{f}	7.8 ± 0.40^{fg}	70.0^{ef}	6.5 ± 0.57^{e}
3.0	0.1		90.0^{d}	9.5 ± 0.29^{de}	77.3^{cd}	8.0 ± 0.65^{d}
3.0	0.5		95.0^{b}	15.0 ± 0.69^{b}	80.0^{bc}	9.3 ± 0.42^{bc}
3.0	1.0		89.3^{de}	10.4 ± 0.57^{d}	74.6^{de}	7.8 ± 0.26^{d}
3.0	0.5	0.1	89.3^{de}	9.5 ± 0.36^{de}	74.6^{de}	8.3 ± 0.35^{cd}
3.0	0.5	0.5	94.6^{bc}	14.8 ± 0.50^{b}	81.3^{b}	10.0 ± 0.43^{b}
3.0	0.5	1.0	100.0^{a}	18.0 ± 0.42^{a}	85.0^{a}	12.5 ± 0.29^{a}
3.0	0.5	2.0	97.3^{ab}	12.5 ± 0.42^{c}	78.6^{c}	9.8 ± 0.34^{b}

*Values are mean of 25 replicates per treatment and repeated thrice. Values with the same superscript are not significantly different at 5 % probability level according to DMRT

4.3.4 Rooting and Acclimatization

The regenerated microshoots and elongated shoots were transferred to the medium containing auxins [NAA, IBA, and IAA (0.1–3.0 mg/l)]. NAA (2.0 mg/l) showed 95 % and 80 % of rooting response with root numbers 8.5 and 7.2 in cotyledon and internode explants, respectively, after 25 days (Table 4.3). IAA and IBA in all concentrations had no effect in producing roots (data not shown). The elongated shoots on transfer for rooting failed to produce roots. The microshoots with the lengthy roots (Fig. 4.3g) were transferred to plastic containers containing mixture of sterilized soil and sand (2:1) and covered with transparent plastic papers punctured for holes to lower down the high atmospheric humidity gradually. After 4 weeks of acclimatization, the plantlets were then shifted to plastic or earthen pots containing soil (Fig. 4.3h) and kept under shade condition for 8 weeks before successfully transplanted to the field; 56.8 % plants survived.

Table 4.2 Shoot regeneration response of callus from *Simarouba glauca* grown on SR medium supplemented with additives, after 25 days

Additives (mg/l)	Cotyledon		Internode	
	Shoot bud induction (%)	Shoot number*	Shoot bud induction (%)	Shoot number*
Sodium citrate				
5	85.0^{cd}	18.3 ± 0.52^{c}	68.0^{d}	10.4 ± 0.57^{d}
10	90.0^{b}	20.0 ± 0.48^{b}	70.0^{c}	13.2 ± 0.28^{c}
15	81.3^{e}	17.3 ± 0.28^{c}	65.3^{e}	9.8 ± 0.26^{d}
20	78.6^{f}	15.4 ± 0.71^{d}	58.6^{fg}	7.5 ± 0.36^{fg}
Glutamine				
5	80.0^{ef}	14.3 ± 0.52^{de}	60.0^{f}	8.4 ± 0.52^{ef}
10	85.0^{cd}	18.0 ± 0.69^{c}	65.3^{e}	9.1 ± 0.34^{de}
15	76.0^{g}	12.4 ± 0.48^{f}	57.3^{g}	7.8 ± 0.50^{efg}
20	69.3^{h}	9.8 ± 0.59^{g}	53.0^{h}	6.5 ± 0.36^{g}
CW (%)				
5	81.3^{e}	13.6 ± 0.42^{ef}	69.3^{cd}	10.0 ± 0.43^{d}
10	86.6^{c}	18.6 ± 0.36^{bc}	74.6^{b}	14.3 ± 0.42^{c}
15	95.0^{a}	25.0 ± 0.87^{a}	80.0^{a}	18.2 ± 0.60^{a}
20	90.0^{b}	19.8 ± 0.67^{b}	77.3^{b}	15.8 ± 0.50^{b}

*Values are mean of 25 replicates per treatment and repeated thrice. Values with the same superscript are not significantly different at 5% probability level according to DMRT

Table 4.3 Rooting response of in vitro raised microshoots from organogenic callus of *Simarouba glauca* grown on MS medium supplemented with auxins, after 25 days

Plant growth regulators (mg/l)	Cotyledon			Internode		
	Percentage of response	Number of roots*	Root length (cm)*	Percentage of response	Number of roots*	Root length (cm)*
NAA						
0.1	77.0^{cd}	4.7 ± 0.28^{d}	2.8 ± 0.26^{e}	61.3^{d}	4.5 ± 0.20^{d}	2.3 ± 0.18^{d}
0.5	81.3^{c}	5.8 ± 0.26^{c}	3.4 ± 0.29^{cd}	68.0^{c}	5.0 ± 0.43^{cd}	3.0 ± 0.30^{c}
1.0	92.0^{ab}	6.3 ± 0.28^{bc}	4.0 ± 0.00^{bc}	73.3^{b}	5.8 ± 0.26^{c}	3.8 ± 0.14^{ab}
2.0	95.0^{a}	8.5 ± 0.36^{a}	5.2 ± 0.38^{a}	80.0^{a}	7.2 ± 0.35^{a}	4.7 ± 0.28^{a}
3.0	89.3^{b}	7.0 ± 0.43^{b}	4.9 ± 0.45^{ab}	77.3^{ab}	6.5 ± 0.42^{ab}	4.0 ± 0.48^{ab}

*Values are mean of 25 replicates per treatment and repeated thrice. Values with the same superscript are not significantly different at 5% probability level according to DMRT

4.4 Discussion

Seedling-derived explants are found suitable for tissue culturing tree species. These explants are found juvenile and result in less contamination rate. When the explants were taken from tree source, many perspectives will interfere with results, such as explant age and environmental conditions. Possibility of active explant availability

is difficult throughout the year, due to that of seasonal changes and plant reactions related to it. During inoculation 70% alcohol and 0.1% $HgCl_2$ result in greater effect on controlling pathogens.

All three auxins (IAA, IBA, and NAA) were found potential in callus induction, but callus morphology varied with different plant growth regulators used. Above all IBA 1.0 mg/l produced maximum frequencies of green compact callus, which is very suitable for caulogenesis. Shoot regeneration was obtained at many levels of treatments, starting from single cytokinins to combined treatments of cytokinins also along with gibberellins. At every level of treatment, regeneration capacity of explants increases significantly. Two cytokinins (BA 3.0 mg/l + KN 0.5 mg/l) along with $GA_3$1.0 mg/l recorded better results. Generally gibberellins tend to help in shoot elongation; surprisingly here we had shoot-promoting results, which are supported by many authors. Many reports found GA_3 as conducive for in vitro shoot regeneration (Chakraborty et al. 2000) or for promotion of growth, biomass production, and xylem fiber length (Ericksson et al. 2000). Furthermore, GA_3 can act as a replacement for auxin in shoot induction, and thus, a ratio of cytokinin-GA_3 may be decisive for differentiation in certain plant tissues (Sekioka and Tanaka 1981).

Organic additives are good enhancers of shoot regeneration when combined with plant growth regulators. Here CW produced the highest shoot number with high percentage in both explants. CW consists of various growth-promoting compounds such as amino acids, organic acids, inorganic compounds, nitrogenous compounds, carbon sources, vitamins, and growth regulators like auxins and cytokinins (George 1993; Yong et al. 2009). The molecular mechanism of CW's compatibility with endogenous and exogenous growth-promoting agents is still unknown. At many cases CW showed inspiring results with shoot regeneration in many species (Boase et al. 1993; Nasib et al. 2008; Daud et al. 2011). All the above results showed only microshoots, whereas GA_3 2.0 mg/l alone produced elongated shoots with reduced shoot numbers. Heidge (1969) reported that higher concentrations of GA_3 tend to inhibit the bud breaking. Elongated shoots from high concentration of GA_3 2.0 mg/l caused some irreversible root inhibition. Antagonistic effect of any auxin or cytokinin was unable to restore the root initiation. It had been clearly demonstrated that the inability of auxin, cytokinin, abscisic acid, and growth retardants CCC and phosfon to counteract or reverse the gibberellin-induced inhibition of adventitious root formation. So the microshoots itself treated for rooting got results with only NAA. Well-rooted plantlets were successfully acclimatized.

This study was undertaken to establish indirect shoot organogenesis using cotyledon and internode explants. Both the explants were standardized for indirect organogenesis, but the cotyledon was found more responsive (25 shoots per explant) than internode (18.2 shoots per explant). GA_3 and CW played a vital role in multiple shoot induction. Exogenous supply of GA_3 can induce endogenous production of auxins. These endogenously produced auxins can combine with cytokinins present in culture medium to produce more number of shoots, whereas high concentration of GA_3 directly involved in shoot elongation rather than multiplication, but it caused some irreversible root inhibition. Other GA_3 treatments resulted with only microshoots, which were later produced sufficient roots upon auxin treatment. Well-rooted plantlets were acclimatized to field condition.

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5 Biotechnological Applications for Characterisation, Mass Production and Improvement of a Nonconventional Tree Legume [*Parkia timoriana* (DC.) Merr.]

Robert Thangjam

Abstract

The loss of global bioresources has been occurring due to increased population accompanied with reduction of agricultural lands, rapid urbanisation, neglect of natural habitats and climate change. Loss of biodiversity and food security are the two of the major worries throughout the world in recent times. Major focus of studies in recent times has been towards the inventorisation of potential plants and plant-based products as sources of food security. Tree bean (*Parkia timoriana*), a nonconventional tree legume widely used as vegetable in the northeast Indian region, is a highly nutrient-rich plant. However, the tree has been facing several issues related to its diseases of biotic and abiotic origin. This leads to serious socio-economic problems and decline in its production. The use of biotechnological tools provides one of the most viable means for addressing the issues related to this plant. This chapter reviews some of the applications of biotechnological tools for the genetic characterisation, sustainable production and improvement in tree beans.

Keywords

Tree bean • *Parkia timoriana* • Biotechnological tools • Genetic diversity • In vitro mass production • Genetic transformation

R. Thangjam (✉)
Department of Biotechnology, School of Life Sciences, Mizoram University, Aizawl 796 004, Mizoram, India
e-mail: robertthangjam@gmail.com

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5.1 Introduction

The northeast region of India is known for its huge reserve of diverse flora and fauna genetic resources including the microbes. Several nonconventional plants and vegetables are also consumed as food in the India among which a tree bearing long and tender pods, commonly known as tree bean or scientifically known as *Parkia timoriana* (DC.) Merr., is consumed as a favourite vegetable by the natives in north-east India. It is a member of the Leguminosae family belonging to the subfamily *Mimosoideae*.

P. timoriana is a widely distributed species and recorded from a variety of forest types on various soils, up to 1300 m (Hopkins 1994). It is usually propagated by seed or cutting. According to Salam and Singh (1997), it requires a well-drained soil for its healthy growth and economic production. The tree is non-deciduous but is without a leaf for a brief period before flowering (Fig. 5.1a). The yellowish drumhead-like inflorescence is a combination of miniature flowers and is found hanging in clusters from branches during the month of September–October. Fruiting starts from November–December onwards. The young fruits are very soft, tender and green in colour which later turns black when fully matured by the end of March–April (Fig. 5.1b, c).

Fig. 5.1 *Parkia timoriana* (DC.) Merr., a multipurpose nonconventional tree legume, commonly known as tree bean. (**a**) Tree bean. (**b**) Green pods in local market in Manipur state of India. (**c**) Variations of pod morphology and characteristics

Parkia, named in the memory of the celebrated African traveller Mungo Park (1771–1805), is a genus of approximately 31 species of leguminous trees distributed through both the New World and the Old World tropics. The genus is taxonomically most diverse in the rainforests of the Amazon basin (Hopkins 1986), but four species are found in Africa and Madagascar (Hopkins 1983) and about ten in the Indo-Pacific region (Hopkins 1994). *P. timoriana* is the most widely distributed species of *Parkia* in the Indo-Pacific region extending from northeast India up to Irian Jaya (Hopkins 1994). It is well adapted to the different agroclimatic regions from colder hilly regions to the hotter plains exhibiting a high degree of morphological variations (Thangjam et al. 2003c).

5.1.1 Economic Uses

The inflorescence and seeds of tree beans are consumed with great delicacy in the northeast Indian region. *P. timoriana* usually flowers during the months of July-August after the monsoon which are used for various local vegetable preparations while the green pods, available from October to March, are scrapped off the outer skin and consumed in all its developing stages till fully matured. When fully matured, the black kernels are also stored for consumption as well as for planting purposes. It is a multipurpose tree, commonly grown in the garden of houses, *jhums* and forests throughout the states of northeast India (Kanjilal et al. 1982). It is also relished as one of the favourite vegetable items among the locals in northeast India for its special smell, taste and flavour. The pungent smell is associated with the presence of thiazolidine-4-carboxylic acid (TCA, thioproline), a cyclic sulphur-containing amino acid, and its consumption in the southern part of Thailand contributes to the lower incidence of stomach cancer (Suvachittanont et al. 1996). Thioproline is associated with the inhibition of the formation of squamous cell carcinomas in the fore-stomach of rats and anti-carcinogenic (Tahira et al. 1984, 1988). Consumption of tender pods is more healthy as the biochemical and nutritional values are superior to other stages (Salam et al. 1992). Longvah and Deosthale (1998) reported that the plant can serve as a potential source of protein and fat to meet the ever-increasing requirements with its 29 % protein content of the kernel and 13–19 % in the pods and 34 % and 1–16 % of fats in the kernel and pods. Unsaturated fatty acids, oleic, linoleic and linolenic acid, make up 63–68 % of the total fat in the pods as well as the kernels. Essential amino acid patterns of the kernel are comparable to FAO/WHO/UNU (1985) amino acid requirement for preschoolers (Longvah and Deosthale 1998). Tree beans are also used in various ethnomedicinal preparations. The seed and tender pods are used for curing of stomach disorders and regulation of liver function, skin remedies, etc. (Burkill 1966; Quisumbing 1951). The oil extract of the plant also possesses insecticidal properties (Salam et al. 1995). The wood of the tree beans can be used as a source of paper pulp (CSIR 1966).

5.1.2 Problems in Production and Utilisation

For sustainable large-scale production and utilisation of tree beans, it is essential to understand the proper genetic resource, means of propagation and improvement techniques. Proper identification and classification of *Parkia* has been associated with problems caused by the confusion of names arising out of various local dialects, difficult geographical location of the habitats, difficult interior regions, etc. Morphological variations such as the characteristics of leaves, capitula and fruit pattern are difficult to ascertain the identity and classification of tree beans (Hopkins 1994). In the northeast Indian state of Manipur, tree beans bearing narrow and uniform-looking light green pods are considered as superior in flavour and form the basis for the identification of 13 cultivars (Meitei and Singh 1990). Salam and Singh (1997) also identified nine varieties of tree beans from different localities in Manipur based on the organoleptic and palatability tests. For a large-scale cultivation and their sustainable production, it is necessary to understand the basis of their evolution, population density, mating system and mechanisms of gene flow that will determine the level and structure of genetic status within and among populations. These informations will be for the effective utilisation and conservation of the tree beans.

The conventional propagation of tree beans is through seeds though in rare instances vegetative cuttings are also used but not popular due to low rooting percentage. On the other hand, the use of seeds for planting is associated with large-scale fungal and pest infestations. The large-scale death of tree beans in the northeast Indian region during the recent years has been associated with the die-back symptoms leading to serious economical problems of the growers (data not published). Infestation by pest like *Cadra cautella* both on the pods and storage has been well reported (Thangjam et al. 2003a). *Anoplophora glabripennis* (Motchulsky) commonly known as Asian long-horned beetle have been found to be associated with the die-back symptoms of tree beans, and a large number of the larvae have been found from the trees across the northeast Indian region (data not shown). Thus, large-scale plantation and sustainable commercial cultivation of tree beans are affected by diseases and other factors such as climatic and diminishing areas of cultivation lands. In order to overcome these problems, it is now a priority to identify the proper genotype for mass production of disease-free planting materials and also use modern biotechnological and conventional breeding techniques for genetic improvement of this important tree species.

5.2 Scope and Application of Biotechnology for Characterisation, Mass Production and Improvement of Tree Beans

In recent times with the advancement of biotechnological tools and techniques, various applications are used for the evaluation, characterisation, mass production and genetic improvement of crops including trees. The scope and application of

biotechnological tools to understand the phytochemical for its pungency, level of genetic diversity of tree beans, in vitro regeneration for mass production of quality planting materials and genetic transformation techniques for improvement are highlighted as follows.

5.2.1 Thioproline Content

Tree beans are associated with a characteristic pungent smell, and its aroma from the fresh pods and seeds is relished by the consumers. Rahnema et al. (1996) identified that the distinctive sulphur aroma in this plant is associated with a principal agent known as thioproline ($C_4H_7NO_2S$, thiazolidine-4-carboxylic acid) which is a cyclic sulphur-containing amino acid. Thioproline is the condensation product of formaldehyde and cysteine (Schubert 1936) and is a natural metabolite which can act as intracellular sulphhydryl antioxidant and free radical scavenger, protecting cellular membranes from damage due to oxygen-derived reactions (Ratner and Clarke 1937; Cavallini et al. 1956). The presence of endogenous formation of thioproline in biological systems such as plant and plant products is considered as a detoxification pathway of formaldehyde (Peres and Dumas 1972). Various reports (Strubelt et al. 1974; Siegers et al. 1978; Brugarolas and Gosalvez 1980) on the importance of thioproline in the protection of the liver against various toxic agents, clinical antitumour effects in cancer patients and anti-ageing properties in drosophila are available widely. Thioproline also showed stimulation of lymphocyte and natural killer (NK) functions in old mice as well as the macrophage functions in vitro when administered in the diet by acting as an effective nitrite-trapping agent in the human body, thereby inhibiting the carcinogenic *N*-nitroso compounds (Tsuda et al. 1988). Various natural sources of thioproline have also been identified such as in cod fish, shiitake mushroom and many kinds of vegetables (Kurashima et al. 1990). It is observed that the level of formaldehyde and thiol contents in the different developmental stages of *P. timoriana* increases with advancing maturity stages of the pod with the maximum concentration detected within fully matured seeds which corresponds to the presence of thioproline content (Thangjam and Maibam 2012). Similarly the thiol groups, i.e. total, nonprotein- and protein-bound composition, in *P. timoriana* increased with the maturity of the pods and decreased upon boiling. Various in vitro assay systems have shown that endogenous formaldehyde formation is genotoxic to the body and their subsequent reduction in content on boiling is due to the volatilisation of formaldehyde and the formation of thioproline endogenously (Takahashi et al. 1985; Frankenberg-Schwager et al. 1980). Thus endogenous formation of thioproline is useful and considered as a detoxification pathway of formaldehyde (Kurashima et al. 1990). The sulphhydryl or thiol groups play an important role for the activities of cellular enzymes as well as keeping the membrane intact (Saez et al. 1990; Vladimirov 1986; Halliwell and Gutteridge 1999). Cellular sulphhydryl groups are the sum of cellular nonprotein SH (NP-SH) and protein-bound SH (P-SH) groups. Cellular NP-SH groups consist of glutathione, L-cysteine, coenzyme A and dipeptides. Protein-bound SH groups are found in

the biomembranes, soluble enzymes and structural proteins. They are important groups having catalytic functions and structural properties. Sulphhydryl groups are the sites of radiation damage. The modulation of thioproline for its sulphur free radical reactivity has been assayed through the determination of its absorbance patterns, using curcumin as a reference material (Thangjam and Maibam 2012). The presence of thioproline in the solution contributed to considerable depletion of curcumin upon gamma irradiation, while the addition of ascorbic acid in the solution containing thioproline and curcumin showed a marked reduction in the depletion level of curcumin (Thangjam and Maibam 2012). Overall the depletion factors of curcumin in the three different experimental conditions are in the order of the following solutions: curcumin + thioproline > curcumin > curcumin + thioproline + ascorbic acid. The characteristic chrome orange-yellow colour of curcumin is due to the presence of two olefinic side chains, which are conjugated to the aromatic ring. Its conjugated systems can be destroyed by thiyl free radicals, and in doing so, it loses its characteristic visible spectrum, i.e. bleaching. Thus, the destruction of curcumin in the presence of thioproline, along with its relatively very low destruction in its absence, clearly indicates the generation and reactivity of thiyl free radicals. In vitro protection of DNA observed in the presence of thioproline against destruction by gamma irradiation also provides a new lead for investigations into protection against radiotherapy and reduction of risk for exposed individuals (Thangjam and Maibam 2012). With increasing doses of gamma radiation, the destruction increased. In the presence of thioproline, considerable protection of DNA depletion from gamma radiation was observed. The radioprotection of DNA by the thiol compounds takes place due to its reaction with the free radicals thereby competing and blocking the free radical-oxygen reaction (Frankenberg et al. 1980). It has been reported that with increasing radiation dose, an increased number of DNA double-strand breaks per cell remain unpaired and that the rate of repair diminishes with increasing radiation dose. It was shown that thioproline, which contains cysteine (sulphhydryl compound), exhibits considerable protection of DNA from gamma radiation. Its protective effect therefore stems from its ability to scavenge free radicals, facilitating the chemical restitution of the original target molecule (DNA).

5.2.2 Status of Genetic Diversity and Population

5.2.2.1 Standardisation of DNA Isolation Protocol

For the use and application of molecular biology techniques, it is a must to isolate and obtain a pure and intact DNA for any downstream purposes such as PCR, sequencing, cloning, etc. However, the available DNA isolation protocols are not always suitable or applicable to all the species or the samples (Porebski et al. 1997). Tree beans contain a high level of polysaccharides and polyphenols; therefore it can hamper the DNA isolation procedures and reactions such as in PCR amplification by inhibiting *Taq* polymerase activity, and DNA also gets degraded in the presence of endonucleases and co-isolation of viscous polysaccharides (Fang et al. 1992; Khanuja et al. 1999). Polyphenols not only inhibit enzymatic reactions (Weishing

et al. 1995) but also reduce the yield and purity of extracted DNA (Loomis 1974; Porebski et al. 1997). Thus a new DNA extraction protocol using imbibed embryos of *P. timoriana* has been *standardised* which allowed easy and rapid extraction of DNA and also minimised the risk of cross-contamination (Thangjam et al. 2003b). This standardised method has been successfully used in various downstream applications using PCR and other techniques (Fig. 5.2).

5.2.2.2 Genetic Diversity

Tree bean (*P. timoriana*) is a diverse species with complex patterns of morphological and evolutionary patterns. Cultivar or varietal identification has been carried out on the pod basis of pod morphology and taste of the individual trees. Since tree bean is an outcrossing species, it is highly heterozygous and morphologically diverse. Morphological traits are influenced by environmental factors, age of the plant and phenology; thus identification and characterisation of genotypes based on these characters are not always reliable. Molecular markers such as RFLP, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter-simple sequence repeat (ISSR), etc., are some of the important tools used for the proper identification and characterisation of crops (Botstein et al. 1980; Beckman and Soller 1983; Tanksley et al. 1989). Using the RAPD technique, considerable genetic variations were detected in the eight tree bean genotypes from Manipur (Thangjam et al. 2003c). The techniques employed are explained using a schematic flow chart (Fig. 5.3).

5.2.2.3 Population Genetic Status

A sound knowledge and understanding of the basis of intraspecific genetic diversity is required to evolve and implement effective conservation strategies. Intraspecies genetic diversity within and among populations is essential for long-term adaptation and future survivability of tree bean. This plays a critical role in the ability of populations to respond to specific adaptations such as resistance to disease or insect, tolerance to certain soil conditions or other attributes that may be of current or future value in tree breeding programmes (Rogers and Ledig 1996). Thus, the conservation of genetic resources involves not only preventing extinction but also ensuring the availability of resources for future use through adaptation to the changing environments (Namkoong 1997).

The use of morphological characters and agronomic traits of *P. timoriana* cannot alone describe the existing level of genetic variations within the species since they represent only a small portion of the plant genome and are also influenced by environmental factors, thus limiting their utility in describing the potentially complex genetic structures (Avise 1976). Molecular markers such as randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSRs) and inter-simple sequence repeats (ISSRs) have been widely used for the detection of genetic variation in many crops (Gupta and Varshney 2000). The advantages of these molecular markers are that they are independent of environmental factors and more numerous than phenotypic characters, thereby providing a clearer indication of the underlying variation in the genome of

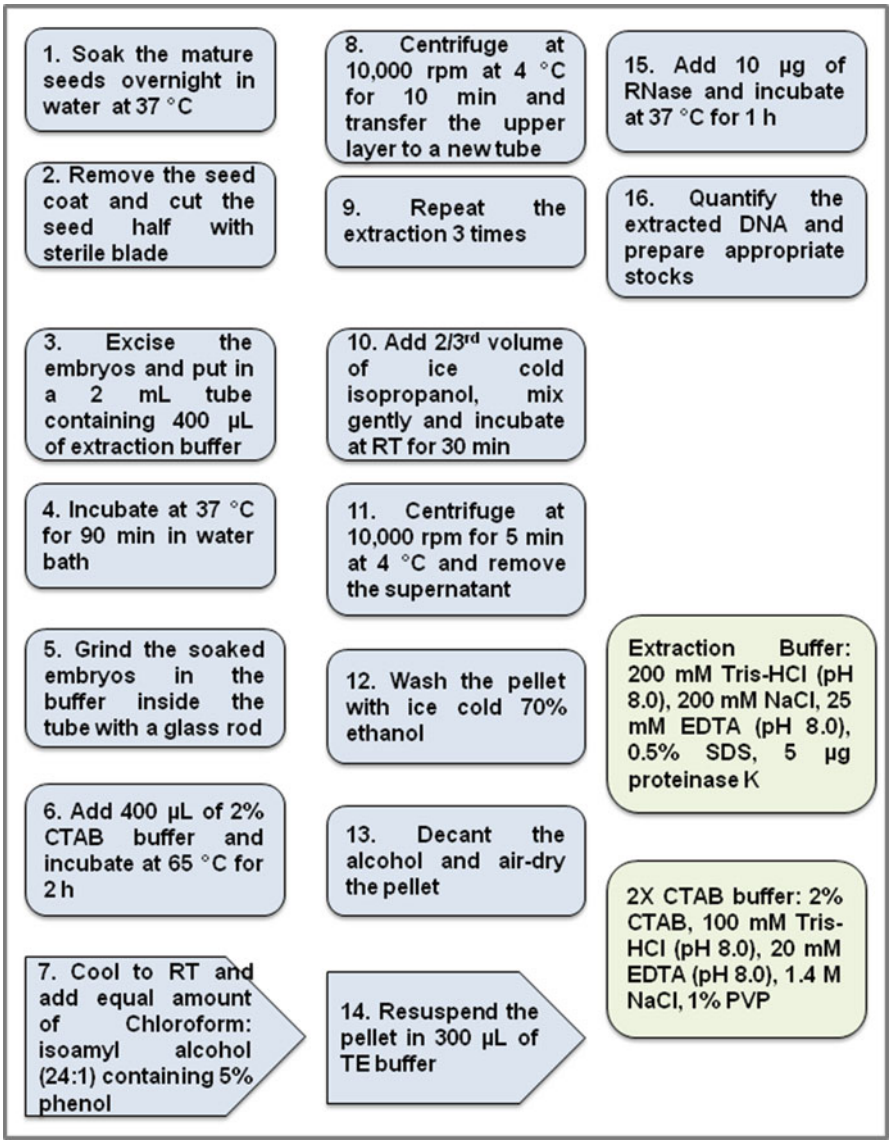

Fig. 5.2 Schematic diagram for extraction of genomic DNA from *P. timoriana* using modified CTAB method (Thangjam et al. 2003b)

an organism (Avise et al. 1989). In *P. timoriana* successful use of RAPD technique in describing the genetic diversity has been reported by Thangjam et al. (2003c) and Suwannarat and Nualsri (2008). However, the use of ISSR markers is effective to describe the complex genetic patterns and therefore more advantageous than the RAPD for studying closely related taxa (Parsons et al. 1997; Chowdhury et al.

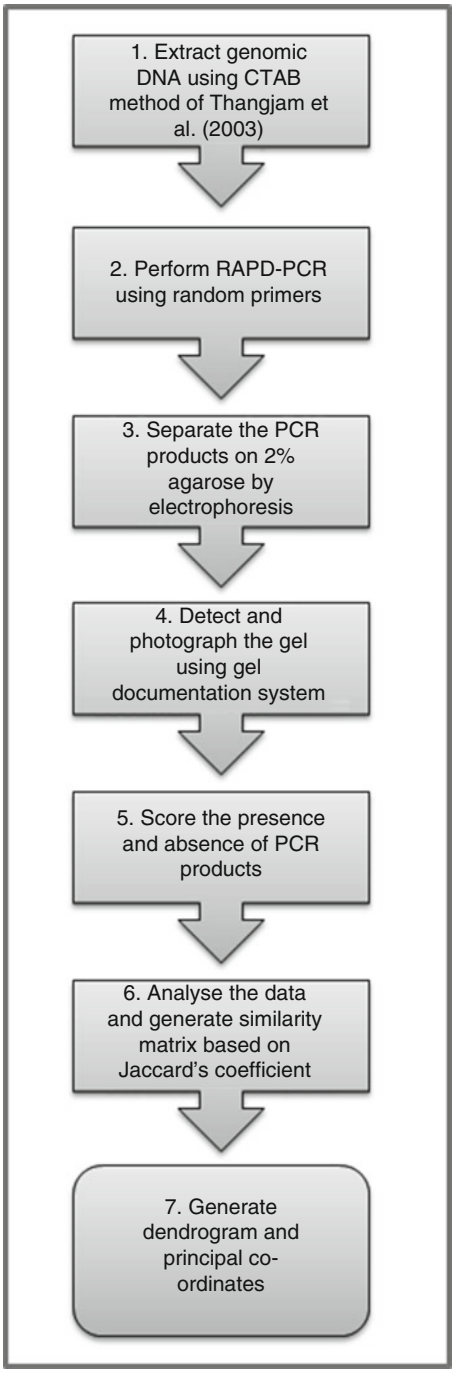

Fig. 5.3 Schematic diagram for the evaluation of genetic diversity in *P. timoriana* using RAPD (Thangjam et al. 2003c)

2002). Goulao and Oliveira (2001) described that ISSR markers are more reproducible than RAPD markers as the ISSR primers. It is also efficiently used for studying genetic diversity and population genetic structure of various plant species (Zietkiewicz et al. 1994; Kantety et al. 1995; Godwin et al. 1997; Archak et al. 2003). Genetic variation in three populations of *P. timoriana* (DC.) Merr. grown in the Manipur was analysed using inter-simple sequence repeat (ISSR) markers (Thangjam 2014). A total of 30 individual trees representing three populations were sampled and studied using ISSR markers which revealed the total genetic variance; 70.04 % were attributed to within-population diversity, while 4.72 % differences to the among populations. The schematic flow chart (Fig. 5.4) shows the procedure and steps involved in the study.

5.2.3 In Vitro Regeneration and Mass Production of Tree Beans

5.2.3.1 Callus Induction and Somatic Embryogenesis

For a large commercial cultivation of tree beans, it would require large amount of superior quality clonal planting materials which are free of diseases which would be difficult to obtain by conventional methods of propagation through seeds and vegetative cuttings. Vegetative propagation by means of in vitro techniques such as cell and tissue culture techniques provides a viable option for plant germplasm conservation and rapid clonal multiplication as well as application in the genetic improvement programmes. The development of somatic embryogenesis techniques has several advantages over organogenesis for propagation as it bypasses the necessity of timely and costly manipulations of individual explants to obtain organogenesis. It does not require the time-consuming subculture steps to increase clonal stock and may overcome difficulties with micropropagation of difficult root species. Somatic embryogenesis may accelerate the introduction of improved clones into commercial production as somatic embryos can be encapsulated and handled as seeds. It may provide a regeneration system amenable to gene transfer techniques.

The basic procedures for the establishment of callus culture were established and described in Fig. 5.5. In this study the ability of callus induction was found to be superior in MS medium than in B_5 medium. Based on this finding, we studied the effect of MS medium supplemented with combinations of different concentrations of 2,4-D (1.0 and 2.0 mg l^{-1}) and BAP (1.0 and 2.0 mg l^{-1}) on *P. timoriana* cotyledon explants, and 100 % callus induction was achieved in the first week. The calli generated turned friable and more nodular in all the treatments by the second week of culture, and then small globular protuberances appeared at the top of these nodular calli by the third week of culture. It was the first report on the collagenic capacity of cotyledon explants of *P. timoriana* and the possibility of inducing somatic embryogenesis from the induced calli (Thangjam and Maibam 2006).

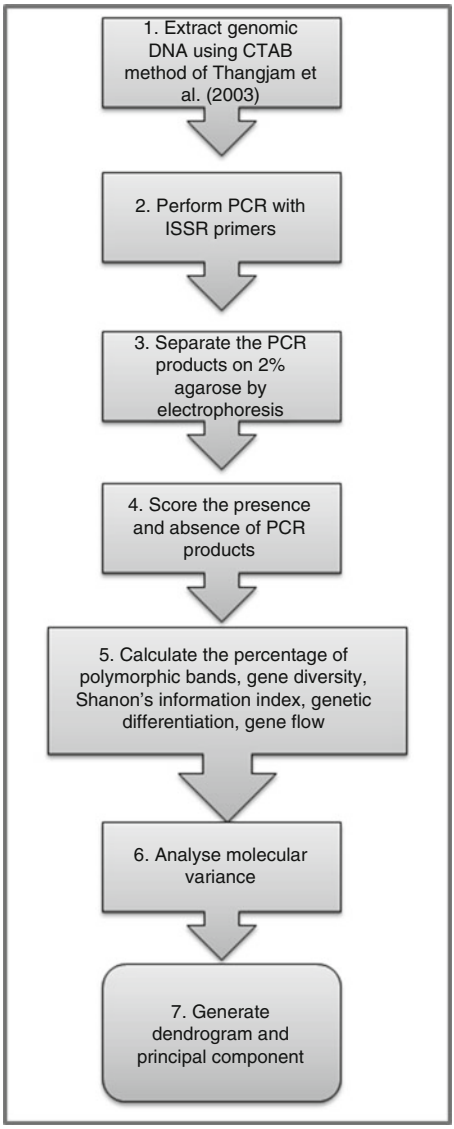

Fig. 5.4 Schematic diagram for the *P. timoriana* population analysis using ISSR markers (Thangjam 2014)

5.2.3.2 In Vitro Regeneration and Genetic Transformation of Tree Bean

As described earlier for a sustainable commercial cultivation of *P. timoriana* tree, it would require large amount of superior quality and genetically improved planting materials that may be difficult to obtain by conventional methods of propagation. The application of tissue culture techniques for mass production of quality planting materials and genetic improvement through genetic transformation using gene(s) coding desired trait/character is the most practical option for any crop. To start with

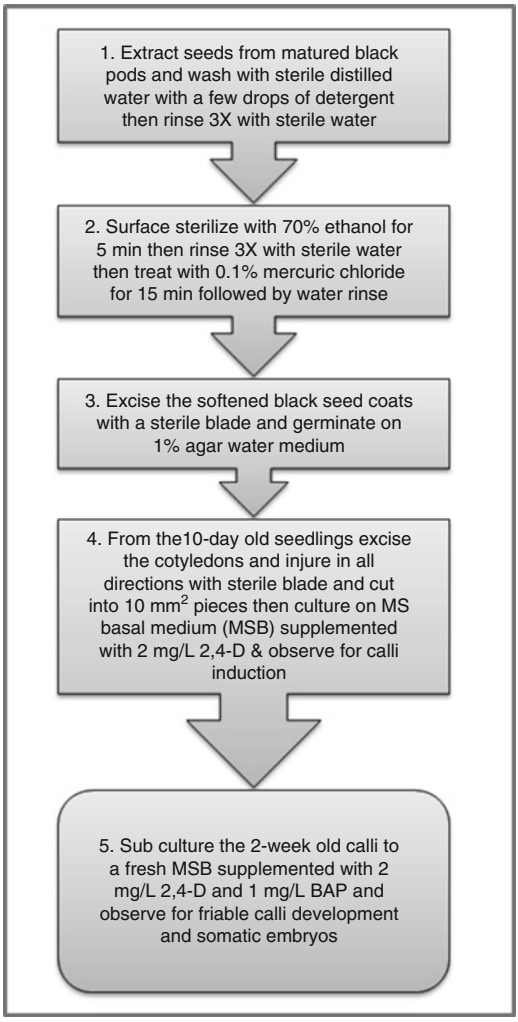

Fig. 5.5 Schematic diagram for the induction of callus and somatic embryos from cotyledonary explants of *P. timoriana* (Thangjam and Maibam 2006)

any genetic improvement programme of a plant requires the development of an efficient transformation and regeneration procedure for further transfer of gene of interest. The conditions for establishing an effective in vitro regeneration and *Agrobacterium tumefaciens*-based transformation through direct multiple shoot organogenesis from cotyledonary node explants without cotyledons and the establishment of an optimal selection system in *P. timoriana* have been successfully established (Thangjam and Sahoo 2012). The schematic flow chart explained the techniques involved (Fig. 5.6).

In vitro regeneration and *Agrobacterium tumefaciens*-mediated genetic transformation of tree bean were achieved using cotyledonary node explants. The explants cultured in MS medium supplemented with combinations of 2.7 μM NAA and 11

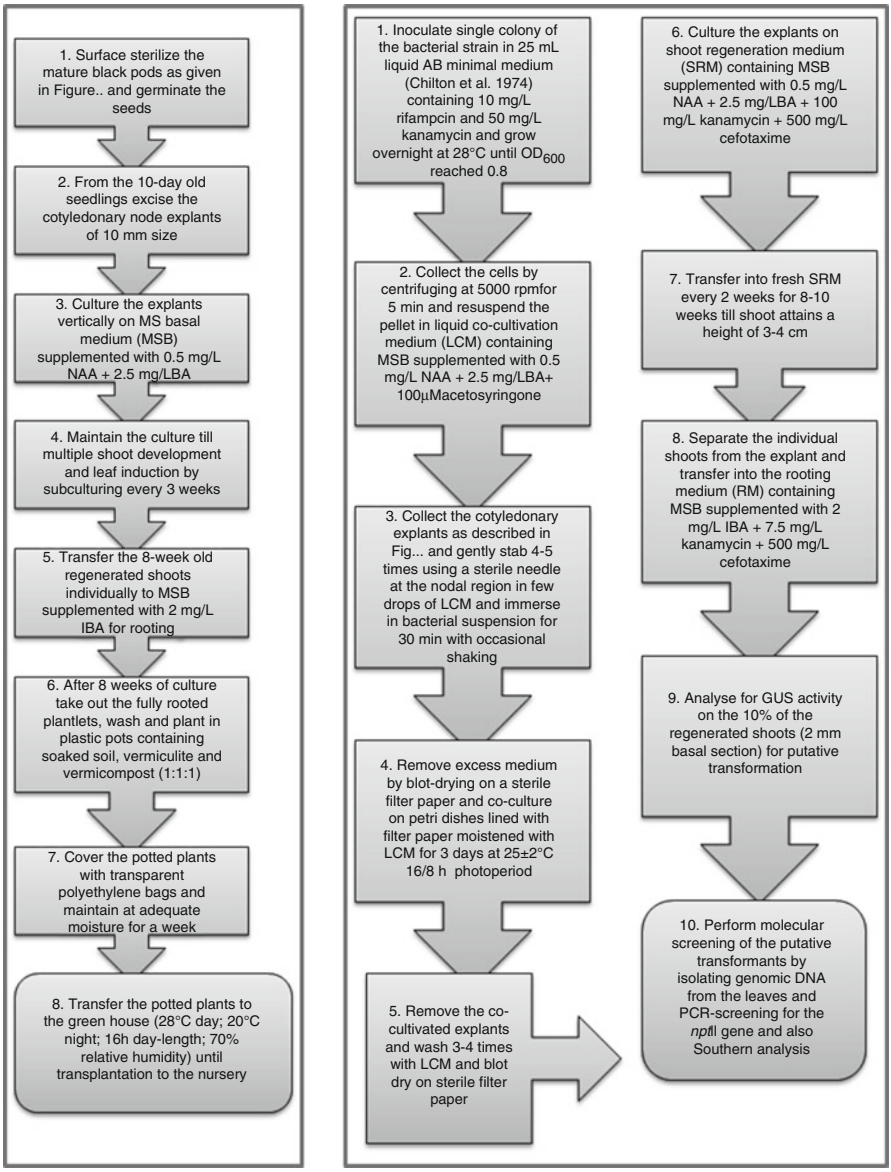

Fig. 5.6 Schematic diagram for the in vitro regeneration and *Agrobacterium*-mediated genetic transformation of *P. timoriana* (Thangjam and Sahoo 2012)

µM BA showed the maximum frequency of multiple shoot (96.66 %) formation and number of shoots per explants (6.60), respectively. For rooting full and half-strength MS medium supplemented with various concentrations of indole-3-butyric acid (IBA) and NAA was studied, and the highest number of root formation was observed

in full-strength MS supplemented with 9.8 μM IBA. Using *A. tumefaciens* strain EHA105 pCAMBIA2301, various optimum conditions for efficient transformation were determined by recording the percentage of GUS^+ explants. Following the optimised conditions, the cocultured explants were cultured on semi-solid shoot regeneration medium (SRM) containing MS medium + 2.7 μM NAA + 11 μM BA + 100 mg/l kanamycin + 500 mg/l cefotaxime. After 8 weeks of culture, the regenerated shoots were rooted in rooting medium (RM) containing MS medium + 9.8 μM indole-3-butyric acid (IBA), 3 % sucrose, 7.5 mg/l kanamycin and 500 mg/l cefotaxime. Successful transformation was confirmed by histochemical GUS activity of the regenerated shoots, *npt*II gene PCR analyses of the regenerated kanamycin-resistant plantlets and Southern analysis of putative transgenic PCR^+ plants.

5.3 Conclusion

Food security has been a challenge for feeding the population globally. This issue has taken more prominence with the worldwide large-scale reduction of agricultural and natural forest due to rapid increase in population and climatic changes. Future food and nutritional requirements can be achieved by integrating and popularising many nonconventional highly nutrient-rich crops like tree beans. Most of these nonconventional crops remained only with the local users limited to the habitats. However many of these plants remain neglected, underutilised and under-researched which leads to their decline or loss. Realising the importance and prospects of such nonconventional crops in supplementing the nutritional and food requirements of the world, the chapter described the application of biotechnological tools for characterisation, mass production and improvement of a nonconventional tree legume [*Parkia timoriana* (DC.) Merr.], which is used as a multipurpose tree vegetable in Southeast Asia.

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A to Z on Banana Micropropagation and Field Practices

6

Norzulaani Khalid and Boon Chin Tan

Abstract

In order to stay competitive in the global banana production, it is important to ensure that the planting materials used have consistent superior agronomic traits and are disease-free and grown in farms with good agricultural practice. The use of suckers from field-grown plants as planting materials may increase the risk in the spread of diseases and inconsistent quality. Hence, the adoption of plant tissue culture technique for mass propagation of banana planting materials has been widely used. Micropropagation of bananas has been successfully established through the use of shoot or meristem cultures and inflorescence either through direct plant regeneration or establishment of regenerable cell suspension cultures. From the cell suspension cultures, single-celled protoplasts have also been isolated and regenerated. Not only the in vitro systems developed through tissue culture provide efficient plant production but also a platform for genetic engineering for agronomic traits improvement. In this chapter, we highlight studies on banana micropropagation and field practices of this important crop.

Keywords

Banana • Biotechnology • Crop improvement • Tissue culture • Pests and diseases

N. Khalid (✉)
Centre for Research in Biotechnology for Agriculture, University of Malaya, 50603 Kuala Lumpur, Malaysia

Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
e-mail: lani@um.edu.my

B.C. Tan
Centre for Research in Biotechnology for Agriculture, University of Malaya, 50603 Kuala Lumpur, Malaysia

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6.1 Introduction

Bananas and plantains belong to the family of Musaceae. They are monocotyledonous and perennial herbs that have been cultivated in nearly 120 countries of the humid and subhumid tropical regions. Bananas and plantains are one of the important staple fruit crops in many developing countries as its nutritional status is higher than other common tropical fruits (Sultan et al. 2011; Chin et al. 2014). Global annual production of bananas and plantains is now exceeding 100 million tonnes (faostat.fao.org). They are mainly grown by local farmers for local consumption and only about 10 % are exported (Čížková et al. 2015). Currently, Cavendish subgroup (AAA) is the most popular commercial cultivar of banana in the international trade (Robinson and Saúco 2010). Banana is traditionally propagated using field-collected suckers or corms (Msogoya et al. 2011). This method is slow as only five to ten suckers are produced per year (Makara et al. 2010) due to its long natural life cycle, sterility, and polyploidy characteristics. Moreover, it is easy to transmit diseases from old to new banana plantations along the generation route (Msogoya et al. 2011; Chin et al. 2014). Bananas and plantains are prone to many pests and diseases such as fungi, viruses, bacteria, insects, and nematodes. Thus, developing an efficient and less labor-intensive method for large-scale production of good quality and disease-resistant banana through biotechnological approaches and good agricultural practices is critical.

6.2 Banana Cultivars

The genus *Musa* is classified into four sections: Callimusa and Australimusa (chromosome number: $2n = 2\times = 20$) and Eumusa and Rhodochlamys ($n = 11$) (Arvanitoyannis et al. 2008). Bananas and plantains are classified in the Eumusa section of the genus *Musa*. Simmonds and Shepherd (1955) classified the edible clones into (AA), (BB), (AB), (AAA), (AAB), (ABB), (AAAA), and (ABBB) based on their ploidy level and genomic origins in relation to two diploid wild species *M. acuminata* (AA genome) and *M. balbisiana* (BB genome) characteristics. *M. balbisiana* is more drought and disease resistant compared to *M. acuminata*, and such characteristics are often found in cultivars containing a "B" genome (Arvanitoyannis and Mavromatis 2009). Most important grown cultivars such as "Gros Michel" and "Cavendish" types (dessert bananas), which constitute most of the world's banana trade, are triploid ($2n = 3\times = 33$) (Ortiz et al. 1995; Osuji et al. 1997; Pillay and Tripathi 2007). In Southeast Asia, most of these triploids have now replaced the original AA diploids due to their vigorous growth and larger fruit (Nasution 1991). To date, the total number of *Musa* cultivars is still uncertain. There are about 300 *Musa* accessions that have been collected and maintained by the Plantain and Banana Improvement Program of the International Institute of Tropical Agriculture (IITA) in a field gene bank located at Onne, Nigeria (Vuylsteke et al. 1993; Arvanitoyannis and Mavromatis 2009).

6.3 Banana Propagation

Banana plants are propagated through vegetative suckers at the base of mother plant. These suckers have a strong vascular connection to the mother, but they can be removed and planted separately to allow rapid vegetative propagation and multiplication (Heslop-Harrison and Schwarzacher 2007). Banana plants propagated through suckers normally remain true to type.

6.4 Tissue Culture-Based Technologies

In vitro culture is an important biotechnological tool to exploit the totipotency nature of plant cells. It can be used to mass propagate uniform and disease-free clones and ideal for gene pool conservation (Rout et al. 2006). This technique has been applied to banana, including shoot regeneration from cultured tissues by organogenesis, somatic embryogenesis, and protoplast culture.

6.4.1 Organogenesis

In vitro propagation via meristem culture is a powerful tool that can produce a large number of disease-free plants in a short span of time (Rout et al. 2006). In banana, various explants such as apical meristems, shoot tips, floral explants, and immature fruits have been used for in vitro propagation (Harirah and Khalid 2006; Tripathi et al. 2008a; Sultan et al. 2011; Ngomuo et al. 2014a). Shoot tip and suckers have been the most commonly used to initiate shoots through direct organogenesis, but cell suspension cultures (indirect regeneration) are also being developed. Most investigators reported cytokinin-dependent shoot multiplication (Table 6.1).

6.4.2 Embryogenic Cell Suspension Culture

Establishment of embryogenic cell suspension culture has become an important step for the banana regeneration through somatic embryogenesis. There have been few explants used to initiate embryogenic callus, such as proliferating meristems (Sholi et al. 2009), immature male and female flowers (Jalil et al. 2003; Husin et al. 2014; Namanya et al. 2014), immature zygotic embryos (Escalant and Teisson 1989), corm tissues, and leaf bases (Novak et al. 1989). Low embryogenic response and long initiation period remain significant limitations as only a few studies reported on somatic embryogenesis from banana and plantain (Table 6.2).

6.4.3 Somatic Embryogenesis

Somatic embryogenesis is the development of embryos from somatic cells. It offers a great potential for large-scale propagation, mainly due to its possibility to scale up the propagation using bioreactor, and serves as a platform for gene transformation

Table 6.1 The recent literatures on the explants and culture medium used to induce in vitro shoot organogenesis

Cultivar	Explant	Culture medium	Reference
Musa beccarii	Suckers and male buds	MS + sucrose, BAP, coconut water, charcoal	Rashid et al. (2012)
Musa spp. cv. Mzuzu	Suckers	MS + sucrose, BAP, ascorbic acid	Ngomuo et al. (2014b)
Musa paradisiaca L. (var. Poovan and Monthan)	Suckers	Macro and micro mixed nutrients + sugar, BAP, IAA	Dhanalakshmi and Stephan (2014)
Musa acuminata (AAA) cv. Vaibalhla	Immature male flowers	MS + Kin, NAA	Hrahsel et al. (2014)
Musa acuminata cv. Berangan	Suckers	MS + sucrose, ascorbic acid, BAP, IAA	Jafari et al. (2011)
Musa paradisiaca L.	Suckers and male inflorescences	MS + BAP, NAA	Ahirwar et al. (2012)
Musa spp. cv. Grand Nain	Shoot meristem with leaf primordial	MS + sucrose, pineapple/ coconut milk	Beshir et al. (2012)
Musa sp. cv. Berangan, Rastali, Nangka, and Abu	Male inflorescence flowers	MS + TDZ, BAP, Kin, Zea, 2-ip, sucrose	Darvari et al. (2010)
Musa sapientum cv. Anupam and Chini champa	Pseudostems from suckers	MS + BAP, Kin, IAA, IBA, sucrose	Mahdi et al. (2014)
Musa spp. cv. Grand Nain	Meristematic shoot tips	MS + BAP, IBA, thiamine, sucrose	Wilken et al. (2014)
Musa cv. Yangambi	Suckers	MS + BAP, IAA, sucrose	Ngomuo et al. (2013)
Musa sp. cv. Agnishwar	Suckers	MS + BAP, Kin, 2-ip, IBA, NAA	Rahman et al. (2013)
Musa spp. cv. Virupakshi and Sirumalai	Immature male flowers	MS + BAP, coconut water	Mahadev et al. (2011)
Musa spp. cv. Grand Nain	Shoot tips	MS + picloram, BAP	Remakanthan et al. (2014)
Musa sapientum L.	Shoot meristem from sucker	MS + BAP, IAA, coconut water	Iqbal et al. (2013)
Musa balbisiana cv. Kluai Hin	Suckers	MS + BAP, coconut water followed by MS + BAP	Kanchanapoom and Promsom (2012)
Musa sp. cv. Awak, Berangan, Rastali, and Raja	Suckers	MS + BAP	Hui et al. (2012)
Musa sp. cv. Mas, Nangka, Berangan, and Awak	Suckers	MS + BAP, IAA	Sipen and Davey (2012)
Musa sp. cv. Grand Nain	Suckers	MS + BAP, Kin	Shankar et al. (2014)

2-ip 2-isopentenyladenine, *MS* Murashige and Skoog, *BAP* 6-benzylaminopurine, *IAA* indole-3-acetic acid, *IBA* indole-3-butyric acid, *Kin* kinetin, *NAA* 1-naphthylacetic acid, *TDZ* thidiazuron, *Zea* zeatin

Table 6.2 Recent examples of explants and culture media used for the establishment of somatic embryos

Cultivar	Explant	Culture medium	Reference
Musa sapientum cv. Anupam	Leaf and stem segments	MS + 2,4-D, NAA	Pervin et al. (2013)
Musa spp. cv. French Sombre	Immature male flowers	MS + biotin, IAA, 2,4-D, NAA, sucrose (callus induction)	Grapin et al. (1996)
Hybrid cultivar FHIA-18		MS + biotin, glutamine, malt extract, proline, NAA, zeatin, kinetin, adenine, sucrose, lactose (embryogenic callus)	Kosky et al. (2002)
Musa spp. cv. Grand Nain			Vishnevetsky et al. (2011)
Musa acuminata cv. Berangan	Male inflorescences	MS + Dhed'a vitamins, myoinositol, biotin, IAA, NAA, 2,4-D, ascorbic acid, sucrose	Chin et al. (2014)
		MS + vitamins, BAP, sucrose	
Pisang Jajee	Embryo from seed	MS + BAP, NAA	Uma et al. (2011)
Musa acuminata cv. Culcutta 4	Meristematic domes of axillary sprouted buds	½ MS + ascorbic acid, 2,4-D, zeatin, malt extract, glutamine, biotin, casein hydrolysate, proline, sucrose	Torres et al. (2012)
Musa spp. cv. Gonja manjaya	Apical shoot tips	MS + sucrose, ascorbic acid, 2,4-D, zeatin (callus induction)	Tripathi et al. (2012)
		SH salts, MS vitamins, glutamine, malt extract, proline, sucrose, lactose, zeatin, kinetin, NAA, 2-ip (embryo development)	
		MS + myoinositol, sucrose, ascorbic acid (embryo maturation)	
		MS salts, Morelc vitamins, sucrose, IAA, BAP (embryo germination)	
Musa sp. cv. Dwarf Cavendish	Immature male flowers	MS + 2,4-D, IAA, NAA, biotin, sucrose (embryogenic callus initiation)	Perez et al. (2012)
		½ MS, ascorbic acid, sucrose (embryogenic cell suspension culture)	
		MS + biotin, BAP, IAA, sucrose (embryo maturation)	

(continued)

Table 6.2 (continued)

Cultivar	Explant	Culture medium	Reference
Musa acuminata cv. Matti, Chingan, and Njalipoovan	Immature male flowers	MS + TDZ, sucrose (embryogenic callus initiation)	Divakaran and Nair (2011)
		MS + biotin (embryo development)	
Musa acuminata cv. Sannachenkadali	Immature male flowers	MS + TDZ, sucrose (embryogenic callus initiation)	Divakaran and Nair (2011)
		MS + glutamine (embryo development)	
Musa spp. cv. Grand Nain	Shoot tips	MS + NAA, BAP/TDZ, glutamine	Remakanthan et al. (2014)
Musa acuminata cv. Berangan	Male inflorescence	MS + Dhed'a vitamins, biotin, IAA, NAA, 2,4-D, ascorbic acid, sucrose (embryogenic callus initiation)	Husin et al. (2014)
		MS + glutamine, sucrose (embryo development)	
Musa acuminata spp. *burmannica*	Embryo from seed	MS + 2,4-D	Uma et al. (2012)
Musa-AAA-EA	Immature male flowers	MS + biotin, IAA, 2,4-D, NAA, ascorbic acid, sucrose (embryogenic callus initiation)	Namanya et al. (2014)
cv. Nakyetengu		MS + 2,4-D, glutamine, malt extract, sucrose (embryogenic cell suspension)	
Musa acuminata cv. Berangan	Immature male flowers	MS + biotin, glutamine, ascorbic acid, sucrose, 2,4-D then transfer to MS + biotin, ascorbic acid, indole-3-acetic acid, NAA, 2,4-D, sucrose (embryogenic callus initiation); liquid MS biotin, ascorbic acid, glutamine, malt extract, 2,4-D, zeatin, sucrose (embryogenic cell suspension); MS + biotin, glutamine, malt extract, proline, sucrose (embryo development)	Jafari et al. (2015)

2-ip 2-isopentenyladenine, *2,4-D* 2,4-dichlorophenoxyacetic acid, *MS* Murashige and Skoog, *BAP* 6-benzylaminopurine, *IAA* indole-3-acetic acid, *IBA* indole-3-butyric acid, *Kin* kinetin, *NAA* 1-naphthylacetic acid, *TDZ* thidiazuron, *Zea* zeatin

(George et al. 2008). In banana, in vitro regeneration can be derived from meristematic tissues via direct organogenesis and from embryogenic cell suspension cultures via somatic embryogenesis (Remakanthan et al. 2014). However, direct organogenesis from a pre-existing meristem is not suitable for transformation as it can lead to the formation of chimeras. This has made somatic embryogenesis as an important prerequisite for genetic engineering.

Somatic embryogenesis in Musaceae was first reported by Cronauer and Krikorian (1983), who produced cell suspension-derived somatic embryos using apices as explants. Although their shoot tips were poorly developed, morphological examination showed that the small cell clusters were competent. In the latter reports, somatic embryos were induced from young male flower buds (Jalil et al. 2003; Kulkarni et al. 2006; Ghosh et al. 2009; Dai et al. 2010; Kulkarni and Bapat 2013), female flowers (Grapin et al. 2000), bracts (Divakaran and Nair 2011), and scalps (Strosse et al. 2006; Sadik et al. 2007; Sholi et al. 2009). Despite the many options, immature male flowers are still the most widely used starting material. Furthermore, the contamination rate of using immature male flowers is generally lower compared to suckers. In our laboratory, we produced cell suspension-derived somatic embryos for Mas and Berangan cultivars using immature male inflorescences (Jalil et al. 2003, 2008; Chin et al. 2014; Husin et al. 2014; Jafari et al. 2015). We developed complete plant regeneration from embryogenic cell suspension and characterized stages of somatic embryogenesis in Mas cultivar through morphological examination to discriminate somatic embryogenesis-specific cellular structures from those emerging through an organogenic route (Fig. 6.1).

The success of somatic embryo formation is largely dependent on factors associated with the donor plant, the culture medium, and the physical culture conditions. Table 6.2 shows the culture media and conditions used by several researchers to

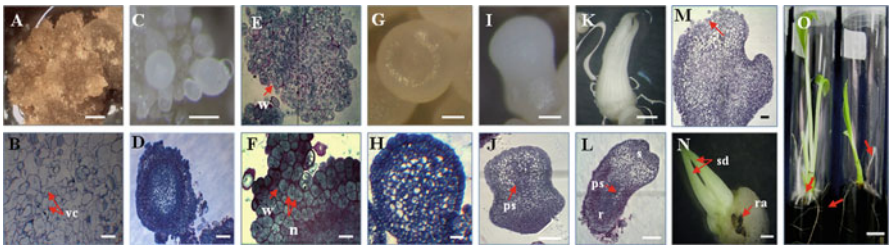

Fig. 6.1 Morphohistological changes in somatic embryogenesis. (**a**) Non-embryogenic callus (bar: 1 mm). (**b**) Histology of non-embryogenic callus (bar: 25 μm). (**c**) Embryogenic callus from male inflorescence (bar: 200 μm). (**d**) Histology of embryogenic callus (bar: 50 μm). (**e**) Cells with indistinct nuclei and poor protein storage when cultured in M2b medium (bar: 50 μm). (**f**) Meristematic cells with distinct nuclei when cultured in M2a medium (bar: 50 μm). (**g**) Globular embryo (bar: 250 μm). (**h**) Histology of globular embryo (bar: 100 μm). (**i**) Mature torpedo stage (bar: 500 μm). (**j**) Histology of mature torpedo embryo (bar: 200 μm). (**k**) Germinated embryo (bar: 1 mm). (**l**) Histology of germinated embryo (bar: 500 μm). (**m**) Irregular protodermal layer (bar: 100 μm). (**n**): Germinated embryo (bar: 1 mm). (**o**) Rooted plantlets derived from somatic embryos (bar: 1 cm). *vc* vacuolated cells, *w* wall, *n* nucleus, *ps* procambial strand, *s* shoot pole, *r* root pole (Reproduced from Jafari et al. 2015)

induce somatic embryos in banana. The current practices for plant regeneration through somatic embryogenesis using embryogenic cell suspension cultures, however, are limited by low rates of embryo germination and long culture duration (18–24 months) in culture medium containing high concentrations of plant growth regulators (Remakanthan et al. 2014). Owing to the strong genotypic influence, the strategy to develop "genotype-independent" embryogenic cell culture is still challenging (Kulkarni et al. 2007; Kulkarni and Bapat 2013). It is thus essential to demonstrate the regeneration of cell suspension cultures in commercially important banana cultivars.

6.4.4 Protoplast Culture

Protoplasts are naked cells where the cell wall has been completely or partially removed either enzymatically or mechanically (Eeckhaut et al. 2013). In principle, each individual protoplast is totipotent and has the potential to reform a cell wall and later proliferate or regenerate into various organs under appropriate chemical and physical stimuli (Khatri et al. 2010). Protoplast is a useful biological system that has been widely used to investigate the mechanism of cell wall formation, cell division, and proliferation (Aoyagi 2011). It facilitates plant genetic improvement technologies, such as somatic hybridization, electroporation, microprotoplast-mediated chromosome transfer, and DNA microinjection (Rezazadeh and Niedz 2015). Protoplast isolation and regeneration have been reported in many fruit species, such as banana (Haïcour et al. 2009), mango (Rezazadeh et al. 2011), grape (Yu et al. 2013), and guava (Rezazadeh and Niedz 2015).

In banana, successful isolation of viable protoplasts was first reported by Bakry (1984). Since then, much work has been reported on banana protoplast culture (Assani et al. 2006; Xiao et al. 2007). Numerous banana cultivars have been successfully regenerated from protoplast. These include banana cv. Bluggoe (Megia et al. 1993; Panis et al. 1993); Brazilian dessert banana (Matsumoto and Oka 1998); Grand Nain (Assani et al. 2001, 2002, 2006); Gros Michel, Currare Enano and Dominico, SF265, IRFA903, and Col49 (Assani et al. 2002); and Mas (Xiao et al. 2007). Despite the tremendous progress made in generating banana protoplast culture, the successes rate for banana protoplast culture is still low, mainly due to its recalcitrant characteristic. Banana plant regeneration from protoplast is also not yet routine for many cultivars. There is no universal method for the isolation and culture of protoplasts. Therefore, developing a simple and efficient protoplast isolation and regeneration protocol is essential. Several factors including cultivars, protoplast isolation, plating density, culture methods, types of digestion enzymes, nurse cells, and media addenda, such as plant growth regulators, must be considered carefully to ensure high success rate (Xiao et al. 2007; Zhou et al. 2008).

Different cultivars and source of materials influence the development of protoplasts. In the past years, Bakry (1984) reported that leaf material was not suitable for protoplast isolation. However, Chen and Ku (1985) successfully generated protoplast from the base tissue of the youngest leaf, but the generated protoplast did not

regenerate cell wall and undergo cell division. Matsumoto et al. (1988) produced protoplast with the ability to form cell wall and undergo cell division using bract tissues. However, the authors did not report any further development of the dividing protoplasts. Megia et al. (1992) reported protoplast regeneration and subsequent callus formation from embryogenic cell suspension cultures of *Musa acuminata* ssp. *burmannica* cv. Long Tavoy. Since then, cell suspension cultures have been used as a source for protoplast isolation (Megia et al. 1992, 1993; Panis et al. 1993; Assani et al. 2002, 2006; Xiao et al. 2007).

The composition, concentration, and incubation period of enzyme mixture have significant effect on the protoplast yield. The enzymes used must be able to degrade cellulose, hemicellulose, pectin, and, in some cases, callose (Bengochea and Dodds 1986). Cellulases, pectolyase, and macerozyme in different combinations and concentrations are commonly used for banana protoplast isolation (Assani et al. 2006; Xiao et al. 2007). The isolated protoplast is subject to osmotic stress after cell wall digestion. Different osmotic stabilizing agents, such as mannitol and sorbitol or combinations of both, are required to adjust the osmotic potential in the bathing incubation medium. For the development of protoplast, calli, and shoot regeneration, both auxins (2,4-dichlorophenoxyacetic acid, 1-naphthylacetic acid, and indole-3-acetic acid) and cytokinins (6-benzylaminopurine and zeatin) are widely included in culture media (Haïcour et al. 2009).

It is now over 120 years since the first crude preparations of plant protoplasts have been made (Klercker 1892). Despite the significant progress that has been achieved in refining the methodologies, several important challenges remain. With the continuing breakthrough in devising regeneration protocols, we anticipate these challenges may finally be resolved especially in the recalcitrant species like banana.

6.4.5 Micropropagation Using Bioreactor

Mass propagation of banana cell suspension cultures is usually carried out in shake flasks. Using modern bioreactors, such as stirred tank reactor, bubble column reactor, balloon-type bubble reactor, and air lift reactor, large-scale propagation of cells, tissues, somatic embryos, and plantlets in liquid suspension in short time is possible (Lee et al. 2011). However, several critical parameters and conditions, such as mixing, gaseous composition, efficient oxygen transfer, pH, and hydrodynamic forces, need to be optimized (Dong et al. 2013). Only a few studies have been reported on propagating banana cell cultures using bioreactor (Chin et al. 2014). For example, Kosky et al. (2002) successfully produced phenotypically normal tetraploid banana hybrid (FHIA-18) using 2 l CMF-100 bioreactor. Recently, we used 5 l balloon-type bubble column bioreactor (BTBCB) to propagate banana cell suspension cultures. We found that the cells incubated in the BTBCB with pH maintained at 5.7 showed higher cell growth rate (7.9×10^{-2} day^{-1}) compared to cells grown in BTBCB system without pH control (6.5×10^{-2} day^{-1}) and shake flasks (5.3×10^{-2} day^{-1}). Furthermore, the yield of cell cultures was increased to 210 % when inoculated in BTBCB over 14 days of culture (Chin et al. 2014). If all necessary culture conditions and physical

parameters are properly controlled, bioreactor may offer good potential for producing high-quality field-ready plants in a one-step process.

6.5 Pests and Diseases

Bananas and plantains are seriously threatened by pests and diseases, including *Fusarium* wilt, black Sigatoka, yellow Sigatoka, Moko disease, *Xanthomonas* wilt, banana bunchy top disease (BBTV), banana streak virus (BSV), weevils, and nematodes (Ploetz et al. 2015; reviewed in Ploetz and Evans 2015; Table 6.3).

Fusarium wilt (Panama disease) is one of the most destructive diseases in banana industry due to its impact on the variety "Gros Michel"-based export trades (Ploetz 2015). This disease was first reported in Australia in 1874. It spread to nearly all banana-growing regions and destroyed the variety "Gros Michel," leading to the replacement of the race 1-resistant "Cavendish" (AAA) cultivars in the 1950s and 1960s. However, in the past years, an extremely lethal pathogen known as "tropical race 4" has been spreading and causing substantial losses to "Cavendish" (Heslop-Harrison and Schwarzacher 2007). At present, there are limited options to protect susceptible cultivars from *Fusarium* wilt. Innumerable control methods have been tested, such as soil fumigation, application of fungicides, crop rotation or soil amendment, and flood fallowing (Ghag et al. 2015). Questionable efficacy and cost ineffectiveness of these methods have been reported. Furthermore, most of the reported control measures have not been tested in real field environments (Ploetz 2015). As there is no resistant variety available against "tropical race 4," the only option is to develop *Fusarium* wilt-resistant banana by genetic engineering. Efforts have been made to develop resistant cultivars against *Fusarium* using single

Table 6.3 The major diseases and pests of banana

Constraint	Caused by
Moko disease	Phylotypes IIA-6, IIB-3, and IIB-4 of *Ralstonia solanacearum*
Xanthomonas wilt (BXW)	*Xanthomonas campestris* pv. musacearum
Blood disease	*Ralstonia haywardii* subspecies celebensis
Fusarium wilt	*Fusarium oxysporum* F. sp. cubense
Anthracnose	*Colletotrichum musae*
Black leaf streak disease (BLSD)	*Mycosphaerella fijiensis*
Eumusae leaf spot	*Mycosphaerella eumusae*
Sigatoka leaf spot	*Mycosphaerella musicola*
Banana bunchy top disease (BBTV)	Banana bunchy top virus
Banana streak disease (BSV)	Banana streak virus
Weevil	*Cosmopolites sordidus*
Burrowing nematode	*Radopholus similis*
Lesion nematodes	*Pratylenchus coffeae* and *Pratylenchus goodeyi*

Table 6.4 Candidate genes used to develop *Fusarium*-resistant transgenic banana

Protein	Candidate gene	Reference
Defensin	*MsDef1*	Abdallah et al. (2010)
	Sm-AMP-D1	Ghag et al. (2014)
Non-expressor of pathogenesis-related proteins-1	*NPR1*	Endah et al. (2008)
Glycosyltransferase	*SsGT1*	Lorenc-Kukuła et al. (2009)
Thionin	*Thi2.1*	Epple et al. (1997)
Antimicrobial peptide	*CaAMP1*	Lee et al. (2008)
	Ace-AMP1	Mohandas et al. (2013)
Thaumatin-like protein	*tlp*	Mahdavi et al. (2012)
Xylem sap protein (silencing)	*XSP10*	Krasikov et al. (2011)

exogenous gene in banana (Ghag et al. 2015). Several candidate genes used for the development of *Fusarium* wilt-resistant banana are listed in Table 6.4.

Black sigatoka (black leaf streak disease), another fungal disease, has been reported to affect subsistence production of various banana cultivars and has become the most pathogenic and of greatest concern to banana growers. It causes premature ripening, alters flavor, and shortens the postharvest green life of fruits (Ploetz and Evans 2015). Black leaf streak disease was first observed in Fiji in the early 1960s (Rhodes 1964) and has spread rapidly to new banana-growing areas. Yield losses due to black leaf streak disease gradually increased to more than 50 % (Kovács et al. 2013), while chemical control of the disease increased production costs to 25–30 % (Marín et al. 2003). Development of resistant, agronomically acceptable cultivars and genomic studies of the pathogen are underway. The banana *Xanthomonas* wilt disease is spreading rapidly and threatens the livelihood of millions of farmers in East Africa (Tripathi et al. 2008b). Affected banana plants usually displayed yellowing and wilting leaves, uneven and premature ripen fruit with yellowish blotches in the pulp, and dark brown placental scars (Tushemereirwe et al. 2004). Although several controls such as cultural practices have been attempted, a long-term solution may again overcome by development of genetic resistance plants. Other viral diseases such as BBTV, BSV, and bract mosaic (caused by banana bract mosaic virus) also constrain banana production. Losses due to nematodes are severe when storms cause toppling of plants that have previously damaged by them (Tripathi et al. 2015). In banana plantations, nematodes are often controlled by periodic application of pesticides. Weevils have been considered as an another important pest for bananas and plantains which can reduce the yield up to 40 % (Tripathi et al. 2015).

6.6 Agricultural Practices

A good cultural practice is necessary to ensure a good crop. One of the recommended practices is pruning or thinning. Pruning the banana mat is essential to ensure best vegetative growth and increase fruit production. This activity is to prune

unnecessary suckers to keep each production unit to only to the mother plant and two or possibly three suckers (Arvanitoyannis and Mavromatis 2009). Pruning is done periodically by removing excess sprouts and old, dried, and sick leaves. Allowing numerous pseudostems to grow besides the mother plant may lead to small bunches of low-quality fruit as well as encourage disease development. Leaving some ample space between plants is important to avoid crowding and competition for water, light, and nutrients. Different ranges of spacing have been reported throughout the world. Common plant spacings range from 2.4×2.4 m to 3.4×3.4 m which result in 360–680 plants per acre (Arvanitoyannis and Mavromatis 2009).

Bananas are usually grown in a nutrient-rich, well-drained, and slightly acidic soil. Similar to other crops, bananas also require a considerable amount of water but are sensitive to waterlogged situations. Protection of fruits against pests is another farm practice to ensure high quality and free of unsightly insect blemishes in banana fruits on arrival. This can be done by placing a perforated polyethylene bag over the fruit bunch when it is approximately 2 weeks old. Besides using chemicals, biological control and the use of natural plant extract that has insecticide and fungicide properties have also been attempted. For instance, farmers in the Dominican Republic found that spraying a solution prepared from the plant extract of anamu (*Petiveria alliacea* L.) is efficient to control bugs and thrips. Tan et al. (2015) reported that the endophytic bacterial strain *Serratia marcescens* ITBB B5-1 isolated from the rubber tree exhibited a high potential for biological control against the *Fusarium* disease in banana. Recent study by Cavero et al. (2015) showed that *Trichoderma atroviride* is a potential biological control agent that was able to reduce severity of black sigatoka as effective as the fungicide, azoxystrobin.

6.7 Conclusion and Future Prospects

Sustainable banana production and agricultural practices are critical to ensure a constant supply of banana fruit to meet the world demand. Strategies that exploit both conventional and biotechnological approaches, such as in vitro culture-based technologies and genetic transformation, are useful in ensuring sustained fruit production for food security.

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In Vitro Plant Regeneration in Dainty Spur [*Rhinacanthus nasutus* (L.) Kurz.] by Organogenesis

7

T. Gouthaman, T. Senthil Kumar, A.S. Rao, and M.V. Rao

Abstract

A proficient organogenesis protocol was standardised for *Rhinacanthus nasutus*, a potential medicinal plant. MS medium supplemented with NAA (2.0 mg/l) + ascorbic acid (30 mg/l) was found to be more effective for callus induction. The highest number of 140.7 shoots/explant with shoot length of 15.5 cm from the callus derived from internode explant and 122.3 shoots/explant with shoot length of 11.0 cm from leaf explant-derived callus were observed on MS medium containing 2.0 mg/l BA + 2.0 mg/l KN + 2.0 mg/l NAA. IAA (1.0 mg/l) has showed effective rooting.

Keywords

Rhinacanthus nasutus • Internode • Leaf explant • Callus • Organogenesis • Plant growth regulators

T. Gouthaman
Department of Plant Science, Bharathidasan University,
Tiruchirappalli 620 024, Tamil Nadu, India

PG & Research Department of Botany, Government Arts College (M),
Krishnagiri 635 001, Tamil Nadu, India

T. Senthil Kumar
Department of Industry University Collaboration, Bharathidasan University,
Tiruchirappalli 620 024, Tamil Nadu, India

A.S. Rao
Department of Biotechnology, Bharathidasan University,
Tiruchirappalli 620 024, Tamil Nadu, India

M.V. Rao (✉)
Department of Plant Science, Bharathidasan University,
Tiruchirappalli 620 024, Tamil Nadu, India
e-mail: mvrao_456@yahoo.co.in

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7.1 Introduction

Rhinacanthus nasutus (L.) Kurz., commonly known as dainty spur, is a potential medicinal plant belonging to the family Acanthaceae. It has been reported to be distributed from the Southeast Asian countries including China, Thailand, Sri Lanka and India (Farnsworth and Bunypraphatasara 1992; Cramer 1998). Several parts of this plant have been used as traditional medicines to cure various ailments such as eczema, herpes, pulmonary tuberculosis, hepatitis, diabetes, hypertension, skin diseases, tinea versicolor, ringworm, itching, fungal infection, allergies, cancers and inflammation and as an antidote to snake venom (Rao et al. 2013; Kodama et al. 1993; Tewtrakul et al. 2009; Farnsworth and Bunypraphatasara 1992; Brimson and Tencomnao 2011). Various bioactive phytochemicals such as anthraquinones, sterols, triterpenes, flavonoids and naphthoquinones have been isolated from *R. nasutus* (Wu et al. 1995, 1998a, b; Sendl et al. 1996).

This plant grows during the rainy season, whereas in summer its aerial parts dry up and the underground root survives. Conventional propagation of *R. nasutus* is done by seeds, roots and cuttings (Cheruvathur and Thomas 2014), in which propagation through seeds is challenging due to higher susceptibility of the seeds to climatic variations. The high medicinal value and indiscriminate harvesting of this plant in the wild lead to loss of natural population at an alarming rate. Hence, there is an imperative need to develop a new approach for mass propagation and conservation of *R. nasutus*. The present study was focused to develop an in vitro protocol for the mass propagation of *R. nasutus* by organogenesis.

7.2 Materials and Methods

7.2.1 Plant Material

Rhinacanthus nasutus plants were collected from the banks of river Kollidam, Tiruchirappalli, Tamil Nadu, India. Internode and leaf explants were collected in a wetted polythene bag; thereafter, explants were washed under running tap water for 10 min to remove debris and further treated with commercial Teepol (detergent) for 2–3 min and thoroughly washed with sterile distilled water under laboratory condition. Surface sterilisation was done with 70% v/v ethanol for 1 min, 0.1% w/v $HgCl_2$ for 5 min and a final wash five times with sterile distilled water.

7.2.2 Medium and Culture Condition

The sterilised internode and leaf explants were cultured on callus induction medium: Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 0.5–5.0 mg/l indole-3- acetic acid (IAA), indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA) and 2–4-dichlorophenoxyacetic acid (2,4-D) (HiMedia, Bengaluru, India) either alone or combination with 2.0 mg/l NAA and

10–50 mg/l ascorbic acid (ASA) or citric acid (CA) (HiMedia, Bengaluru, India) or 2.0 mg/l NAA + 10–50 % coconut water (CW) or 2.0 mg/l NAA + 5–25 mg/l adenine sulphate (ADS) or glutamine (GLU) (HiMedia, Bengaluru, India). Green compact nodular callus derived from both explants was cultured on shoot regeneration medium: MS medium with various concentrations (1.0–5.0 mg/l) and combinations of 6-benzyl adenine (BA), 6-furfuryl amino purine (KN) and N-isopentenyl amino purine (2ip) (HiMedia, Bengaluru, India) and NAA (2.0 mg/l). In vitro-raised elongated shoots were transferred to rooting medium: MS medium with IAA, IBA and NAA (0.5–5.0 mg/l). All the cultures were maintained at 25 ± 2 ° C with 16 h photoperiod under white cool fluorescent light (Philips, India) with 35 μE M^{-2} S^{-1} light intensity and subcultured at 2-week intervals.

Regenerated plantlets were transferred to sterilised earthen pots (6 × 8 cm) containing sterile soil/sand/compost (150 g/pot) (3:1:1 v/v/v) and irrigated with 50 ml of tap water. The potted shoots were covered with transparent polythene bags to prevent desiccation and they were maintained in the culture room conditions. The relative humidity was reduced gradually, and after 1 month, plants were transferred to earthen pots (16 × 16 cm) filled with 2:1 (v/v) mixture of soil and organic manure (750 g/pot) and maintained in a greenhouse, subsequently planted in the field.

7.2.3 Statistical Analysis

Each experiment was repeated thrice with 20 replications each. The experimental design was random and factorial. The data were presented as mean ± S.E. and mean separation was performed using one-way analysis of variance (ANOVA) followed by Duncan's new multiple range test (DMRT). All the above calculations and analysis were carried out using SPSS statistical software ver. 11.5.0 (2002) for Windows (software package from SPSS Inc., 1989–2002; www.spss.com).

7.3 Results

7.3.1 Callus Induction

Auxin-induced callus from internode as well as leaf explants after 4 weeks of culture on MS medium (Figs. 7.4a and 7.5a). Among four auxins, NAA was the most effective for callus induction followed by 2, 4-D, IAA and IBA. Compact green nodular callus was obtained after the first subculture (Fig. 7.1).

Green compact callus was formed and initiated from the cut ends of the explants. MS medium with 2.0 mg/l NAA showed best response of 70.4 % of callus (dark green and compact) in internode explant and 66.5 % in leaf explant; 2, 4-D at 3.0 mg/l showed 45.6 % callus induction in internode explant and 39.2 % in leaf explant; IAA 2.0 mg/l induced 24.8 % white, loose or friable callus in internode explant with limited proliferation, followed by 22.6 % in leaf explant and IBA 2.0 mg/l showed 15.8 % brownish callus with minimal proliferation rate, followed by leaf explant (14.1 %) (Fig. 7.1).

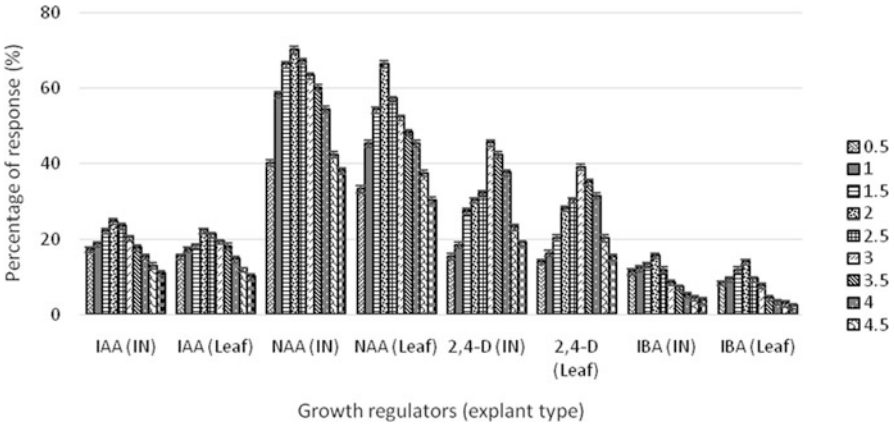

Fig. 7.1 Callus induction from internode and leaf explants of *Rhinacanthus nasutus* on MS-supplemented auxins, after 4 weeks. IN- Internode

The explants exude phenolic compounds in the culture medium during callus induction. The exudation was reduced by ASA and CA (10–50 mg/l). ASA 30 mg/l and CA 20 mg/l showed better callus induction with no phenolic exudation. The callus was greenish, compact and nodular with 86.4 % response from internode on ASA and 78.4 % response on CA (Fig. 7.5b, c) followed by 75.5 % from in ASA (Fig. 7.4b) and 72.6 % in CA from leaf explants (Figs. 7.2 and 7.3).

Coconut water (10–50 %) was supplemented to the medium towards improving callus induction and proliferation. Coconut water at 20 % showed 70.0 % green colour callus in internode explant, followed by 66.3 % brown callus in leaf explant (Figs. 7.2 and 7.3).

Along with the optimal concentration of NAA (2.0 mg/l), ADS (5–25 mg/l) and GLU (5–25 mg/l) presented improved callus induction and proliferation than CW. ADS at 15 mg/l showed the second-best response of 80.4 % callus in internode and 74.4 % in leaf explant (Figs. 7.2 and 7.3).

7.3.2 Shoot Regeneration

Shoot bud regeneration from primary callus (27 days old) of both explants was observed on MS medium fortified with various plant growth regulators, after 2 weeks of culture. The callus with and without tiny shoot buds were subcultured onto the medium with same treatment for further shoot proliferation, till a maximum number of shoots were obtained. The multiple shoots were subcultured at a 2-week interval for further proliferation. All the calluses obtained from internode and leaf explants produced maximum number of shoots in 8 weeks of culture. The rate of shoot multiplication was high in the initial subculture on the fresh medium, but later there was a gradual decline.

The mean number of shoot production was increased with the increase in cytokinin concentrations. BA played a key role in inducing regeneration of shoot buds and

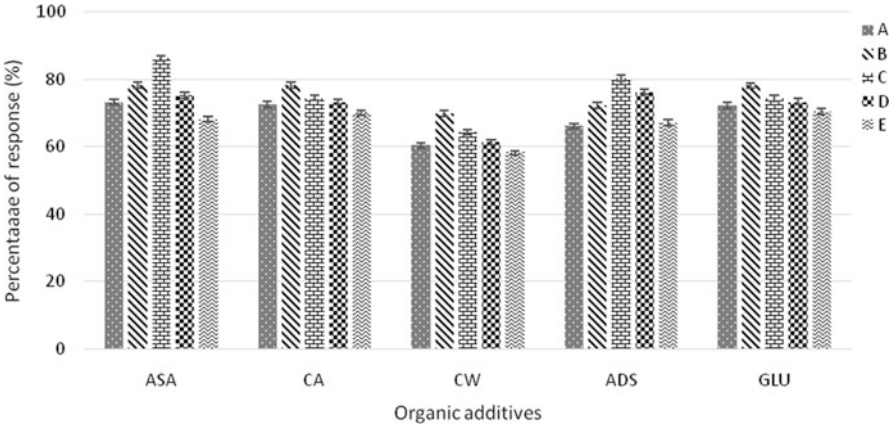

Fig. 7.2 Callus induction from internode explants of *Rhinacanthus nasutus* cultured in MS medium + 2.0 mg/l NAA and organic additives, after 4 weeks. ASA and CA concentrations of A–E were 10, 20, 30, 40 and 50 mg/l respectively; CW concentrations of A–E were 10, 20, 30, 40 and 50 % respectively; ADS and GLU concentrations of A–E were 5, 10, 15, 20 and 25 mg/l

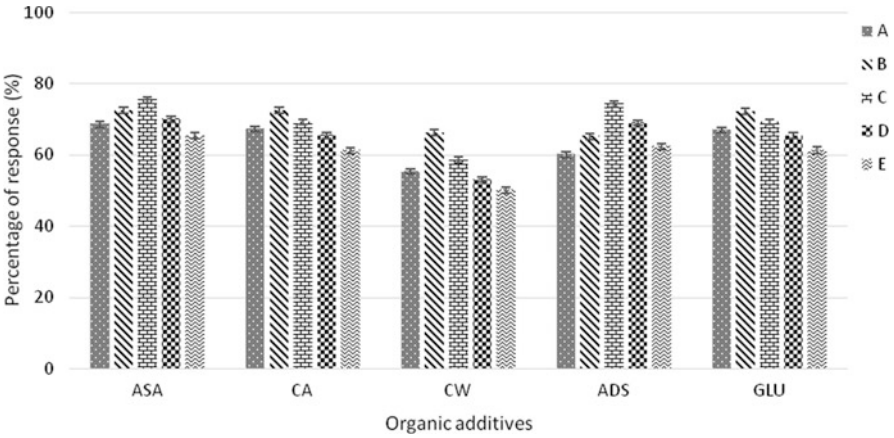

Fig. 7.3 Callus induction from leaf explants of *Rhinacanthus nasutus* cultured in MS medium + 2.0 mg/l NAA and organic additives, after 4 weeks. ASA and CA concentrations of A–E were 10, 20, 30, 40 and 50 mg/l respectively; CW concentrations of A–E were 10, 20, 30, 40 and 50 % respectively; ADS and GLU concentrations of A–E were 5, 10, 15, 20 and 25 mg/l

proved essential for multiple shoot induction and proliferation. The number of shoot formation varied with the concentrations of BA supplemented in the medium. BA treatments suppressed callus formation and rooting and produced the maximum number of shoot buds. BA 2.0 mg/l showed the best shoot regeneration from both explants within 8 weeks of culture (Figs. 7.4c and 7.5d). Maximum shoot regeneration (69 %) with 64.5 shoots and shoot length of 5.7 cm was observed in the callus elicited from internode explant, followed by leaf explant-derived callus (59.3

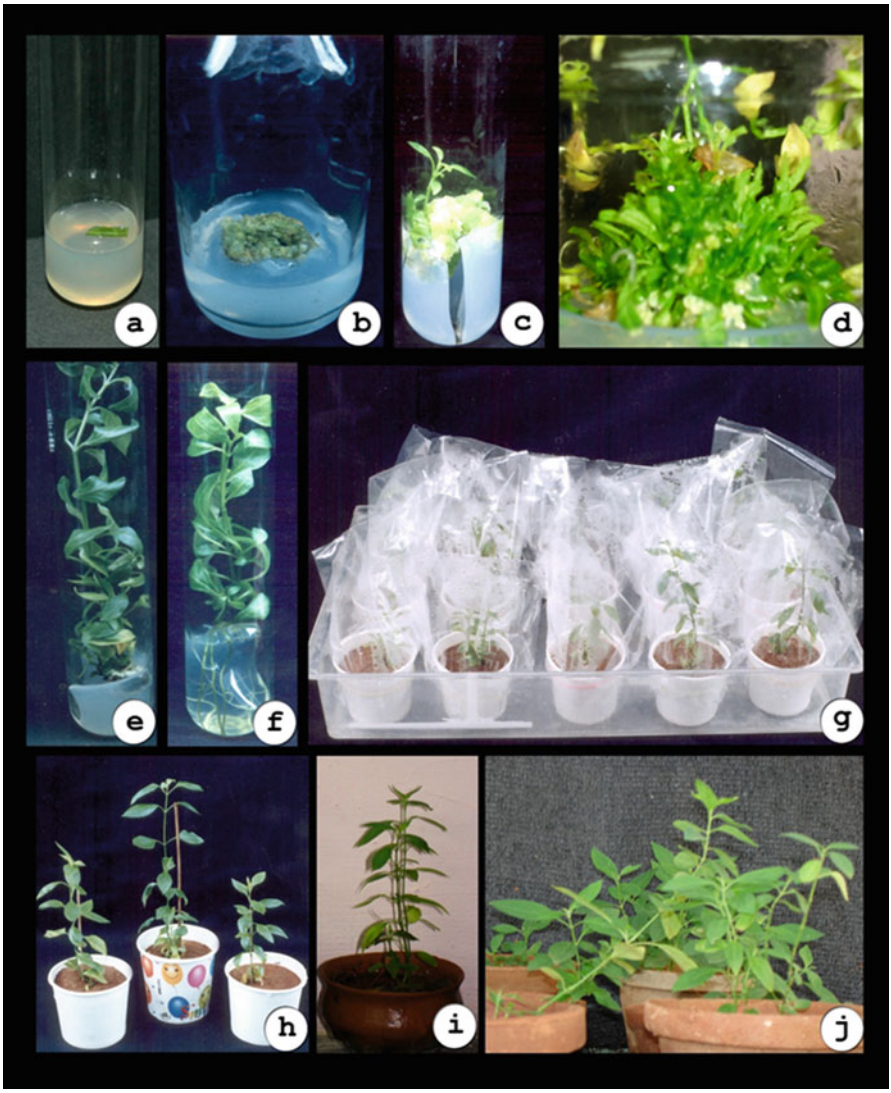

Fig. 7.4 Indirect organogenesis of *Rhinacanthus nasutus* via leaf explant. (**a**) Leaf. (**b**) Green compact callus. (**c**) Regeneration of shoots. (**d**) Multiple shoots induction. (**e**) Shoot elongation. (**f**) Rooting. (**g**) In vitro acclimatisation using polybags. (**h, i**) In vitro acclimatisation. (**j**) Hardened plantlets in the greenhouse

shoots/callus with shoot length 5.5 cm) (Table 7.1). KN treatments showed less number of shoots compared to BA, while 2iP treatment showed callus proliferation along with the multiple shoot induction.

Combinational treatments of BA and KN resulted in an increase in shoot production. The highest shoot bud regeneration (78 %) with 98.3 shoots and shoot length of 13.3 cm was observed on 2.0 mg/l BA and 2.0 mg/l KN from callus induced from

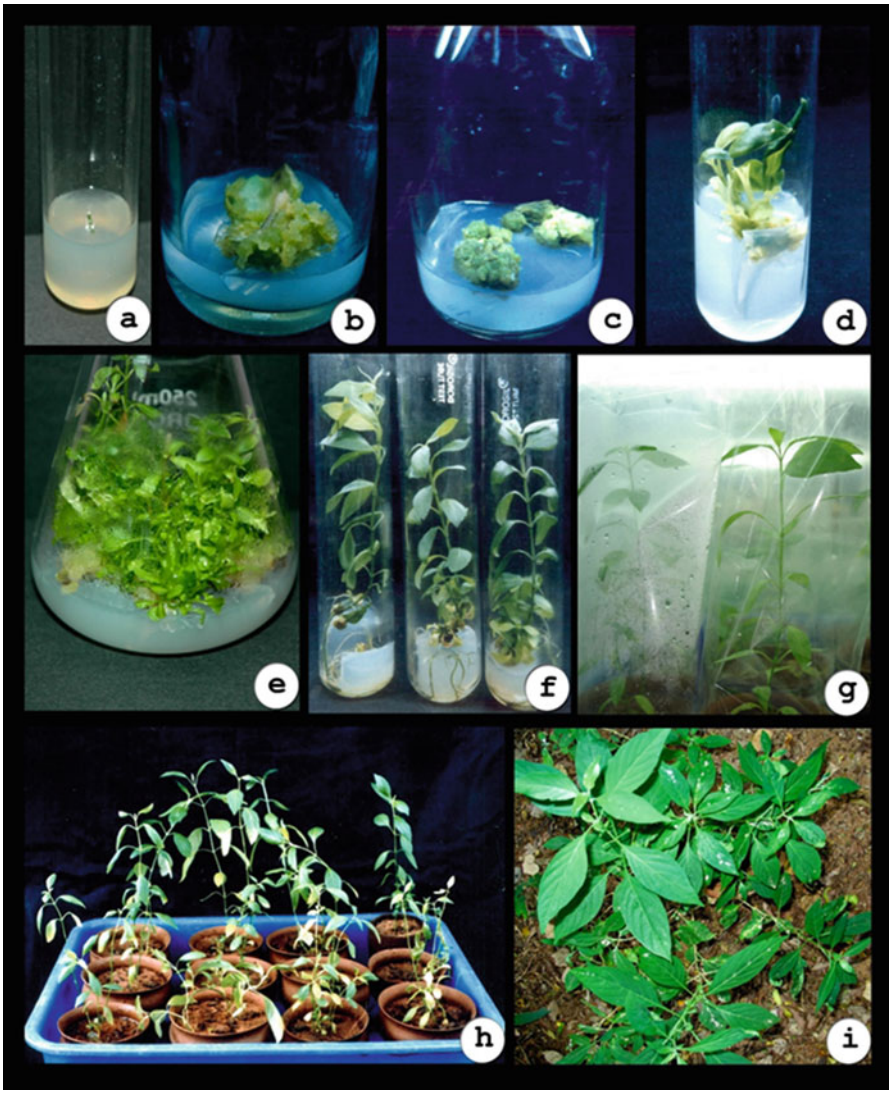

Fig. 7.5 Indirect organogenesis of *Rhinacanthus nasutus* via internode explant. (**a**) Internode. (**b, c**) Green compact callus. (**d**) Regeneration of shoots. (**e**) Multiple shoots. (**f**) Shoot elongation and Rooting. (**g, h**) In vitro acclimatisation. (**i**) Field-established plant

internode followed by leaf explant-derived callus (69 %; 81.6 shoots/callus; shoot length 9.4 cm), within 8 weeks of culture (Table 7.2). Increase in concentration of BA along with KN enhanced shoot regeneration 60 %.

Synergistic treatment of NAA (2.0 mg/l) with BA and KN combinations enhanced the shoot bud production, compared to BA + KN and BA alone treatments. In these treatments, the secondary multiple shoots produced on subcultures showed little

Table 7.1 Multiple shoot induction from the callus derived from internode and leaf explants of *Rhinacanthus nasutus* on MS medium supplemented with cytokinins, after 8 weeks

	Multiple shoots/explant		Shoot length (cm)	
Cytokinin (mg/l)	Internode	Leaf	Internode	Leaf
BA				
1.0	59.5 ± 0.80^{c}	48.4 ± 0.67^{c}	5.4 ± 0.72^{ab}	5.4 ± 0.72^{ab}
2.0	64.5 ± 0.78^{a}	59.3 ± 0.52^{a}	5.7 ± 0.56^{a}	5.5 ± 0.69^{a}
3.0	60.5 ± 0.54^{b}	57.4 ± 0.78^{b}	5.2 ± 0.61^{b}	4.5 ± 0.64^{b}
4.0	55.3 ± 0.40^{d}	52.4 ± 0.77^{d}	4.3 ± 0.56^{cd}	4.3 ± 0.66^{c}
5.0	52.4 ± 0.72^{e}	49.4 ± 0.78^{e}	4.2 ± 0.70^{d}	4.2 ± 0.70^{bc}
KN				
1.0	40.4 ± 0.36^{g}	39.6 ± 0.96^{h}	3.3 ± 0.70^{f}	3.3 ± 0.61^{e}
2.0	44.6 ± 0.75^{f}	43.4 ± 0.65^{f}	3.4 ± 0.65^{ef}	3.6 ± 0.56^{de}
3.0	36.4 ± 0.62^{h}	40.4 ± 0.62^{g}	3.5 ± 0.72^{e}	3.6 ± 0.55^{de}
4.0	32.1 ± 0.58^{i}	33.3 ± 0.60^{i}	3.3 ± 0.69^{f}	3.3 ± 0.66^{e}
5.0	26.5 ± 0.79^{j}	24.3 ± 0.56^{j}	3.0 ± 0.60^{g}	3.2 ± 0.63^{ef}
2iP				
1.0	18.4 ± 0.75^{m}	15.4 ± 0.67^{m}	3.1 ± 0.60^{fg}	3.3 ± 0.78^{e}
2.0	19.4 ± 0.65^{l}	18.6 ± 0.66^{l}	4.2 ± 0.47^{d}	3.7 ± 0.66^{d}
3.0	25.6 ± 0.52^{k}	23.5 ± 0.56^{k}	4.5 ± 0.55^{c}	4.2 ± 0.72^{bc}
4.0	18.4 ± 0.65^{m}	18.4 ± 0.69^{l}	3.4 ± 0.43^{ef}	3.2 ± 0.66^{ef}
5.0	16.3 ± 0.76^{n}	15.4 ± 0.77^{m}	1.8 ± 0.40^{h}	2.5 ± 0.58^{f}

Each value represents the mean ± S.E. of 20 replicates per treatment for stem and leaf explants and repeated three times. Values with the same superscript are not significantly different at 5 % level probability level according to Duncan's new multiple range test

Table 7.2 Multiple shoot formation from the callus derived from internode and leaf explants of *Rhinacanthus nasutus* on MS medium supplemented with BA and KN, after 8 weeks

		Multiple shoots/explant		Shoot length (cm)	
Cytokinin (mg/l)		Internode	Leaf	Internode	Leaf
BA	KN				
1.0	1.0	60.2 ± 0.64^{d}	55.4 ± 0.78^{e}	11.1 ± 0.46^{b}	7.4 ± 0.68^{b}
2.0	2.0	98.3 ± 0.62^{a}	81.6 ± 0.55^{a}	13.3 ± 0.64^{a}	9.4 ± 0.76^{a}
3.0	3.0	73.5 ± 0.34^{b}	65.5 ± 0.70^{b}	9.3 ± 0.84^{a}	8.3 ± 0.63^{c}
4.0	4.0	56.3 ± 0.63^{c}	52.4 ± 0.75^{c}	7.4 ± 0.72^{c}	6.4 ± 0.78^{bc}
5.0	5.0	48.3 ± 0.64^{e}	46.4 ± 0.80^{d}	5.4 ± 0.83^{c}	4.8 ± 0.53^{d}

Each value represents the mean ± S.E. of 20 replicates per treatment for stem and leaf explants and repeated three times. Values with the same superscript are not significantly different at 5 % level probability level according to Duncan's new multiple range test

basal callus. Initially, both the explants swelled to enlarge in size and then green compact callus initials formed at the margins. The highest percentage of regeneration (92 %) was observed in 2.0 mg/l BA + 2.0 mg/l KN + 2.0 mg/l NAA from the callus induced from internode explant, followed by leaf explant-derived callus 80 %. At these treatments, the highest number of 140.7 shoots with shoot length

Table 7.3 Multiple shoot formation of callus derived from internode and leaf explants of *Rhinacanthus nasutus* on MS medium + 2.0 mg/l NAA and BA + KN, after 8 weeks

PGR (mg/l)		Multiple shoots/explant		Shoot length (cm)	
		Internode	Leaf	Internode	Leaf
BA	KN				
1.0	1.0	93.2 ± 0.74^{d}	79.7 ± 0.67^{d}	12.1 ± 0.66^{ab}	9.1 ± 0.42^{b}
2.0	2.0	140.7 ± 0.52^{a}	122.3 ± 0.42^{a}	15.5 ± 0.34^{a}	11.0 ± 0.66^{a}
3.0	3.0	87.5 ± 0.37^{b}	73.4 ± 0.68^{b}	13.7 ± 0.44^{b}	8.9 ± 0.45^{c}
4.0	4.0	66.5 ± 0.57^{c}	60.2 ± 0.67^{c}	9.4 ± 0.82^{bc}	7.5 ± 0.62^{cd}
5.0	5.0	51.2 ± 0.70^{e}	49.1 ± 0.55^{e}	8.9 ± 0.37^{c}	5.0 ± 0.52^{d}

Each value represents the mean ± S.E. of 20 replicates per treatment for stem and leaf explants and repeated three times. Values with the same superscript are not significantly different at 5 % level probability level according to Duncan's new multiple range test
PGR plant growth regulator

15.5 cm was recorded from internode-derived callus and 122.3 shoots with shoot length 11.0 cm was recorded from leaf-derived callus, within 8 weeks of culture (Table 7.3; Figs. 7.5e and 7.4d). This combination was so effective to get a quick response in inducing the shoot formation. The regenerated shoots were subcultured on the same medium for further shoot growth. The shoot elongation was *de novo* in the respective concentrations and combinations of the medium. It was observed that the maximum shoot length (15.5 cm) was also obtained on the above treatment, after 8 weeks of culture period (Table 7.3; Fig. 7.4e and 7.5f).

7.3.3 Rooting

In vitro-raised shoots were transferred to rooting medium – MS medium supplemented with auxins: IAA, IBA and NAA (0.5–5.0 mg/l). The rooting response was varied with auxin type and their concentrations. The highest number of 8.6 roots/shoot with an average length of 15.5 cm was recorded on IAA 1.0 mg/l (Table 7.4; Figs. 7.5f and 7.4f) followed by IBA 2.0 mg/l (6.2 roots/shoot with root length 4.8 cm) and NAA 2.5 mg/l (5.8 roots/shoot with root length 3.9 cm) (Table 7.4).

7.3.4 Hardening

Regenerated plantlets transferred to the sterilised earthen pots for hardening acclimatised well under in vitro and green house conditions (Figs. 7.4g–j and 7.5g–i). The survival rate of hardened plantlets under filed transformation was 85 %.

Table 7.4 Root induction of shoots raised by organogenesis of internode and leaf explants of *Rhinacanthus nasutus* on MS medium supplemented with auxins, after 8 weeks

Auxin (mg/l)		Roots/shoot	Root length (cm)
IAA	0.5	6.2 ± 0.62^{cd}	12.3 ± 0.67^{ef}
	1.0	8.6 ± 0.59^{a}	15.5 ± 0.72^{d}
	1.5	8.0 ± 0.48^{b}	13.4 ± 0.87^{b}
	2.0	7.2 ± 0.55^{c}	10.5 ± 0.54^{a}
	2.5	6.0 ± 0.67^{de}	9.0 ± 0.72
	3.0	5.4 ± 0.44^{e}	8.8 ± 0.66^{de}
	3.5	4.0 ± 0.72^{fg}	7.2 ± 0.35^{e}
	4.0	4.0 ± 0.49^{gh}	6.7 ± 0.57^{f}
	4.5	2.8 ± 0.57^{i}	5.4 ± 0.68^{g}
	5.0	1.5 ± 0.61^{lm}	4.3 ± 0.47^{hi}
NAA	0.5	2.0 ± 0.37^{ij}	2.2 ± 0.24^{j}
	1.0	3.6 ± 0.42^{h}	2.8 ± 0.28^{jk}
	1.5	4.2 ± 0.48^{i}	3.0 ± 0.32^{l}
	2.0	4.4 ± 0.57^{j}	3.4 ± 0.37^{lm}
	2.5	5.8 ± 0.70^{jk}	3.9 ± 0.24^{m}
	3.0	5.0 ± 0.63^{kl}	3.5 ± 0.28^{mn}
	3.5	4.6 ± 0.54^{kl}	2.7 ± 0.36^{no}
	4.0	3.9 ± 0.50^{lm}	2.2 ± 0.37^{o}
	4.5	2.5 ± 0.44^{m}	1.8 ± 0.22^{p}
	5.0	2.0 ± 0.32^{mn}	1.1 ± 0.29^{pq}
IBA	0.5	4.8 ± 0.66^{ij}	3.7 ± 0.43^{j}
	1.0	5.0 ± 0.58^{h}	3.9 ± 0.36^{jk}
	1.5	5.7 ± 0.44^{i}	4.3 ± 0.48^{l}
	2.0	6.2 ± 0.36^{j}	4.8 ± 0.42^{m}
	2.5	5.9 ± 0.39^{k}	4.5 ± 0.38^{m}
	3.0	5.3 ± 0.45^{kl}	4.0 ± 0.47^{mn}
	3.5	4.0 ± 0.51^{kl}	3.6 ± 0.33^{no}
	4.0	3.6 ± 0.37^{lm}	3.0 ± 0.26^{o}
	4.5	2.4 ± 0.38^{m}	2.8 ± 0.20^{p}
	5.0	1.7 ± 0.27^{mn}	2.0 ± 0.23^{pq}

Each value represents the mean ± S.E. of 20 replicates per treatment for stem and leaf explants and repeated three times. Values with the same superscript are not significantly different at 5 % level probability level according to Duncan's new multiple range test

7.4 Discussion

7.4.1 Callus Induction

From the results, it was inferred that NAA induced maximum callus in both explants followed by 2, 4-D, IAA and IBA. The internode explants showed better callus induction followed by leaf explants. The effect of auxin and their concentration

have a significant role for callus initiation using various explants. The effective response of NAA followed by 2, 4-D was also reported by Famelaer et al. (1996) in *Tulipa gesneriana*. Sudhakar et al. (2006) observed callus induction from leaf and nodal explants of *R. nasutus* on MS media supplemented with 0.5 mg/l 2, 4-D, whereas Meena and Dennis (2014) observed from root explant of *R. nasutus* on MS medium supplemented with 3.0 μM KN and 0.4 μM IBA. We observed maximum callus induction in NAA followed by 2, 4-D. Liu et al. (1997) also reported that the exogenous NAA caused the accumulation of endogenous IAA through reducing the activity of IAA oxidase and vice versa in soybean. The influence of NAA on callus growth may be due to regulation of polyamine metabolic enzymes through activation of the genes coded for these enzymes (McClure and Guilfoyle 1989) or through regulating these enzymes' activities.

To increase the efficiency of callus formation and to control the phenolic exudation, MS medium was supplemented with ASA (10–50 mg/l), CA (10–50 mg/l), coconut water (10–50 %) and ADS and GLU (5–25 mg/l) in combination with 2.0 mg/l NAA. Among all the treatments, 30.0 mg/l ASA showed the best response of 86.4 % of callus induction and proliferation. However, there are only very few reports supporting the role of ASA and CA in morphogenesis (Dhar and Upreti 1999). Importance of ASA in plant cells has long been recognised initially in controlling the redox system of cells and such processes as seed germination, growth, oxidative photophosphorylation, stimulation of RNA synthesis, bud development and prevention of senescence (Sweet and Guruprasad 1997). Organic acids and their potassium and sodium salts stabilise the pH of hydroponic solution (George and Sherrington 1984) or in vitro media (Arnow et al. 1953). Murashige and Tucker (1969) observed pronounced growth stimulation in CA treatment. CA is an absolute prerequisite for respiration and biological oxidation. The major fate of citrate in plant tissues appears to be metabolism via TCA cycle or via glyoxylate cycle. The beneficial effect of CA may be due to its antioxidant property due to the increase in morphogene-specific storage proteins by organic acids (Nichol et al. 1991). ADS was effective in organogenesis from callus in suspension cultures in garlic (Cid et al. 1999). Beneficial effects from adenine addition are found in media containing both ammonium nitrate and cytokinins (Nickerson 1978; Pyott and Converse 1981). Adenine can serve as a precursor for zeatin synthesis, but the rate is low (Dickinson et al. 1986). Addition of coconut milk improved regeneration of garlic callus (Ayabe et al. 1995). Komalavalli and Rao (2000) observed that the nature of the explant, seedling age, medium type, plant growth regulators, complex extracts (casein hydrolysate, coconut milk, malt extract and yeast extract) and antioxidants (activated charcoal, ASA, CA and polyvinylpyrrolidone) markedly influenced in vitro propagation of *Gymnema sylvestre*.

7.4.2 Shoot Regeneration

Green, compact nodular callus induced shoot buds rather than other types. The balance between auxin and cytokinins is necessary for callus initiation and growth. A

high cytokinin-to-auxin ratio is generally required (George 1993). BA was the most effective cytokinin for induction of shoots in *Ophiorrhiza prostrata* from leaf and internode explants via shoot organogenesis (Beegum et al. 2007). Multiple shoot regeneration from other medicinal plant species such as *Coleus forskohlii* (Reddy et al. 2001), *Echinacea purpurea* (Koroch et al. 2002), Roman chamomile (Echeverrigaray et al. 2000), *Silene vulgaris* (Jack et al. 2005) and *Thapsia garganica* on MS medium supplemented with different concentrations of NAA, IAA, 2,4-D and BA, KN (Makunga et al. 2005). In *Phaseolus lunatus* callus induction and shoot regeneration was achieved using epicotyl, cotyledon and hypocotyl explants on MS medium supplemented with TDZ (0.5 mg/l), IAA (0.05 mg/l) for callus formation and BA (1.0 mg/l) for shoots induction (Kanchiswamy and Maffei 2008).

A combination of two or more growth regulators is required for organogenesis, either applied simultaneously or sequentially (Evans et al. 1981). A balanced ratio of growth regulators plays an important role in differentiation of shoot buds (Wilson et al. 1996). The results obtained were similar with the abovesaid references. The shoots produced were grown luxuriantly in combination with cytokinin and auxin treatments. Similar results were reported by Bhati et al. (1992) who observed enhanced production of shoots per explant with incorporation of auxins with optimal concentrations of cytokinins. Depending on the concentration of BA, KN and NAA either inhibited, stimulated or did not affect shoot multiplication, which also depended on the cytokinin level (Al-Bahrany 2002).

For the induction of shoot buds, BA is superior to other cytokinins, which may be due to the plant tissues which readily metabolise than the other synthetic growth regulators, and also BA has the ability to produce zeatin endogenously (Barna and Wakhlu 1993). In the present study, BA and KN combination showed better response than individual cytokinin treatment. Synergistic effect of auxin along with cytokinins improved shoot regeneration potential. BA and KN in combination with NAA produced maximum number of shoots.

Sudhakar et al. (2006) reported only 34.1 shoots/explant from the callus induced from nodal explant of *R. nasutus* on MS medium with 1.5 mg/l KN, wherein GA_3 0.4 mg/l showed maximum shoot length of 8.0 cm. Whereas, Meena and Dennis (2014) reported 28.3 shoots on MS medium with 5.0 μM BA and 0.7 μM NAA. Our study showed improved shoot development of 140 shoots/explant from internode explant followed by 122.3 shoots/explant from leaf explant. This clearly indicates the auxin-cytokinin combination is superior in the shoot regeneration. Shoot number per explant was strongly influenced by genotype, culture medium and application of growth regulators (Burbulis et al. 2005).

7.4.3 Rooting

Auxins (IAA, IBA and NAA) were tested for in vitro rooting. IAA and IBA were found to be more effective for root induction. Roots were greenish in colour and longer with numerous branches with secondary hairs on IAA containing medium,

whereas on IBA and NAA treatments, the roots were light greenish and white with lesser secondary hairs. Rooting usually follows treatment with auxin alone, or with mixtures containing more auxin than cytokinin, exogenous cytokinins are more inhibitory. Auxin-induced root formation is thought to require, or induce, the promotion of polyamine synthesis (George 1993) was of the opinion that auxin promotes polyamine synthesis which leads further to root formation. Sudhakar et al. (2006) reported best rooting of shoots induced from the callus of nodal explants of *R. nasutus* on half-strength MS medium containing 1.0 mg/l IBA. Meena and Dennis (2014) reported maximum rooting (3.4 roots/ shoot) in shoots derived from the callus of root explants of *R. nasutus* on MS medium with 4.0 μM IBA. In the present study, the best root induction was achieved in IAA 1.0 mg/l. The rooted plantlets appeared to be phenotypically normal.

7.4.4 Acclimatisation

Since in vitro-raised plantlets lack epicuticular wax and ineffective stomata function (Sutter 1985), they have to be transferred to acclimatisation process (Murali and Duncan 1995). Hence, the plantlets were potted and kept in the same microenvironment (culture room) by covering it with polybags and subsequently removed and continued in the same environment. The physical and environmental condition of culture and the medium composition and the timing of treatment applications are also important for obtaining shoot multiplication from tissues of species exhibiting low competence (Vasil and Thorpe 1994). The survival rate was 85 % in the present study.

7.5 Conclusion

The present study reported an efficient and improved organogenesis protocol for *R. nasutus*. Internodal explant yielded 140.7 shoots/explant with shoot length of 15.5 cm from the callus, whereas leaf explant showed 122.3 shoots/explant with shoot length of 11.0 cm on MS medium with 2.0 mg/l BA + 2.0 mg/l KN + 2.0 mg/l NAA. Effective rooting was observed on IAA (1.0 mg/l). This protocol can be helpful for conservation strategies and phytochemical studies of *Rhinacanthus nasutus*.

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Application of Tissue Culture for *Laburnum anagyroides* Medik. Propagation

8

S.N. Timofeeva, L.A. Elkonin, O.I. Yudakova, and V.S. Tyrnov

Abstract

Laburnum anagyroides, a small tree or shrub from the Fabaceae family, is a promising object for its use in decorative landscaping and as a source of pharmaceuticals. The conventional propagation methods are not always successful for *L. anagyroides*. Herein, we describe an approach involving the application of tissue culture techniques for its micropropagation. This approach is based on activation of the pre-existing meristems from the axillary buds taken from a mature tree or seedling explants and includes the following phases: (1) preparation of primary explants and their cultivation on the Murashige and Skoog (MS) medium supplemented with 2.22 μM 6-benzylaminopurine (BAP), hot water pretreatment with subsequent cultivation of the seeds on a MS medium used to overcome the physical dormancy of seeds, (2) proliferation of the initiated explants on the full-strength (for seedling explants) or ½ MS medium (for axillary buds) with 2.22 μM BAP, (3) rooting of individual shoots on the ¼ MS medium supplemented with 2.68 μM α-naphthaleneacetic acid, and (4) acclimatization of the plants by spraying with the Emistim® elicitor. It was found that the explanting season also affected the initiation frequency from axillary buds but did not influence the culture initiation from seedling explants. In the tissue culture of both buds and seedlings, BAP not only stimulated higher number of shoots but also ensured the

S.N. Timofeeva
Botanical Garden, Saratov State University, Navashina str., 1, Saratov 410010, Russia

L.A. Elkonin (✉)
Department of Biotechnology, Agricultural Research Institute of South-East Region, Tulaikov str., 7, Saratov 410010, Russia
e-mail: lelkonin@gmail.com

O.I. Yudakova • V.S. Tyrnov
Department of Genetics, Saratov State University, Astrakhanskaya str., 83, Saratov 410012, Russia

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development of normal shoots compared with the thidiazuron-containing medium. The results of our study can be used for the mass propagation of *L. anagyroides* and for obtaining good-quality seedlings suitable for gardening and for pharmaceutical industry.

Keywords
Laburnum anagyroides • Micropropagation • Plant growth regulators • Acclimatization • Seed dormancy

8.1 Introduction

Laburnum anagyroides Medik. is a large shrub or small tree from the Fabaceae family. It is native to the Central and Southern Europe mountains and is well adapted to temperate climates. The total life span of the tree is about 20 years; it is fast growing and comes into fruition in 3–5 years. It can fix atmospheric nitrogen within its specialized root nodules. It is quite demanding to the growing conditions (light, soil composition, moisture) and grows on a range of soils provided the soil is well drained and does not dry out for extended periods. In the sharply continental climate conditions (the Lower Volga region of Russia), it freezes in the winter, but recovers quickly.

L. anagyroides is a very beautiful ornamental shrub, especially during flowering period, when its golden-yellow flowers gathered into graceful, pendulous-flowered brushes up to 30 cm, creating the illusion of "golden rain" for which it has received a second title of Golden Chain Tree. Owing to its attractive appearance, it is used as a popular ornamental plant in parks and gardens throughout Europe (Szentesi and Wink 1991; Hewood 1993).

L. anagyroides is well known as a poisonous plant. Various parts of the plant contain an alkaloid cytisine, which is especially abundant in mature seeds (up to 5 %). In small doses, cytisine causes respiration excitation, raises blood pressure, and slows down the heart rate. In toxic doses, cytisine causes loss of consciousness and respiratory arrest. In addition, cytisine being a nicotinic receptor is used in the medicinal product Tabex® for smoking cessation (Tutka and Zatonski 2006; Tzancova and Danchev 2007).

Traditionally, the tree has been propagated through seeds and also by layering or grafting (Hartmann et al. 2010). However, conventional propagation methods are not always successful for the tree, and therefore, clonal propagation through tissue culture can be a reliable alternative to the traditional methods of propagation. Stimulation of the pre-existing meristems of the axillary bud has already been recommended as a most successful clonal technique for the in vitro plant regeneration of trees and shrubs (Harry and Thorpe 1994).

There are numerous reports on micropropagation of the species belonging to Leguminosae family such as various *Acacia* species (Beck and Dunlop 2001; Vengadesan et al. 2002), *Ceratonia siliqua* (Romano et al. 2002), the tropical tree

legumes (Hong and Bhatnagar 2007), *Robinia pseudoacacia* (Kanwar et al. 2007), *Vitex negundo* (Ahmad and Anis 2007), *Albizia odoratissima* (Rajeswari and Paliwal 2008), and *Cassia siamea* (Parveen et al. 2010). The purpose of our study was to elaborate a technology for *L. anagyroides* propagation by activation and proliferation of the pre-existing meristems from axillary buds taken from a mature tree or seedling explants. In the present study, we summarize the results of our experiments on the development of in vitro culture protocols for *L. anagyroides* micropropagation.

8.2 Micropropagation Using Axillary Buds

A single 12-year-old tree of *L. anagyroides* grown in the Botanical Garden of Saratov State University, Russian Federation (latitude 51°32′26″ north, longitude 46°00′30″ east), was used as a donor plant. Defoliated branches were washed under running tap water, cut to 2–3 cm long segments, and then immersed into 1 % (w/v) solution of commercial washing powder for 15–20 min. After that, the twigs were washed in running tap water for 20 min, surface disinfected with 0.1 % (w/v) mercuric chloride solution for 15 min, and washed with sterile distilled water (three to five changes at least).

8.2.1 Culture Initiation

8.2.1.1 Primary Explants

One-year-old shoots were used as explant source. Two types of primary explants were dissected from the shoots: single axillary buds and nodal explants (5–7 mm) consisting of a dormant axillary bud and a stem segment.

After inoculation into the initiation medium, the axillary buds (Fig. 8.1a) started to grow and elongated, whereas the nodal segments turned brown and died. After 4 weeks, the number of survived and initiated explants was significantly ($P \leq 0.01$) higher for axillary buds than for nodal explants (72.5 % and 9.1 %, respectively). Perhaps, the survival of the nodal explants was less effective because they produced higher amounts of phenolic compounds which inhibited tissue proliferation. This problem is a common phenomenon in mature tree micropropagation to significantly reduce the efficiency of initiation and proliferation of axillary buds and shoots (Minocha and Jain 2000; Hong and Bhatnagar 2007). In contrast, the axillary buds cultured concurrently produced no phenolic compounds, thus being preferred for the establishment of aseptic cultures. Other factors could be possibly involved, since nodal segments in some species are preferred explants for the micropropagation of woody plants (Ahuja 1993), including Leguminosae species (Vengadesan et al. 2002).

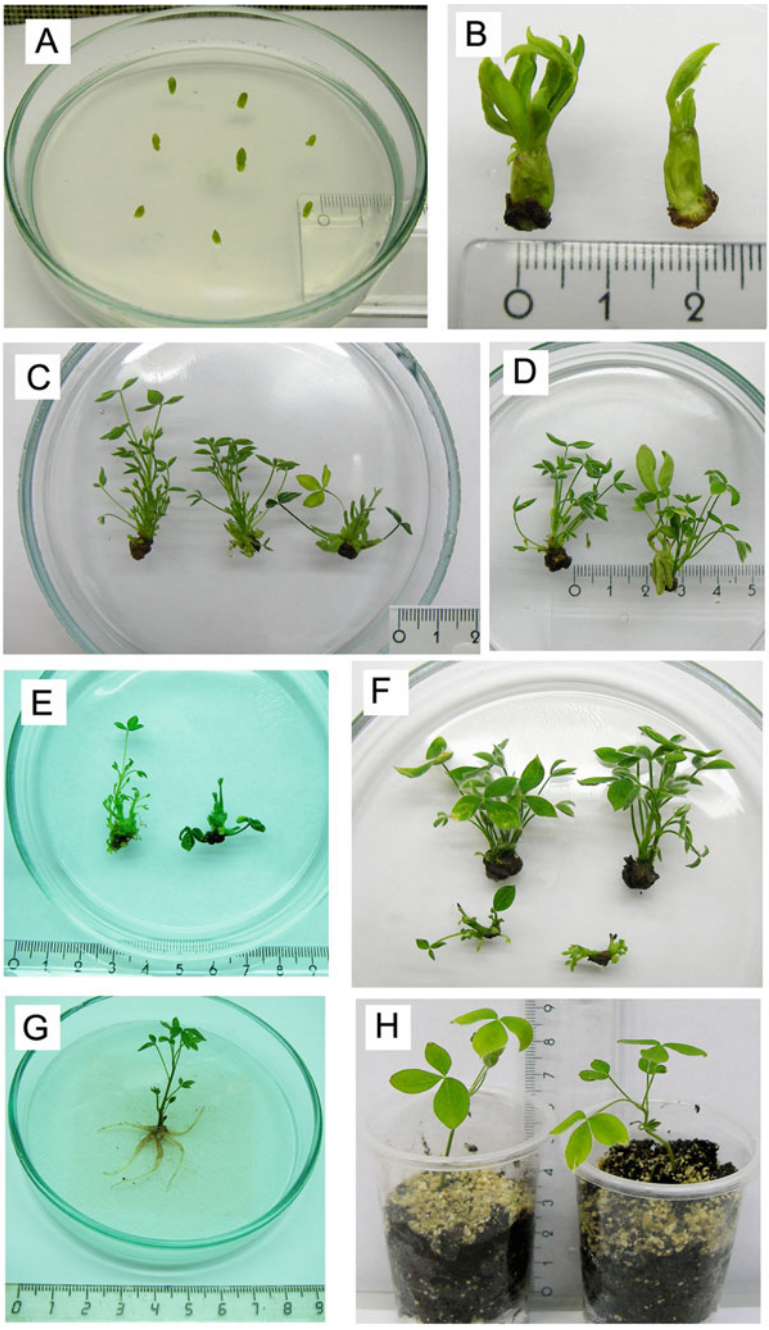

Fig. 8.1 In vitro propagation of *L. anagyroides* through axillary bud explants. (**a**) Axillary buds after inoculation in the MS medium; (**b**) shoot developed from an axillary bud after 4 weeks of cultivation in the media supplemented with 2.22 μM BAP (left, the MS medium; right, WPM); (**c**) shoots developed after 8-week cultivation in the ½ MS (*left*), MS (*center*), and WPM media (*right*), each medium

8.2.1.2 Initiation Medium

The MS (Murashige and Skoog 1962) (full or half strength) and WPM, Woody Plant Medium (Lloyd and McCown 1980), media were used for culture initiation. All the media contained vitamins according to the corresponding medium protocol, 20 g L^{-1} sucrose, and 7 g L^{-1} agar (Panreac) and were supplemented with 2.22 μM of 6-benzylaminopurine (BAP). The medium pH was adjusted to 5.9 before autoclaving at 121 °C for 20 min. The primary explants were cultured in Petri dishes (dia. 10 cm) containing 25 mL of the induction medium. Further, cultivation phases were performed in glass jars (100 or 200 mL) containing 25 mL of the medium, capped with aluminum foil, and sealed with Parafilm™. The cultures were incubated in the growth room at 24 ± 2 °C under a 14 h photoperiod from Osram Fluora lamps (2 klux).

After inoculation into the initiation medium, the axillary buds started to grow and elongated. The number of the responding axillary buds was recorded after 14 days of inoculation. The relative number of initiated buds on the media with a different mineral composition ranged from 61.5 % to 80.0 %. Although no significant effect of the medium on the initiation frequency was revealed, the MS medium was more favorable for bud development than the WPM one (Fig. 8.1b).

To test the effect of phytohormones on the axillary bud response, the MS medium supplemented with various cytokinins in several concentrations was used. BAP (2.22 and 8.88 μM), 6-furfuryl-aminopurine (kinetin) (2.32 and 4.64 μM), and thidiazuron (TDZ) (2.25 μM) were used. Initial response of the buds was noticed in all experiments irrespective of the cytokinin type (BAP, kinetin, or TDZ). However, the subsequent axillary shoot formation as well as shoot elongation was observed only in the explants derived from the BAP-containing media (2.22 μM). Similar results were observed in *Tylophora indica* (Faisal et al. 2007) and *Eclipta alba* (Ray and Bhattacharya 2008), in which the explants from a BAP-containing medium had better potential for shoot regeneration than those from media with kinetin or TDZ.

The proliferation rate of responsive buds was low. After 6–8 weeks of cultivation, small primary shoots with lengths of 8–10 mm developed.

8.2.1.3 Season

Our experimental material was collected from April to November, three times, in total, in two replications, containing 15–20 samples each. The frequency of responded buds in the spring and autumn experiments (77.8 and 72.5 %, respectively) was significantly ($P \leq 0.001$) higher than that in our summer experiments (18.6 %) (Fig. 8.2). The explants initiated in the summer had greatly delayed the

supplemented with 2.22 μM BAP; (**d**) shoots developed after 8-week cultivation in ½ MS supplemented with 2.22 μM BAP (*left*) and 8.88 μM BAP (*right*); (**e**) shoots developed after8-week cultivation in ½ MS supplemented with 2.22 μM BAP (*left*) and 2.25 μM TDZ (*right*); (**f**) shoots developed from tip segments (*upper row*) and nodal segments (*lower row*) cultured on the MS basal medium supplemented with 2.22 μM BAP; (**g**) rooted shoots after 4-week cultivation in the ¼ MS medium supplemented with 2.68 μM NAA; (**h**) plantlets in pots after 1.5 months in a greenhouse

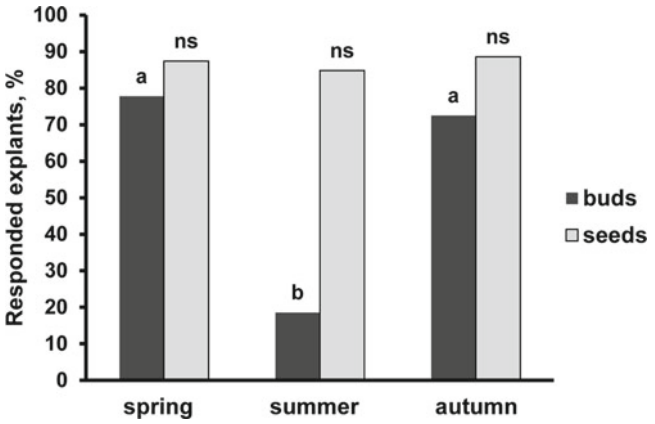

Fig. 8.2 Effect of the explanting season on the frequency of *L. anagyroides* responded explants of axillary bud or seedling cultures on the MS medium supplemented with 2.22 μM BAP. Data for axillary buds followed by different letters are significantly different at $P \leq 0.05$ (one-way ANOVA, Duncan's Multiple Range Test, $F = 82.37$, $P \leq 0.001$); *ns* nonsignificant ($F = 1.27$)

pace of development and eventually died. At the same time, there were no significant differences observed between the numbers of explants initiated in the spring and autumn (75.3 % and 70.7 %, respectively).

The influence of the explanting season on the establishment of aseptic cultures is a common phenomenon in woody species. A good amount of data on the best explanting season among woody species is described in the literature. For example, in *Ceratonia siliqua* (Romano et al. 2002), *Holarrhena antidysenterica* (Kumar et al. 2005), *Robinia pseudoacacia* (Kanwar et al. 2007), and *Populus tremula* (Peternel et al. 2009), spring is the best explanting season. For *Morus australis* (Pattnaik et al. 1996), *Gymnema sylvestre* (Komalavalli and Rao 2000), and *Crateva adansonii* (Sharma et al. 2003), autumn is the best season. In contrast to these reports, the aseptic *L. anagyroides* cultures were equally successful in initiating during both the spring and autumn seasons.

It is generally accepted that the effect of explanting season on the establishment of aseptic cultures depends upon (1) the physiological, morphological, and physical states of the donor tissue tree, (2) changes in these states in different seasons, (3) the environmental conditions required for these changes to occur, and (4) the environmental conditions occurring in the habitat between the time of maturation and germination (Baskin and Baskin 1998). During the annual ontogenetic tree cycle, after the end of the dormancy period, various phenological events were observed, such as leaf unfolding, flowering, juvenile vegetative growth, adult vegetative growth, seed maturation, leaf fall, and, finally, the dormancy period (Kramer and Haännine 2009). The selection of explants at a specific responsive stage of the mature tree's life cycle is a most important factor in the establishment of aseptic cultures. The importance of the physiological status of donor plants for in vitro culture initiation was reported and disputed in numerous papers (Benson 2000; Minocha and Jain 2000; Giri et al. 2004).

It is clear that the composition, ratio, and levels of endogenous plant growth regulators, as well as the primary and secondary metabolites in these stages of tree vegetation, are different and variable. Evidently, such season-dependent metabolism takes place both before and during in vitro culture induction. There are several examples in the literature supporting this supposition. For example, comparison of the antioxidants in dormant and sprouting buds has shown that the activity of antioxidant enzymes like SOD and glutathione reductase is lower in the sprouting buds than in the dormant ones (Polle and Rennenberg 1994). The antioxidant status of the buds may play an important role in their ability to sprout under tissue culture conditions. Thakar and Bhargava (1999) reported seasonal changes in the antioxidant enzyme activities, namely, ascorbate peroxidase, superoxide dismutase, and guaiacol-dependent peroxidase before and after the in vitro bud growth induction of *Gmelina arborea*. Label et al. (1988) showed that hormonal changes in the endogenous levels of abscisic acid, indole-3-acetic acid, and benzyladenine take place both before and during the in vitro bud growth induction of *Prunus avium*. Hohtola (1988) found that the season-dependent metabolism in *Pinus sylvestris* is retained in the tissue culture conditions.

At the same time, the reduced frequency of summer initiation in our experiments may be explained by environmental conditions. In summer, the donor tree was subjected to stressful environmental conditions (extreme atmospheric temperatures and soil moisture deficit). As a general rule, plants have a lot of typical physiological, biochemical, and metabolic adaptive responses to abiotic stress. Among them are accumulation of protein, enzymes, amino acids, and hormones, modulation of sugar metabolism, and a lowered photosynthetic activity. In addition, drought stress inhibits cell division leading to decreased plant growth (Thakar and Bhargava 1999; Kramer and Haännine 2009; Thara et al. 2011).

In our case, the reduced frequency of summer initiation may be explained by the physiological status of shoot tissues of the donor tree, which may be caused both by the effect of drought stress upon the donor tree and by changes of the phenological event.

8.2.2 Multiplication Phase

After 6–8 weeks of primary cultivation, the axillary buds developed a single shoot with few small leaves. During subsequent subculturing of the responded explants to a fresh medium, alongside the development and growth of the primary shoot, the stimulation of axillary pre-existing meristems also took place. As a result of this process, shoot clusters with several shoots (3–10 mm length) were developed.

At the same time, a small pale-yellow callus is formed from the basal part of the explants. To reduce the genetic instability of regenerated shoots, the callus was discarded during subsequent multiplication cycles.

Table 8.1 Effect of the basal media on shoot multiplication of *L. anagyroides* after 60 days of culturing

Medium	Shoot sprouting frequency (%)	Mean no. of shoots per explant	Shoot length (mm)
MS	45.0 b	1.1a	9.7 a
½ MS	85.9 c	3.0 b	9.5 a
WPM	26.9 a	1.1 a	8.3 a
F	93.5**	10.6*	1.9 *ns*

Each medium was supplemented with 2.22 μM BAP
Values are the mean of two replications, each consisting of ten explants. Data followed by different letters are significantly different at $P \leq 0.05$ (one-way ANOVA, Duncan's Multiple Range Test)
ns no significant differences
*$P \leq 0.05$; **$P \leq 0.01$

8.2.2.1 Proliferation Medium

For shoot multiplication, three different media were compared, namely, MS, half-strength MS medium salts (½ MS), and WPM. Each medium contained vitamins according to its protocol, 20 g L^{-1} sucrose, and 7 g L^{-1} agar (Panreac) and was supplemented with 2.22 μM of BAP.

The best results of shoot proliferation and elongation were observed on the ½ MS medium which produced 3.0 shoots per explant in 85.9 % of cultures (Table 8.1). No significant difference was observed in the shoot length between MS, ½ MS, and WPM media, but dwarf shoots were noted on the WPM (Fig. 8.1c).

The ½ MS medium was used to study the effect of the plant growth regulators on shoot multiplication and elongation. Different plant growth regulators were tested, namely, BAP (2.22, 4.44, 8.88, and 17.76 μM), BAP together with kinetin (2.22 μM and 2.32 μM, respectively), kinetin (2.32 μM), thidiazuron (2.25 and 9.00 μM), and zeatin (2.28 μM). The shoot sprouting frequency, shoot number, and shoot length varied according to the type and concentration of the cytokinin used (Table 8.2). Kinetin and zeatin alone showed poor regeneration, inducing the formation of 1–1.2 shoots with lengths of 6–7 mm. The best response of shoot regeneration was observed for the BAP-containing media. The effectiveness of BAP relative to axillary shoot regeneration has been reported for other woody species such as *Ximenia americana* (Aloufa et al. 2003), *Holarrhena antidysenterica* (Kumar et al. 2005), *Vitex trifolia* (Hiregoudar et al. 2006), and *Tylophora indica* (Faisal et al. 2007). The maximum number of shoots per explant was observed for the BAP-containing media (2.22 and 8.88 μM) (3.0 each), in ~86 % of cultures. The newly regenerated shoots were well developed and had normal appearance (Fig. 8.1d). However, prolonged subculturing on the medium with 8.88 μM BAP caused symptoms of vitrification and hyperhydricity of the tissues. In addition, the shoots developed on this medium were rooted less successfully than those from the medium with 2.22 μM BAP (data not shown).

In our experiments, TDZ in 2.25 and 9.00 μM produced fewer numbers of shoots per explants (1.6 and 2.3, respectively) than BAP did in 2.22 and 8.88 μM (3.0

Table 8.2 Effect of various cytokinins on shoot multiplication of *L. anagyroides* after 60 days of culturing on the ½ MS medium

Cytokinin (μM)	Shoot sprouting frequency (%)	No. of shoots per explant	Shoot length (mm)
BAP, 2.22	85.9	3.0 f	9.5 de
BAP, 4.44	81.8	1.7 abcd	9.1 cd
BAP, 8.88	85.8	3.0 ef	9.2 cde
BAP, 17.76	80.3	2.0 bcd	11.5 f
BAP, 2.22 + kinetin, 2.32	54.4	2.5 def	7.8 bc
Kinetin, 2.32	65.9	1.2 ab	6.0 a
Zeatin, 2.28	34.8	1.0 a	6.9 ab
TDZ, 2.25	62.0	1.6 abc	8.5 cd
TDZ, 9.00	72.3	2.3 cdef	10.7 ef
F	1.3 *ns*	7.0*	11.1*

Values are the means of three replications, each consisting of ten explants
Data followed by different letters in the same column are significantly different at $P \leq 0.05$ (one-way ANOVA, Duncan's Multiple Range Test)
ns no significant difference
*$P \leq 0.05$

each), although TDZ for other woody species from the Leguminosae family was shown to be more efficient for shoot formation than other growth regulators (Hong and Bhatnagar 2007). Besides, in the case of *L. anagyroides* micropropagation, the TDZ-containing media produced morphologically stunted shoots with abnormally developed leaves (Fig. 8.1e). Similar inhibitory effects of TDZ on the growth and elongation of shoots were also observed for other woody trees such as *Albizia chinensis* (Sinha et al. 2000), *Vitex negundo* (Ahmad and Anis 2007), *Balanites aegyptiaca* (Anis et al. 2010), and *Cassia siamea* (Parveen et al. 2010). The formation of stunted shoots or the inhibition of internode elongation may be due to the high cytokinin activity of TDZ (Huetteman and Preece 1993).

8.2.2.2 Explant Effects

In the course of our preliminary analysis, differences in the multiplication rate between different explant types were noticed. Detailed analysis revealed that the shoot tips produced a significantly ($P \leq 0.01$) higher number of axillary shoots per explant (3.9) than the nodal explants (2.1) (Table 8.3 and Fig. 8.1f).

The superiority of shoot tips in comparison with nodal explants was also observed in the micropropagation of some other woody species, such as *Sorbus domestica* (Arrilaga et al. 1991), *Acacia catechu* (Kaur and Kant 2000), *Ulmus minor* (Conde et al. 2008), and *Arbutus unedo* (Gomes and Canhoto 2008).

Thus, optimum shoot multiplication and elongation were achieved when shoot tip explants were subcultured on the ½ MS medium supplemented with 2.22 μM BAP. These cultures maintained shoot regeneration ability for 2 years without any significant loss of viability.

Table 8.3 Effect of the explant type and BAP concentration on shoot multiplication of *L. anagyroides* after 60 days of culturing on ½ MS

Type of explants	Concentration of BAP (μM)	Shoot sprouting frequency (%)	No. of shoots per explant	Shoot length (mm)
Tip segments	2.22	100.0 b	4.0 c	12.1 e
	8.88	100.0 b	3.5 bc	10.1 b
Nodal segments	2.22	69.4 a	2.0 a	7.3 a
	8.88	72.8 a	1.9 a	8.2 ab
Average for explant type (factor A)				
Tip segments		100.0 b	3.9 b	11.1 b
Nodal segments		72.3 a	2.1 a	7.5 a
F		13.8*	77.8**	42.0**
Average for BAP concentration (factor B)				
2.22		84.7 a	3.1 a	9.7 a
8.88		87.6 a	2.8 a	8.9 a
F		0.1 *ns*	1.8 *ns*	2.2 *ns*

Values are the mean of three replications, each consisting of 5–7 explants taken from 2-year-old cultures

Data followed by different letters in the same column are significantly different at $P \leq 0.05$ (one-way ANOVA, Duncan's Multiple Range Test)

ns no significant differences

*$P \leq 0.05$; **$P \leq 0.01$

8.2.3 In Vitro Rooting

In vitro derived shoots (10–15 mm in length) were isolated from shoot clusters and transferred onto the MS medium (½ or ¼ strength) supplemented with 10 g L^{-1} sucrose. Adventitious rooting of woody plants is usually induced by auxin. Among the auxins, IAA and IBA are the most frequently applied for rooting (Harry and Thrope 1994). We have studied the effects of different auxins (IAA, NAA, and IBA) on rhizogenesis. For root induction in media, various auxins were added in several concentrations, namely, IAA (2.85 μM), IBA (2.45 and 9.80 μM), and NAA (2.68 and 10.74 μM). The medium without auxins was used as a control. The rooting frequency, the number, and the length of developed roots were counted after 1 month of cultivation.

Shoot cultivation in the ½ or ¼ MS medium without auxins resulted in discoloration of the shoots and the absence of their subsequent development. Addition of various auxins (IAA, IBA, and NAA) in different concentrations to the ½ MS medium also did not stimulate rhizogenesis. The root development was observed only in the media containing ¼ MS mineral salts, supplemented with the abovementioned auxins. Similar data were obtained by Kaur and Kant (2000) for *Acacia catechu* micro-shoots, who also reported that the best results among rooting media were registered in the ¼ MS in comparison with the full or half-strength MS media.

Table 8.4 Effect of various auxins on the root formation of *L. anagyroides* after 30 days of culturing on ¼ MS

Auxin (μM)	Rooting (%)	No. of roots per shoot	Root length (mm)
IAA, 2.85	24.7 a	1.5 a	8.0 a
IBA, 2.45	29.7 a	5.8 b	21.6 b
NAA, 2.68	61.3 b	5.4 b	21.3 b
F	11.2*	7.0*	19.8**

Values are the means of three replications, each consisting of ten shoots
Data followed by different letters in the same column are significantly different at $P \leq 0.05$ (one-way ANOVA, Duncan's Multiple Range Test)
*$P \leq 0.05$; **$P \leq 0.01$

IBA and NAA at high concentration (9.80 μM and 10.74 μM, respectively) stimulated abundant callus development from the basal end of the shoots. Similar results were observed in other Leguminosae species (Parveen et al. 2010). Apparently, in Leguminosae, high concentrations of exogenous auxins in the medium stimulate callus development, which results in a poor vascular connection between the shoot and roots, thus reducing the survival percentage of plants.

A lower level of IAA (2.85 μM) or IBA (2.45 μM) or NAA (2.68 μM) resulted in normal root formation. Among the tested auxins, NAA in 2.68 μM induced the highest percent of rooted shoots (61.3 %), 5.4 roots per shoot with a root length of 21.3 mm (Table 8.4 and Fig. 8.1g).

The growth and development of adventitious roots stimulated shoot elongation and the formation of new leaves; and the plantlets were ready for transferring into non-sterile conditions after 30–45 days of cultivation on the rooting medium.

8.2.4 Acclimatization

This phase was most difficult and labor consuming. Regenerated plants with their fully expanded leaves and well-developed root systems were transplanted into pots with a soil mixture consisting of three peats/two leaf grounds/one sand (v/v/v) and were transferred to a greenhouse. Unfortunately, the majority of our plantlets died after 7–10 days.

In order to increase their survival rate, we used a combination of the following approaches: incubation of plantlets in tubes with non-sterile water for 10–14 days before their transferring into the soil, shading of plantlets during 2 weeks after potting into the soil, and covering the hotbed with a transparent polythene membrane to ensure high humidity. In order to acclimatize our plants to the greenhouse conditions, the polythene membrane was opened for 10–15 min every day. After 1 month, the polythene membrane was completely removed.

Additional positive influence was caused by spraying of the plantlets by the commercial elicitor Emistim® (two drops per 100 ml of distilled water). In such a case, the survival rate of the plants was approximately 60 %; the regenerated plants exhibited normal growth and did not show any noticeable signs of abnormal phenotypic variation (Fig. 8.1h).

8.3 Micropropagation Using Seedling Explants

Seedling tissues are generally considered as a reliable alternative to vegetative tissues of mature plants (Benson 2000). Therefore, we developed a technique for micropropagation of *L. anagyroides* by using seedlings as a starting material.

Seed pods appeared in June and mature by the end of August. These pods are dehiscent and remain on the plant for some time. Some pods opened in late September, but many open in the following spring. Seeds either fall out of the open pods or remain in the pods for a year or longer (Szentesi and Wink 1991). The seeds have a large embryo, surrounded by a fairly thick (especially from the cotyledon) layer of the endosperm. They have a hard, impermeable coat.

The pods were shelled and shrunken and contained hard-coated seeds. These were collected at the end of September and stored at room temperature in darkness until used. The dark-colored seeds were discarded. Only plump light-yellow seeds were used in the laboratory germination and further establishment of experiments.

8.3.1 Seed Germination

In our preliminary experiment, seed germination was low and did not exceed 10 %. One of the causes of this poor seed germination is the hard seed coat that may be a physical barrier to water uptake and/or gas exchange. The phenomenon is sometimes referred to as "seed coat hardiness" because the seed coats remain hard and impenetrable during exposure to normal germination conditions (Nikolaeva et al. 1985; Baskin and Baskin 2004). Seeds may be dormant upon release from their mother plant (primary or innate dormancy), and dormancy can also be induced in seeds after they have become nondormant (secondary or induced dormancy), if the conditions become unfavorable for germination (Lambers et al. 2008).

The Leguminosae family exhibits some of the most advanced morphological structures of the seed coat to regulate physical dormancy. The seed coat consists of four distinct layers, namely, (1) the outermost layer which is the cuticle, which has a waxy and water-repellent character; (2) the macrosclereids or palisade layer, which consists of long, narrow, tightly packed, vertical cells; (3) the osteosclereids, which is a layer of more loosely packed cells; and (4) the parenchyma layer, which is made up of a layer of little differentiated cells. Impermeability is caused by the cuticle and the palisade layer; scarification through the cuticle and halfway through the palisade layer is sufficient to overcome this impermeability, and the seeds start to absorb water (Lambers et al. 2008).

8.3.1.1 Pretreatment to Overcome Dormancy

There are few reports available on the removal of *L. anagyroides* seeds out of dormancy by pretreatment with concentrated H_2SO_4 for 0.5–2 h (Nikolaeva et al. 1985; Hartmann et al. 2010). Stratification is another method used to break dormancy; the positive effect is caused by both low and high temperatures, as well as alternating low and high temperatures. Stimulation of dormant seed germination by culturing

them on nutrient media under sterile conditions is also used (Nikolaeva et al. 1985). Plant growth regulators (such as kinetin, BAP, gibberellic acid) and various nitrogenous compounds (such as potassium nitrate) are known to have a stimulating effect on breaking dormancy and promote the germination process of forest tree seeds (Leadem 1987).

We have tested a range of temperature pretreatments for stimulation of seed germination on a particular media.

Seeds were stratified by three regimes, namely, (1) cold stratification (seeds were kept at −18 °C for 1 month), (2) warm pretreatment (seeds were soaked in hot water (≈90 ° C) for 20–30 min), (3) an alternate temperature regime (cold stratification for 1 month; thereafter, the seeds were soaked in hot water for 20–30 min). After the pretreatment, the seeds were surface disinfected with 0.1 % (w/v) mercuric chloride solution for 15–20 min, washed with at least three changes of sterile distilled water, and cultured in different media in a growth chamber at 24 °C for 1 month.

The media used for seed germination were the full-strength PGR-free MS medium, MS supplemented with 2.22 μM BAP, and WPM supplemented with 2.22 μM BAP. All media contained vitamins according to the corresponding medium protocol, 20 g L^{-1} sucrose, and 7 g L^{-1} agar (Panreac). pH was set at 5.8–6.0 before autoclaving at 120 °C for 20 min.

The seeds began to germinate after 10–14 days of cultivation on a nutrient medium, and after 3–4 weeks, the seedlings had normal appearance.

The seeds not subjected to temperature pretreatment (control) had a low germination ability, 11–13 % (Table 8.5). The temperature pretreatments had a significant

Table 8.5 Effect of temperature pretreatment and culture media on in vitro germination of *L. anagyroides* seeds after 30 days of culturing

Medium (factor A)	Temperature pretreatment of dry seeds (factor B), number of germinated seeds (%)				Mean
	Control	Cold stratification	Warm pretreatment	Alternate temperature	
MS PGR-free	13.5 a	26.0 a	70.0 bcdefg	80.7 defg	47.5 a
MS + 2.22 μM BAP	12.5 a	24.2 a	82.3 g	79.8 cdefg	49.0 a
WPM + 2.22 μM BAP	11.2 a	27.2 a	80.8 efg	82.1 fg	50.3 a
Mean	12.4 a	24.9 b	77.7 c	80.8 c	
$F_{(A)}$					0.2 *ns*
$F_{(B)}$					118.0**
$F_{(A \times B)}$					0.92 *ns*

Values are the means of two replications, each consisting of ten explants. The number of germinated seeds was counted after 1 month of cultivation

Data followed by different letters in the same column are significantly different at $P \leq 0.05$ (one-way ANOVA, Duncan's Multiple Range Test)

**$P \leq 0.01$

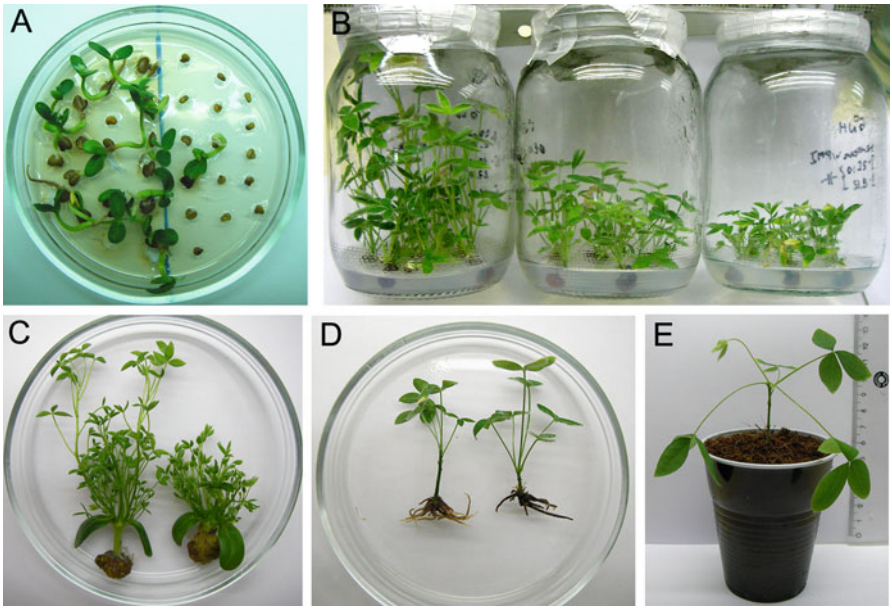

Fig. 8.3 In vitro propagation of *L. anagyroides* through seedling explants. (**a**) Germination of seeds after 3 weeks of cultivation on the MS medium supplemented with 2.22 μM BAP (seeds after hot water pretreatment (*left*) and control (*right*)); (**b**) multiple shoot regeneration in the MS (*left*), ½ MS (*center*), and WPM media (*right*), each medium supplemented with 2.22 μM BAP; (**c**) shoot clusters developed after 8 weeks of cultivation in the MS medium supplemented with 2.22 μM (*left*) and 17.76 μM BAP (*right*); (**d**) rooted shoots after 4 weeks of cultivation in the ¼ MS medium supplemented with 2.68 μM NAA (*left*) and 2.45 μM IBA (*right*); (**e**) plantlet in a pot after 1 month in a greenhouse

effect on the evaluations of germinated percentage ($P \leq 0.01$). The cold stratification of seeds at –18° resulted in increasing of their germination percentage up to 24–27 %. However, the seeds subjected to cold stratification produced about 10–15 % abnormal seedlings with morphological deviations in their leaf and shoot development. A significant increase of the germination frequency was observed after the hot water pretreatment of dry seeds (70.0–82.3 %) (Fig. 8.3a). The highest germination percentage (77.8–88.9 %) was observed after the alternate temperature regime, but this method of pretreatment delayed obtaining sterile seedlings for 2 months, whereas the period of stratification by hot water was significantly shorter, 3–4 weeks.

The mean germination percentage on various media varied within 47–50 % and did not depend on the nutrient medium composition (Table 8.5). However, we noticed that the seedlings developed in the PGR-free MS medium were thin, elongated, and prone to vitrification as compared to the seedlings in other media (MS and WPM) supplemented with 2.22 μM BAP. Moreover, after transfer to a medium for micropropagation, the PGR-free MS-derived seedlings produced fewer shoots. Therefore, only the seedlings initiated on the MS medium supplemented with 2.22 μM BAP were used as explants for further multiplication.

Table 8.6 Effect of pretreatment and growing conditions on the seed germination of *L. anagyroides*

Growing conditions (factor A)	Pretreatment of dry seeds (factor B), number of germinated seeds (%)		Mean
	Without pretreatment (control)	Warm pretreatment	
In vivo (soil substrate)	2.1 a	18.9 b	8.4 a
In vitro (MS+2.22 μM BAP)	14.8 b	73.6 c	46.2 b
Mean	10.5 a	44.2 b	
FA			433.8***
FB			344.2***
FA_xB			133.7**

L. anagyroides seeds were germinated with or without hot water pretreatment under in vivo conditions (a soil mixture consisting of three peats/two leaf grounds/one sand) and under in vitro conditions (the MS medium supplemented with 2.22 μM BAP). Germination frequency was counted after 1 month. Values are the means of three replications, each consisting of 10–15 seeds
Data followed by different letters in the same column are significantly different at $P \leq 0.05$ (one-way ANOVA, Duncan's Multiple Range Test)
***$P \leq 0.001$; **$P \leq 0.01$

Remarkably, contrary to axillary bud cultures, where the frequency of responded explants initiated in summer was significantly low, we observed no significant differences between the frequencies of seed germination in our experiments conducted in spring, summer, and autumn (87.4, 84.8, and 88.6 %, respectively) (Fig. 8.2).

8.3.1.2 Comparison of In Vitro and In Vivo Germination

It was also found that seed pretreatment yielded high germination frequency only in in vitro conditions, after germination on the nutrient medium. The seeds subjected to hot water pretreatment and sown in the soil had a low germination frequency (19 % as compared to 74 %, Table 8.6). The cause of such a difference is unclear yet. Clearly, the components of the nutrient medium (vitamins and sucrose) and hormones provide advantageous conditions for the growth and development of seedlings at the early stages of development. Such additives are apparently missing in the composition of the soil substrate.

In fact, the use of in vitro culture conditions was to intensify the process of obtaining aseptic seedlings. Well-developed seedlings can be obtained with a high frequency within 3–4 weeks. In addition, such seedlings further showed the best growth and development in non-sterile conditions as compared to those obtained through germination directly in the soil.

8.3.2 Multiplication of Axillary Shoots

After 4 weeks, elongation of the hypocotyl and root and full expansion of the cotyledonary leaves were observed for the in vitro germinated seedlings. After the appearance and unfolding of the first true leaf, the seedling roots were cut off, and explants, consisting of the hypocotyl, cotyledon, and epicotyl, were transferred to a medium for propagation. Three different media were tested, namely, MS, ½ MS, and WPM; two concentrations of BAP were tested in each variant of the nutrient medium, namely, 2.22 and 8.88 μM. All tested media contained 20 g L^{-1} sucrose and 7 g L^{-1} agar (Panreac).

The seedling-derived cultures grew fast. Unlike axillary bud-derived cultures, where growth and development were slowed down, in the seedling-derived cultures, the bunch of shoots with various lengths was developed already in primary cultures after 6–8 weeks of cultivation as a result of activation of the axillary meristems. The shoot cultures in different media looked differently, showing changes in the number of emerging shoots and their length (Table 8.7 and Fig. 8.3b).

The shoot sprouting frequency on all tested media reached 100%. The best results of shoot proliferation (6.0–6.4 shoots per explant) and shoot elongation (21.5–29.8 mm) were observed on the MS medium with addition of 2.22 and 8.88 μM BAP (Table 8.7). It was interesting to note that half-strength MS medium is optimal for shoot proliferation in axillary bud-derived cultures, whereas the full-strength MS medium is good for seedling-derived cultures. The superiority of the MS medium over other ones, such as WPM or B5, for shoot proliferation was also reported for other seedling-derived cultures of other woody species such as *Sterculia urens* (Hussain et al. 2008) and *Arbutus andrachne* (Mostafa et al. 2010).

Therefore, the MS medium was used to study the effect of plant growth regulators on shoot multiplication and elongation. In this study, various cytokinins were added to the MS basal medium, namely, BAP (2.22, 8.88, and 17.76 μM), TDZ (2.25, 9.00, and 18.00 μM), and two combinations of BAP and TDZ (1.11 + 1.12 and 2.22 + 2.25, respectively).

Table 8.7 Effect of the basal media on the shoot multiplication of *L. anagyroides*

Medium	BAP (μM)	Mean no. of shoots per explant	Shoot length (mm)
MS	2.22	6.0 c	23.4 d
	8.88	6.4 c	17.7 c
½ MS	2.22	3.5 a	16.8 bc
	8.88	3.2 a	16.9 c
WPM	2.22	5.4 a	10.1 a
	8.88	4.8 abc	11.5 a
F		6.7*	36.5*

Values are the means of two replications, each consisting of ten explants
Data followed by different letters in the same column are significantly different at $P \leq 0.05$ (one-way ANOVA, Duncan's Multiple Range Test)
*$P \leq 0.05$; **$P \leq 0.01$

Table 8.8 Effect of various cytokinins on the shoot multiplication of *L. anagyroides* after 60 days culturing on the MS medium

Cytokinin (μM)		No. of shoots per explant	Shoot length (mm)
BAP	TDZ		
2.22	–	4.7 c	21.5 b
8.88	–	3.1 a	29.8 c
17.76	–	3.4 ab	14.5 a
–	0.90	4.4 bc	16.7 a
–	3.60	3.1 a	15.9 a
–	7.20	3.2 a	15.5 a
1.11	1.12	4.0 abc	13.3 a
2.22	2.25	4.2 abc	13.3 a
F		3.9*	24.3*

Values are the means of three replications, each consisting from ten explants
Data followed by different letters in the same column are significantly different at $P \leq 0.05$ (one-way ANOVA, Duncan's Multiple Range Test)
*$P \leq 0.05$

The maximum number of shoots per explant was observed both on the 2.22 μM BAP-containing media (4.7) and 2.25 μM TDZ (4.4). An increased concentration of BAP (8.88 and 17.76 μM) and TDZ (9.00 and 18.00 μM) in the MS medium did not stimulate the development of increased number of shoots as compared to 2.22 μM BAP or 2.25 μM TDZ. Moreover, higher concentrations of both BAP and TDZ reduced the shoot number as well as the shoot length (Table 8.8 and Fig. 8.3c). A similar observation was made with in vitro cultured hypocotyl explants of *Feronia limonia* (Hiregoudar et al. 2005). It was noticeable that higher concentrations of BAP (5.0 μM) in the MS medium are not beneficial in producing more adventitious shoots as compared to 2.0 μM BAP. Stevens and Pijut (2012) also reported TDZ concentrations higher than 4.5 μM to have a deleterious effect on *Fraxinus profunda* adventitious shoot formation.

In our study, the combination of two active cytokinins, BAP and TDZ, did not stimulate the development of a higher number of shoots as compared to both BAP and TDZ alone. Addition of TDZ to the BAP-containing medium significantly reduced the length of the developing shoots, while morphologically stunted shoots with abnormally developed leaves were developed on the TDZ-containing medium.

Like in axillary bud cultures, the seedling tips used as secondary explants had higher ability to form new shoots than the nodal explants (data not shown). In all variants, a compact pale-yellow callus was developed from the basal part of the explants (Fig. 8.4a).

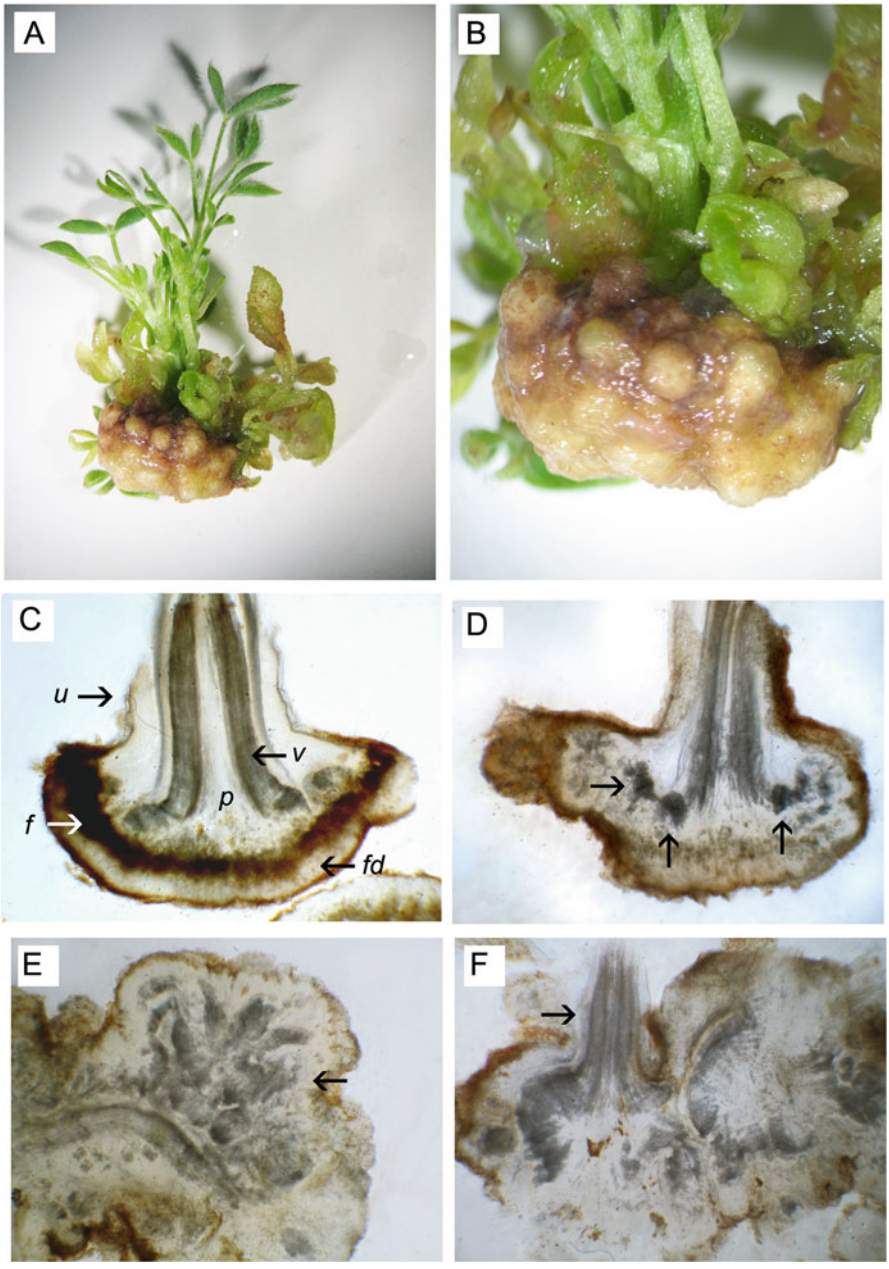

Fig. 8.4 Shoot regeneration from the callus of *L. anagyroides*. (**a**) A callus formed from the basal part of the shoots on the proliferation medium; (**b**) a shoot developed from the individual globular structures; (**c**) the histological structure of the callus after 4 weeks of cultivation (*u* undifferentiated tissue, *p* parenchyma tissue, *v* vascular bundle, *f* fellema, *fd* felloderma); (**d**) the zones of meristematic activity (*arrows*) formed in the vascular bundle; **e** a shoot bud (*arrow*); (**f**) the zones of meristematic activity and an adventitious shoot (*arrow*) in the 3-month-old callus. All *scale bars*: 1 mm

8.3.3 Rooting and Acclimatization

The shoots longer than 10 mm were isolated from the shoot bunch and used in further experiments on rooting. It was found that the rooting processes were almost identical as in the regenerants obtained from axillary bud-derived cultures (Sect. 1.3). The roots started emerging on 10–14 days after transfer to the rooting medium, and 5–7 roots with a length of 15–20 mm were formed after 1 month (Fig. 8.3d). Root development was observed only on the ¼ MS supplemented with auxins, and NAA was more efficient as compared to IBA.

It was noticed that the rooting frequency on the ¼ MS supplemented with 2.68 μM NAA varied widely from 0 to ~70 %. Our detailed analysis has revealed that root formation depends not only on the composition of the rooting medium but also on the multiplication medium for shoot development. The shoots developed on the ½ MS supplemented with a low level of BAP (2.22 μM) or TDZ (2.25 μM) had the maximum frequency of rooting, while the shoots developed on the media with a higher level of cytokinins showed a lower frequency of rhizogenesis.

For example, the maximum rooting frequency (63.3 %) was observed in the shoots transferred from MS with 2.22 μM BAP, while the shoots transferred from the medium with 17.76 μM BAP developed no root system (Table 8.9). In addition to the differences in the rooting frequency, noticeable differences were observed in the root system morphology. The root system of the shoots taken from the 2.22 μM

Table 8.9 Effect of the multiplication media on the rooting frequency (%) and acclimatization of *L. anagyroides* after 30 days

Multiplication medium				
	Cytokinin (μM)			
Medium	BAP	TDZ	Rooting (%)[a]	Acclimatization[b]
MS	2.22	–	63.3 d	+
	8.88	–	34.3 b	±
	17.76	–	0.0 a	–
	–	2.25	59.1 cd	+
	–	9.00	21.1 ab	±
	–	18.0	18.3 ab	–
	1.11	1.12	32.5 b	±
	2.22	2.25	16.1 ab	–
WPM	2.22	–	16.6 ab	–
	8.88	–	11.1 ab	–
F			9.3*	

Values are the means of two replications, each consisting of ten shoots

Data followed by different letters in the same column are significantly different at $P \leq 0.05$ (one-way ANOVA, Duncan's Multiple Range Test)

*$P \leq 0.05$

[a]Rooting medium: ¼ MS supplemented with 2.68 μM NAA

[b]+, survived more than 50.0 % regenerants; ±, survived less than ≤50.0 % regenerants; – , all regenerants died

BAP apparently looked normal, whereas the shoots taken from the 8.88 μM BAP produced small thickened roots. It is likely that during in vitro cultivation, the exogenous cytokinin used in high concentration is not fully utilized and accumulates in the shoot tissues, which complicates the rooting process.

Acclimatization of rooted plants was performed according to some methods described in Sect. 1.4. The regenerants derived from seedling cultures tolerated to acclimatization stress better than the plants derived from axillary bud-derived cultures, and they were characterized by higher adaptive potentials. After 6–8 weeks of adaptation to non-sterile conditions, the regenerants looked normal and were ready to transfer into field conditions (Fig. 8.3e).

8.4 Shoot Regeneration from Callus

Micropropagation of woody plants using callus cultures is a common practice. Basal callus was a source of adventitious shoot production in *Ceratonia siliqua* propagation (Romano et al. 2002). Moreover, as in the case of the European aspen (*Populus tremula* L.) propagation, shoot regeneration from callus outnumbered the direct shoot formation from apical meristems; therefore, it was used to increase the efficiency of micropropagation (Peternel et al. 2009).

In our experiments with *L. anagyroides*, concomitantly with shoot development, a small pale-yellow callus was formed from the basal part of the shoots on the proliferation medium (Figs. 8.4a and 8.5). This callus had a globular compact appearance, and at the end of each passage (8 weeks of cultivation), shoots were developed from the individual globular structures (Fig. 8.4b).

As far as the basic prerequisite for the application of tissue culture technology for the clonal propagation of plants is maintaining their genetic identity, we performed a histological study to reveal whether these shoots develop from the preexisted meristems of explants or they form from the apical meristems developing de novo in this callus.

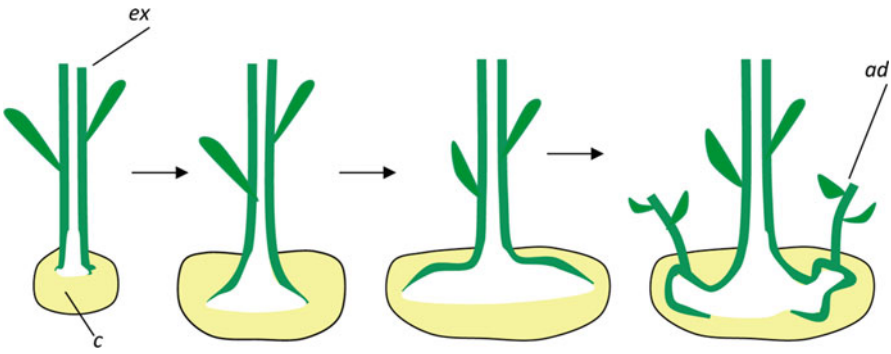

Fig. 8.5 Schematic development of shoot formation in callus cultures of *L. anagyroides*; *ex* explant, *c* callus, *ad* adventitious shoots

Histological preparations were performed according to our method based on a clearing technique of plant tissues (Herr 1971). Explants were fixed in aceto-alcohol (3:1) temporarily in 4, 5, 6, 7, and 8 weeks, after 3 months from the beginning of cultivation on the MS medium supplemented with 2.22 μM BAP. The fixed material was rinsed with water for 24 h and then incubated in glycerin for 5 weeks. Its sections (with a thickness of about 0.5 mm) were placed on a slide in a drop of the Herr clearance solution (Herr 1971) for 3–5 days.

It was found that the callus developed in the base of the shoots after 4 weeks of cultivation had a heterogeneous structure. The upper part (distal to the medium surface) of the callus consisted from undifferentiated tissue (Fig. 8.4c, *u*). The central area of the callus consisted of the overgrown basal part of the shoot. It consisted of the core parenchyma tissue (Fig. 8.4c, *p*) and vascular bundles (Fig. 8.4c, *v*). The lower part of the callus consisted of the dark-colored cells of fellema and the light-colored cells of felloderma.

After 6–7 weeks of cultivation, zones of meristematic activity got formed on the vascular bundles (Fig. 8.4d). Subsequently, shoot buds got differentiated from these zones (Fig. 8.4e). After 3 months of cultivation, the number of the zones with meristematic activity increased, and some of them gave rise to adventitious shoots (Fig. 8.4f).

These results testify to the following sequence of developmental events. The proliferative activity of the meristematic tissues of the primary shoot led to the formation of callus tissue. During cultivation, zones of high proliferative activity were formed on the vascular bundles of the initial shoot, from which adventitious shoots developed. In histological sections, in the area of the formation of an adventitious shoot, the connection of the vascular bundles of the adventitious shoot with the vascular bundles of the primary shoot was clearly visible.

Thus, the shoots regenerated from the callus developed on the basal part of the primary shoot arose as a result of proliferation of the meristematic tissues of the original explant. It can serve as one of the evidences of genetic identity between the shoots which developed by direct organogenesis from epicotyl meristems and adventitious shoots appeared “within” the callus tissue. These findings allow using such shoots in the technology of micropropagation of *L. anagyroides* to improve its effectiveness.

8.5 Conclusion

Woody plants are perennial and complex organisms, whose reproductive potentials decline with age. Clonal propagation through tissue culture is a reliable alternative to the traditional methods of propagation of adult plants. The results of our experiments on the application of tissue culture techniques for the propagation of *L. anagyroides*, a shrub, showed that the mass and reliable plant regeneration in this species can be achieved by careful observance of the known regularities specific to the tissue culture of woody species. In our work, we used the basic strategy for woody tree micropropagation, i.e., direct regeneration by activation and

proliferation of the pre-existing meristems from axillary bud explants taken from a mature tree.

Choosing of the explant type and the explanting season mainly determines the efficiency of micropropagation of woody species. The axillary bud explants taken from a mature tree were more preferable for the establishment of aseptic cultures than nodal explants. The juvenile tissues of seedlings were more responsive starting material than explants taken from a mature tree. However, seeds are characterized by physical dormancy. Hot water pretreatment with subsequent cultivation of seeds on the MS medium allowed overcoming this phenomenon and significantly increased the seed germination (up to 70–82 %) in comparison to non-treated seeds (11–13 %).

The explanting season caused effect on the initiation of regenerable aseptic cultures from the mature tree explants in contrast to those from the juvenile tissue of seedlings. The physiological status of the donor plants is evidently the main cause of seasonal variations of the initiation frequency in axillary bud cultures.

The mineral composition of the basal medium is another important factor of the effectiveness of micropropagation. During the initiation phase of micropropagation, both in the axillary bud culture and in the seedling culture, the mineral composition of the culture medium caused no considerable effect on the initiation frequency. However, at the multiplication phase, significant differences have been revealed. The MS medium was found to be more efficient for shoot proliferation as compared to WPM. It should be noted that the full-strength MS medium is more favorable for the proliferation of seedling-derived cultures, whereas the ½ MS medium is more suitable for axillary bud-derived cultures. Perhaps, this is due to the higher regeneration and adaptive capacity of juvenile tissues as compared to adults with their higher demand for nutrients.

Cytokinins are known to be one of the key factors of woody plants during their in vitro propagation. In our experiments, BAP had better potential for shoot regeneration than kinetin or TDZ. Both in the tissue culture of buds and seedlings, BAP not only stimulated a higher efficiency of proliferation of shoot cultures but also ensured the development of normal shoots as compared with the TDZ-containing medium.

The results showed that the reduced content of mineral salts in the basal medium (¼ MS) and low level of auxin were are key factors for successful rooting of regenerated shoots. NAA was a more efficient inducer of root development as compared to IAA and IBA. It should be noted that the shoots developed on the 2.22 μM BAP-containing or 2.25 μM TDZ-containing medium were rooted and acclimatized significantly better than those taken from their proliferation media with a high cytokinin level.

Although no significant differences in the efficiency of micropropagation were recorded between the cultures derived from buds and seedlings, in general, micropropagation via seedling-derived cultures was a more affordable, fast, and effective method.

The histological study has showed that newly formed shoots were linked with the primary explants by a common vascular system and, therefore, arose as a result of

proliferation of the meristematic tissues of the explant. It can serve as one of the evidences of genetic identity among the shoots and the original shoot and could be used to increase the efficiency of micropropagation.

The results of our investigation can be used for the mass propagation of *L. anagyroides* and for obtaining seedlings for decorative landscaping and as a source of pharmaceuticals.

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Recent Advances in Asteraceae Tissue Culture

9

Jyothi Abraham and T. Dennis Thomas

9.1 Introduction

Asteraceae (earlier known as Compositae) family has several other names such as sunflower family, thistle family or daisy family. The name Asteraceae originated from the term 'Aster' means 'composite', and it refers to the characteristic inflorescence of this family. The head or capitellum inflorescence in Asteraceae has flower heads composed of many small flowers, called florets, that are surrounded by bracts (Bisht and Purohit 2010). This is one of the most evolved and largest families of flowering plants representing approximately 10 % of all flowering plants worldwide (Funk et al. 2009). The family Asteraceae comprises 43 tribes, 1600–1700 genera and about 24,000–30,000 species (Funk et al. 2005). The members of this family are distributed worldwide and show rich diversity of habit and habitat. Asteraceae members occupy almost every environment and continent including in the temperate regions and tropical mountains except Antarctica (Bayer et al. 2007). In India this family is represented by nearly 177 genera and 1052 species (Rao et al. 1988). The various taxa that come under Asteraceae exhibit a spectrum of life forms such as annual, biennial or perennial herbs, undershrubs, shrubs, trees, scramblers and aquatic plants (Bisht and Purohit 2010). In addition to the above categories, perennial species which are adapted to survive in cold or dry seasons with underground storage organs and spiny and succulent plants with milky saps are also present in

J. Abraham
Postgraduate and Research Department of Botany, St. Thomas College, Palai, Arunapuram (P.O), Kottayam, Kerala 686 574, India

T.D. Thomas (✉)
Postgraduate and Research Department of Botany, St. Thomas College, Palai, Arunapuram (P.O), Kottayam, Kerala 686 574, India

Department of Plant Science, School of Biological Sciences, Central University of Kerala, Padannakkad, 671314 Kasaragod, Kerala, India
e-mail: den_thuruthiyil@yahoo.com

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this family (Bisht and Purohit 2010). The members of this family include edible, noxious, medicinal, endangered and invasive species (Heywood et al. 2007). *Senecio* is the largest genera (1500 species) in this family, followed by *Vernonia* (1000 species), *Cousinia* (600 species) and *Centaurea* (600 species). Several members of Asteraceae are famous for their medicinal properties and are used against a variety of diseases. Medicinal plants such as calendula, tansy, camomile, wormwood, arnica, coltsfoot, echinacea, elecampane, milk thistle and chicory are routinely used for various medicinal purposes.

Traditionally, Asteraceae members are used for the treatment of wounds, bleedings, headache, pains, spasmodic diseases, flatulence, dyspepsia, dysentery, lumbago, leucorrhoea, haemorrhoids, gangrenous ulcer and disorders causing cachexia (Achika et al. 2014). Moreover, the astringent, antipyretic, anti-inflammatory, hepatoprotective, diaphoretic (in fevers), smooth muscle relaxant, nerve tonic and laxative properties of various members of this family have been reported (Achika et al. 2014). Phytochemical investigations of the Asteraceae family have revealed that many components from this family are highly bioactive. Asteraceae members are also well known for their beautiful flowers and are often seen in most of the botanical gardens. The familiar ornamental plants of Asteraceae include dahlia, zinnia, cosmos, aster, sunflower, marigold and chrysanthemum.

Plant tissue culture is the science of growing plant cells, tissues or organs isolated from the mother plant, on artificial media in aseptic condition (Thorpe 2007). Plant tissue culture technology has been widely employed for the rapid multiplication and micropropagation of several endemic, endangered and threatened plants (Thomas and Sankar 2009; Thomas and Hoshino 2010; Cheruvathur et al. 2010; Abraham et al. 2012). The other applications of this technique include plant regeneration via somatic embryogenesis (Kumar and Thomas 2012; Cheruvathur et al. 2013a, b), shoot organogenesis from callus (Cheruvathur and Thomas 2011; Jose and Thomas 2015), haploid plant production through androgenesis (Bajaj 1990) or gynogenesis (Bhojwani and Thomas 2001), triploid plant production via endosperm culture (Thomas et al. 2000; Thomas and Chaturvedi 2008; Hoshino et al. 2011), production of secondary metabolites (Cheruvathur and Thomas 2014) and protoplast isolation, culture and plant regeneration (Thomas 2009). Medicinal and aromatic plants of this family are widely overexploited for various purposes, and hence there is an urgent need for standardizing in vitro micropropagation protocols for important members of this family. Researchers have already developed micropropagation protocols for a number of taxa in this family (Table 9.1). In this review we examine the application of in vitro technology and production of large number of plants in various Asteraceae members.

9.2 *Elephantopus scaber*

E. scaber is an important medicinal herb which is distributed in the tropical regions of Southeast Asia and Latin America (Cabrera and Klein 1980). It is well known as a Chinese folk medicine since the whole plant is used for the treatment of various

Table 9.1 A summarized account of some selected reports on recent tissue culture works in Asteraceae members

Name of the plant	Explant	Type of medium and plant growth regulators	Response	References
Senecio candicans	L	MS + BA (2.0 mg/l) + IAA (2.0 mg/l) + NAA (3.0 mg/l)	MT	Hariprasath et al. (2015)
Spilanthes acmella	N	MS + BA (2.0 mg/l) + Kn (2.0 mg/l) +I BA (2.0 mg/l)	MT	Kurian and Thomas (2015)
Spilanthes acmella	N	MS + BA (0.5 mg/l) + Kn (1.0 mg/l) + IBA (1.0 mg/l)	MT	Joshi et al. (2015)
Elephantopus scaber	Seed, L	MS + 2,4-D (1.1 mg/l) + Kn (0.1 mg/l) + NAA (0.3 mg/l) + IBA (1.2 mg/l)	MT	Abraham and Thomas (2015b)
Elephantopus scaber	CN	MS + TDZ (1.5 mg/l) + NAA (0.5 mg/l) + IBA (1.0 mg/l)	MT	Abraham and Thomas (2015a)
Plectranthus barbatus	L	MS + NAA (1.0 mg/l) + Kn 1.5 mg/l) + BA(2.0 mg/l) + GA_3 (0.6 mg/l) + IBA (1.5 mg/l)	MT	Thangavel et al. (2014)
Leuzea carthamoides	L	MS + BA (0.5 mg/l) + IAA (0.5 mg/l) + IBA (1.0 mg/l) + NAA (1.0 mg/l) + 2,4-D (1.0 mg/l)	MT	Zand et al. (2014)
Matricaria chamomilla	L, AB, stem	MS + Kn (1.0 mg/l) + NAA (1.0 mg/l)	MT	Sayadi et al. (2014)
Pluchea lanceolata	N	MS + BA (2.5 mg/l) + Kn (2.5 mg/l) + TDZ (0.5 mg/l) + IBA (0.5 mg/l) + 2iP (2.5 mg/l)	MT	Kher et al. (2014)
Achillea millefolium	Seed	MS + BA (0.9 mg/l) + Kn (0.6 mg/l) + IAA (1.2 mg/l) + IBA (1.2 mg/l) + NAA (0.3 mg/l) + Zt (0.3 mg/l)	MT	Shatnawi (2013)

(continued)

Table 9.1 (continued)

Name of the plant	Explant	Type of medium and plant growth regulators	Response	References
Artemisia annua	Seed	MS + BA (0.1 mg/l) + NAA (0.1 mg/l) + GA_3 (0.5 mg/l)	MT	Tahir et al. (2014)
Blumea mollis	N	MS + BA (1.0 mg/l) + NAA (1.0 mg/l) + Kn (1.0 mg/l)	MT	Tamilarasi and Thirugnanasmpandan (2013)
Gerbera jamesonii	SA	MS + BA (2.0 mg/l) + IAA (0.5 mg/l) + IBA (2.0 mg/l) + 2,4-D (2.0 mg/l) + Kn (2.0 mg/l)	MT	Minerva and Kumar (2013)
Arnica montana	L, P	MS + BA (1.mg/l) + Kn (1.0 mg/l) + IAA (0.1 mg/l) + IBA (0.5 mg/l) + 2,4-D (0.1 mg/l) + zeatin (1.0 mg/l) + 2iP (1.0 mg/l)	MT	Petrova et al. (2011)
Eupatorium triplinerve	N	MS + BA (1.0 mg/l)	MT	Janarthanam et al. (2011)
Vernonia anthelmintica	CN	MS + BA (1.1 mg/l) + Kn (1.1 mg/l) + NAA (1.1 mg/l) + 2,4-D (0.2 mg/l)	MT	Subhan and Agrawal (2011)
Senecio macrophyllus	ST	MS + BA (1.0 mg/l) + Kn (2.5 mg/l) + ZEA (2.5 mg/l) + NAA (0.11 mg/l)	MT	Trejgell et al. (2010)
Sphaeranthus indicus	L	MS + BA (1.0 mg/l) + IAA (0.3 mg/l) + IBA (0.5 mg/l) + Kn (0.5 mg/l)	MT	Yarra et al. (2010)
Echinacea angustifolia	FS	MS + BA (3.0 mg/l) + IBA (0.5 mg/l)	MT	Lucchesini et al. (2009)
Leontopodium nivale	C	MS + BA (2.0 mg/l)	MT	Pace et al. (2009)
Anthemis nobilis	ST	MS + BA (0.5 mg/l) + IAA (0.1 mg/l) + IBA (0.1 mg/l)	MT	Echeverrigaray et al. (2008)
Sphaeranthus amaranthoides	ST, AB	MS + BA (4.0 mg/l) + Kn (4.0 mg/l) + IBA (2.0 mg/l)	MT	Ravipaul et al. (2008)
Lychnophora pinaster	Seed, E	MS + BA (1.1 mg/l) + GA_3 (2.5 mg/l) + NAA (2.0 mg/l)	MT	De Souza et al. (2007)

(continued)

Table 9.1 (continued)

Name of the plant	Explant	Type of medium and plant growth regulators	Response	References
Pentanema indicum	ST, N	MS + BA (2.0 mg/l) + IBA (2.0 mg/l) + IAA (1.0 mg/l)	MT	Sivanesan and Jeong (2007)
Vernonia amygdalina	N	MS + BA (0.5 mg/l) + NAA (2.0 mg/l) + Kn (4.0 mg/l) + 2,4-D (0.5 mg/l)	MT	Khalafalla et al. 2007
Eclipta alba	CN	MS + BA (1.0 mg/l) + Kn (1.0 mg/l) + GA_3 (0.5 mg/l) + IBA (2.0 mg/l) + isopentenyl adenine (0.9 mg/l)	MT	Baskaran and Jayabalan (2005)
Achillea filipendulina	Meristem, N	MS + BA (2.0 mg/l) + TDZ + IAA (1.0 mg/l) + NAA (0.1 mg/l) + IBA (0.12 mg/l)	MT	Evenor and Reuveni (2004)
Spilanthes acmella	AB	MS + BA (2.0 mg/l) + IBA (0.6 mg/l)	MT	Haw and Keng (2003)
Artemisia judaica	Seeds, hypocotyl	MS + BA + TDZ (0.2 mg/l) + IBA (0.15 mg/l) + NAA (0.1 mg/l)	MT	Liu et al. (2003)
Echinacea pallida	L	MS + BA (1.0 mg/l) + NAA (0.01 mg/l) + IBA (0.1 mg/l)	MT	Koroch et al. (2003)
Hymenoxys acaulis	ST, stem	MS + BA (0.45 mg/l) + IBA (0.1 mg/l)	MT	Ault (2002)
Santolina canescens	ST	MS + BA (0.25 mg/l) + Kn (0.3 mg/l) + IBA (0.5 mg/l) + IAA (1.0 mg/l) + NAA (0.5 mg/l)	MT	Casado et al. (2002)
Arnica montana	N	MS + 2iP (1.4 mg/l) + zeatin (1.0 mg/l) + NAA (1.0 mg/l)	MT	Keul and Deliu (2001)
Tagetes erecta	L	MS + GA_3 (4.0 mg/l) + BA(1.0 mg/l) + NAA (0.5 mg/l)	MT	Misra and Datta (2001)

AB axillary bud, *C* cotyledon, *CN* cotyledonary node, *FS* flower stalk, *L* leaf, *MT* multiple shoot, *N* node, *P* petiole, *SA* stem apices, *ST* shoot tip

diseases. It is also used to cure diabetes, enteritis, flatulence and diuresis and is adopted as an analgesic, astringent and antiemetic agent (Hiradeve and Rangari 2014; Daisy et al. 2007; Lee et al. 2008). It is used against cardiovascular diseases, bronchitis and smallpox (Kiritikar and Basu 1991). The healing properties of roots of this plant are useful for heart as well as liver problems, and the hot water extract of roots is used to combat filariasis and diarrhoea (Hammer and Johns 1993). The aqueous extract of leaves is applied externally to treat eczema and ulcers (Chopra et al. 1956). The whole plant is macerated and applied on the surface of wounds to promote wound-healing activity (Vaidya 1999). It is reported that this species is also used for its antitumour, hepatoprotective, wound-healing and anti-inflammatory activity (Rajesh and Latha 2001; Sankar et al. 2001; Rajkapoor et al. 2002; Singh et al. 2005; Xu et al. 2006).

The seeds of *E. scaber* were aseptically cultured on half-strength Murashige and Skoog (1962) medium without any plant growth regulators (Rout and Sahoo 2013). The germinated seeds were transferred to callus induction medium fortified with different concentrations (0.25–2.0 mg/l) of 2,4-dichlorophenoxyacetic acid (2,4-D) or 6-benzyladenine (BA) alone and in combination with kinetin (Kn; 0.25–2.0 mg/l). The proliferated calli were transferred to shoot regeneration medium. A combination of 2.0 mg/l BA and 1.0 mg/l α-naphthalene acetic acid (NAA) was the most effective hormone for shoot regeneration from callus (13.7 shoots/callus). The elongated shoots transferred to half-strength MS medium supplemented with different concentrations of auxins like NAA, indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) for root induction. NAA was most suitable for root induction (6.2 roots/shoot) when compared to IAA (3.5 roots/shoot) and IBA (3.7 shoots/callus). The in vitro regenerated plantlets were transplanted to greenhouse for acclimatization. The survival of the plantlets was recorded as 77 %. The antioxidant activity of *E. scaber* was analysed from in vitro and in vivo grown leaf and root samples, and result showed that antioxidant enzymes like superoxide dismutase (SOD) and guaiacol peroxidase (GPX) were extensively more concentrated in the leaf and root samples of in vitro grown plants, whereas the catalase (CAT) concentration was maximum recorded in leaf samples of in vivo plants (Rout and Sahoo 2013).

Abraham and Thomas (2015a) standardized an efficient protocol for the rapid micropropagation of *E. scaber* using cotyledonary node explants. Direct multiple shoot induction from cotyledonary node explants at various age groups was induced on MS medium fortified with different plant growth regulators. The optimum shoot induction was observed when 20-day-old cotyledonary node explants were inoculated on MS medium fortified with 1.5 mg/l thidiazuron (TDZ) and 0.5 mg/l NAA. Here, 98 % of the cultures produced shoots, with a mean number of 33.7 shoots per explant. The maximum frequency of rooting (100 %) and average number of roots (3.3 per shoot) were obtained on MS medium supplemented with 1.0 mg/l IBA. The plantlets were acclimatized and transferred to soil with 92 % success (Fig. 9.1).

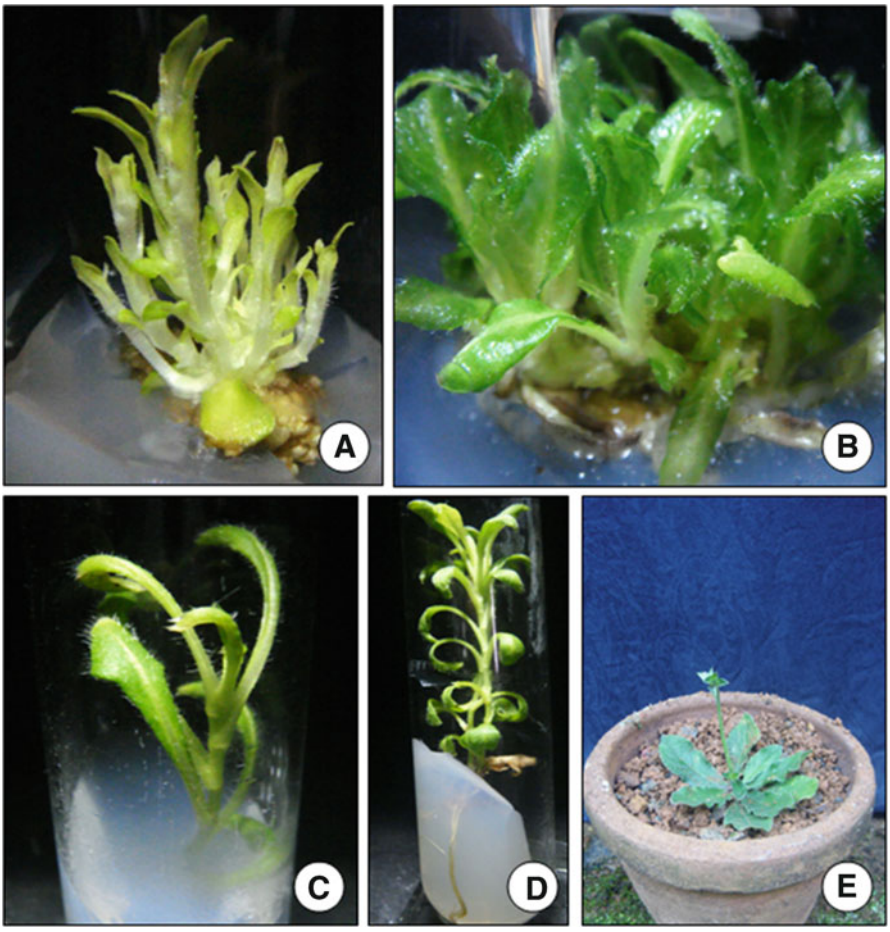

Fig. 9.1 Various stages of micropropagation in E. scaber. (**a**) Multiple shoot induction from nodal segments of E. scaber 3 weeks after culture on MS medium supplemented with 3 mg/l BA. (**b**) Same as in Fig. 9.1a 6 weeks after culture. (**c**) Shoot elongation on MS medium supplemented with 1.0 mg/l BA. (**d**) Rooting of shoots on ½ MS medium with 1.5 mg/l IBA. (**e**) An 8-month-old transplanted plant during flowering period

In another report, a plant regeneration procedure via callus organogenesis has been developed for *E. scaber* by Abraham and Thomas (2015b). The calli were induced from seeds and leaf segments. The optimum callus induction (89 %) was observed on MS supplemented with 1.1 mg/l 2,4-D and 0.1 mg/l Kn from seed explant. Further, the highest callus regenerating frequency (91 %) and number of

shoots (56) per culture were noted on MS medium supplemented with 1.4 mg/l BA and 0.25 mg/l NAA. Optimum rooting of regenerated shoots was observed on half-strength MS medium supplemented with 1.1 mg/l IBA. On this medium, 100 % shoots produced roots with a mean number of 3.2 roots per shoot. The use of vesicular arbuscular mycorrhizae (VAM) during acclimatization along with potting mix has been confirmed in this study, and the highest response of 100 % plant survival was obtained in a mixture of autoclaved garden soil and sand (2:1) and VAM was utilized as potting mix. Molecular studies (inter-simple sequence repeats (ISSR) analysis) have confirmed the true-to-type nature of regenerated plants.

9.3 *Anthemis xylopoda*

A. xylopoda is an endemic plant which falls in the critically endangered category in the *Red Data Book* of Turkey (Ekim et al. 2000). Uzel et al. (2004) have recently reported the chemical composition of the essential oils isolated from air-dried leaves and flowers of *A. xylopoda*. According to Uzel et al. (2004), the phytochemical components of *A. xylopoda* include borneol, 1,8-cineole, 2,5,5-trimethyl-3,6-heptadien-2-ol, α,β-thujone and carvacrol from flowers and leaves, of which borneol was the major constituent. Further, their results showed that the oil extracted from *A. xylopoda* exhibited significant antimicrobial activity.

A protocol was optimized in *A. xylopoda* for the direct adventitious shoot induction from in vitro leaf explants (Erdag and Emek 2009). MS medium supplemented with BA, Kn and TDZ was employed for regeneration experiments. BA at 0.5 mg/l was chosen because it yielded the highest number of adventitious shoots (6.70 shoots/explant). However, the highest mean shoot length was noted on MS medium containing 0.2 mg/l BA (4.30 cm). The MS medium with 0.5 mg/l IBA was optimum for rooting of regenerated plantlets. Another interesting observation noticed during the study was the induction of flower buds during rooting. IBA was found to be inducing more flower buds than IAA. The maximum flowering percentage was recorded on MS medium containing 1.0 mg/l IBA. The yellow flowers produced in vitro were morphologically normal and similar to field-grown plants.

9.4 *Dendranthema grandiflorum*

Chrysanthemum (*D. grandiflorum*) is one of the most popular ornamental plants in the world and it includes about 40 species. Despite its ornamental value, these plants have both aesthetic and medicinal uses in many countries. Chrysanthemum is also a reservoir of various useful secondary metabolites including essential oils which are biologically active (Schwinn et al. 1994).

D. grandiflorum garden cultivars ‘Yes Morning’ and ‘Hi-Maya’ and pot cultivar ‘Peace Pink’ were used for anther culture by Khandakar et al. (2014). Callus induction rate among these cultivars did not show much variation on MS medium supplemented with 1.0 mg/l of 2,4-D, 2.0 mg/l of BA, 250 mg/l of casein hydrolysate and 45.0 g/l of sucrose, solidified by 2.75 g/l gelrite (Khandakar et al. 2014). The

pretreatment of anther at 4 °C for 48 h had significantly enhanced the callus induction as well as shoot regeneration rate. The highest shoot induction frequency (79 % callus inducing shoots) was obtained when androgenic calli were differentiated on MS medium supplemented with 2.0 mg/l of BA and 0.1 mg/l of NAA. The elongated shoots were rooted on MS medium supplemented with 0.1 mg/l of NAA. The plantlets were acclimatized and transferred to soil. The haploid nature of the plants was confirmed by ploidy analysis using cytology.

9.5 *Echinacea purpurea*

The phytochemistry of *Echinacea* species is well studied and caffeic acid derivatives, flavonoids, polyacetylenes, alkamides, pyrrolizidine alkaloids, polysaccharides and glycoproteins were isolated and characterized from this plant (Bauer and Foster 1991; Bauer and Wagner 1991; Bauer and Reminger 1989; Bauer et al. 1988, 1989). In the last few years, there is an increased demand for natural remedies, and therefore, the value of this medicinal plant has increased.

E. purpurea is the most widespread (McGregor 1968) and extensively cultivated medicinal species of the genus *Echinacea* (McKeown 1999). In America and Canada, this plant is traditionally used for the treatment of respiratory ailments, sores, wounds and a variety of other ailments (Tyler 1993; Hobbs 1994). Additionally, this plant is also used for the treatment of cold, influenza, wound and candidiasis (Bauer 1999).

Koroch et al. (2002) optimized an efficient plant regeneration protocol via organogenesis from callus derived from leaf tissue of *E. purpurea*. BA alone produced green organogenic callus. The highest shoot organogenesis from callus was obtained on MS medium supplemented with BA (1.0 mg/l) and NAA (0.01 mg/l). This medium gave the highest shoot regeneration frequency (100 %) associated with a high number of shoots (7.7 shoots/explant). An increase in NAA concentration resulted in increased callus production and low shoot initiation. The optimum rooting of shoots was observed on MS medium supplemented with 0.4 or 1.0 mg/l IBA. The rooted plantlets were successfully transplanted to the field after acclimatization.

9.6 *Eclipta alba* and *Eupatorium adenophorum*

Eclipta alba is a small, annual herb distributed in the tropical and subtropical regions of the world. This plant is used as tonic and diuretic in hepatic and spleen enlargement and in jaundice and skin diseases (Anonymous 1952). Wedelolactone and dimethyl wedelolactone are the two important active phytochemicals present in *E. alba*, and both these constituents have antihepatotoxic activity (Wagner et al. 1986; Franca et al. 1995). The emetic and purgative properties of roots are reported, and the root is applied externally as an antiseptic to ulcers and wounds of cattle (Anonymous 1952). The antibiotic activity of shoot extract against *Staphylococcus aureus* and *Escherichia coli* was reported (Anonymous 1952). Conventionally, *E. alba* is propagated by seeds. *Eupatorium adenophorum* is a herb usually distributed

in higher elevations (1000–2000 m above mean sea level) and is used in the treatment of stomach ache and to prevent bleeding (Uniyal 1980). *E. adenophorum* is traditionally propagated by vegetative cuttings.

Nodal segment culture was effectively used to micropropagate *E. alba* and *E. adenophorum* by Bothakur et al. (2000). Proliferated microshoots were obtained through axillary branching from cultured nodal segments on modified MS and half-strength of MS medium supplemented with BA and Kn. The highest shoot induction frequency (16.0 shoots/explant) and root growth was obtained on MS medium supplemented with 0.05 mg/l Kn. However, only satisfactory result (6.0 shoots/explant) was obtained with BA 0.05 mg/l. Regenerated shoots were rooted and successfully acclimatized in soil. The in vitro raised plants grew normally and did not show any morphological variations.

Singh et al. (2012) standardized a protocol for the efficient in vitro regeneration of *E. alba* through transverse thin cell layer (tTCL) culture. Nodal segments were transversely cut and were used as tTCL explants for plant regeneration. BA plays a crucial role in inducing multiple shoots and its interaction with Kn or NAA influenced the shoot yield. The most effective media combination for multiple shoot induction was 3.0 mg/l BA and 1.0 mg/l Kn. Here, 100 % explants responded with a mean number of 32.6 shoot buds per tTCL nodal explant. The regenerated shoots were rooted on MS basal medium. The rooted plantlets were acclimatized and transplanted to soil with a survival frequency of 90–100 %. Genetic fidelity of regenerated plants was assessed by using random amplified polymorphic DNA (RAPD) markers and confirmed the genetic similarity of regenerated plants with the mother plants.

9.7 *Carthamus tinctorius*

The common name of *C. tinctorius* is safflower or false saffron. It produces the characteristic red/orange pigment which is used for colouring rice and bread and for dyeing cloths (Wang and Li 1985). Due to the rich content and high nutritional value of its edible oil, it has become an important crop in some parts of the world including Turkey and Iran (Kumar and Kumari 2011). *C. tinctorius* is a good purgative, antipyretic, analgesic and an antidote to poisoning (Weiss 1983). Its use in haemorrhage, whooping cough, chronic bronchitis, rheumatism and sciatica is well established (Bae et al. 2002).

Walia et al. (2007) studied the morphogenic response of endosperm of *C. tinctorius* cv. HUS-305. The endosperm was isolated from seeds of plant at globular to heart-shaped stages of zygotic embryo development. The excised endosperm was cultured on MS medium containing various concentrations of BA, Kn, TDZ, 2,4-D or NAA. Although callusing was optimum on 2,4-D medium, there was no shoot regeneration. Endosperm embryos were formed only from calli developed on media supplemented with BA, Kn or TDZ with the last eliciting highest response. The addition of adenine sulphate (100 mg/l) to the medium further improved the induction of endosperm embryos. For embryo elongation and plantlet production, MS basal medium or 0.2 mg/l gibberellic acid (GA_3) was employed.

Ghasempour et al. (2014) was successful in inducing callus from various explant types such as shoot, root and leaf segments of in vitro grown seedlings. The sterilized seeds were germinated on MS medium. The various explants were then isolated and cultured on MS medium supplemented with different concentrations of BA, Kn, NAA and 2,4-D. The leaf explant was producing the highest calli (97.79 %) on MS medium fortified with 1.0 mg/l BA. The calli were transferred to regeneration medium containing various concentrations of BA and NAA after 4 weeks. The highest shoot regeneration from calli was obtained on MS medium supplemented with 0.1 mg/l NAA and 1.0 mg/l BA.

9.8 *Centaurea* spp.

9.8.1 *Centaurea ultreiae*

Centaurea is one of the largest genera in the family Asteraceae. *C. ultreiae* is a critically endangered species in Spain (Banares et al. 2003). A protocol was developed for the efficient shoot regeneration from leaves and roots of *C. ultreiae* (Mallon et al. 2011). Direct shoot induction from leaf and root explants was obtained when the explants were cultured on half-strength MS medium in the presence of BA, zeatin, Kn or N6-(2-isopentenyl)adenine (2iP), each provided at five different levels. On the MS medium supplemented with 0.1 mg/l IBA, 90 % leaf explants produced a mean number of 2.48 shoots per explant, whereas 94.3 % of root explants produced an average number of 5.60 shoots per explant, on a medium supplemented with 0.1 mg/l BA. Further, histological analysis confirmed the connection between vascular tissues of regenerated shoots and cambial cells of leaf explants. The origin of adventitious shoots was found to be from pericycle cells of root explants.

9.8.2 *Centaurea arifolia*

C. arifolia is a data deficient (DD) plant according to IUCN category (Ekim et al. 2000; IUCN 2001). A successful in vitro micropropagation technique via callus-mediated shoot organogenesis was developed for *C. arifolia* (Yuzbasioglu et al. 2012). The leaf explants were obtained from germinated seedlings on growth regulator-free half-strength MS medium. Leaf explants excised from 6-week-old seedlings were cultured on MS medium supplemented with 1.0 mg/l BA, 0.1 mg/l NAA and 2.0 mg/l BA and 0.2 mg/l NAA for callus induction and shoot organogenesis. Adventitious shoot induction from calli was obtained within 3 weeks of culture. The callus-derived shoots were rooted on MS medium with 1.0 mg/l IBA. This protocol provides a successful propagation technique through indirect in vitro organogenesis from leaf segments of *C. arifolia*.

9.8.3 *Centaurea cyanus* and *Centaurea montana*

European traditional medicine uses the *C. cyanus* flower heads in the treatment of minor ocular inflammation (Bruneton 1995). Anti-inflammatory and immunological properties of flower extracts of this plant have been experimentally demonstrated by Garbacki et al. (1999). It also contains major flavonoid glycosides that could be used in various pharmacomedicinal assays (Gonnet 1996). An important dimeric indole alkaloid called montamine is extracted from the seeds of *C. montana*, and it exhibited cytotoxic activity against Caco-2 colon cancer cells (Shoeb et al. 2006).

In vitro regeneration and flowering in *C. cyanus* had been reported (Alaiwi et al. 2012). Young leaf explants were used as explants. The explant was cultured on MS medium supplemented with 2.0 mg/l BA and 0.1 mg/l IAA for 4 weeks regenerated 50–60 shoots per explant. The shoots produced in vitro flowering and seed set when incubated on MS basal medium supplemented with B5 vitamins, 100 mg/l myo-inositol and 30 g/l sucrose for 4 weeks under 16 h photoperiod. The callus was induced when young leaves of *C. montana* are inoculated on MS medium with 1.0–6.0 mg/l 2,4-D alone or in combination with 0.5 mg/l BA. Greater fresh weight (FW), dry weight (DW) and packed cell volume (PCV) were observed when liquid MS medium amended with 2.0 mg/l 2,4-D compared to MS semisolid medium. The authors concluded that MS medium supplemented with proper phytohormones could be used to obtain effective shoot regeneration and in vitro flowering in *C. cyanus*.

9.8.4 *Centaurea cineraria*

This species is endemic and the distribution is limited to Circeo Mountain (Lazio, Italy). The plant population was estimated to be very low (Valletta et al. 2015). An ex situ conservation strategy was developed for *C. cineraria* subspecies *circae* which include achene collection and in vitro plant propagation in order to carry out restoration programmes. The study showed that 5.5 % of achenes were morphologically healthy (Valletta et al. 2015). There was no seed dormancy since most of the fresh seeds germinated and didn't need any treatments. Under a photoperiod of 12/12 h (light/dark) and temperature regime +20/+10 °C, higher seed germination rate (67.5 %) was achieved. In vitro micropropagation studies including shoot induction, rooting, acclimatization and field transfer were achieved. On MS medium containing 0.5 mg/l benzylaminopurine and 2 mg/l Kn, 74 % of shoot explants formed multiple shoots. On MS medium supplemented with 0.5 mg/l IBA, 100 % shoots rooted and over 90 % survived the acclimatization phase. After field transfer, the in vitro derived plants bloomed and showed no morphological differences from wild plants. Similarly, preliminary phytochemical analysis indicated a comparable profile for in vitro propagated and wild plants (Valletta et al. 2015).

9.9 *Lactuca sativa*

The leafy vegetable lettuce (*L. sativa*) is grown on all continents. The whole plant has been utilized in the treatment of stomach problems, to stimulate digestion, to enhance appetite and to relieve inflammation (Sayyah et al. 2004). The triterpene lactones isolated from this plant showed anti-inflammatory activities (Araruna and Carlos 2010). *L. sativa* gives protection against D-galactose-induced oxidative stress and reduces accumulation of lipofuscin granules (Deshmukh et al. 2007). This plant is a rich source of antioxidants such as quercetin, caffeic acid, vitamin C (Brunk and Terman 2002), carotenoids (Kim et al. 2007) and phytols (Bang et al. 2002). The antioxidant activity of *L. sativa* has been reported to prevent chronic diseases related to oxidative stress such as cancer (Chu et al. 2002).

A reproducible protocol for the production of uniform shoots in a wide range of lettuce (*L. sativa*) genotypes was reported (Hunter and Burritt 2002). This study indicated a strong influence of genotype on regeneration capacity. The per cent explants producing shoots and mean number of shoots varied with the genotype, and it can be doubled by culturing cotyledon explants on MS medium supplemented with 0.54 mg/l NAA and 0.44 mg/l BA. It was believed that in this plant, the loss of regeneration ability occurs in cotyledons due to its age. However, in this study it was confirmed that this regeneration ability is highly genotype depended and not related to cotyledon age. In most studies cotyledon explants were isolated 2–4 days after germination, whereas in this study for many cultivars, the cotyledons were isolated 14 days after germination and it still showed regeneration ability and produced shoots.

9.10 *Stevia rebaudiana*

S. rebaudiana is a perennial herb of Asteraceae family. This natural sweetener is indigenous from higher elevations of northern Paraguay (Soejarto et al. 1983; Lewis 1992). However, it could be found growing in other habitats like semi-arid environment, ranging from grassland to scrub forest to mountain terrain. The leaves of stevia yield several phytochemicals mainly diterpene glycosides, such as stevioside and rebaudiosides, which are estimated to be about 300 times sweeter than sucrose (Tanaka 1982). In China, Taiwan, Thailand, Korea, Japan, India and Malaysia, this plant is commercially cultivated (Jain et al. 2009). Stevia is also important as a natural non-caloric sweetener since its leaves contain stevioside. It has been widely employed in a wide range of processed foods as a substitute for conventional sugars or artificial dietetics especially in Japan (Handro and Ferreira 1989).

El-Zaidy et al. (2010) established an efficient shoot regeneration protocol in *S. rebaudiana* using adult leaf explants. Both MS medium and Linsmaier and Skoog (LS; Linsmaier and Skoog 1965) medium were suitable for callus induction. The most suitable combination for callus induction includes 1.0 mg/l 2,4-D, 0.5 mg/l BA and 1.0 mg/l GA_3, and the genotype 4 produced the highest frequency of callus. The calli were developed into embryos, and the green embryo development was highest

in genotype 4 on MS medium supplemented with 2.0 mg/l BA. High-frequency plant multiplication and elongation of shoots were achieved in genotypes 5 and 3 on MS medium supplemented with 0.1 mg/l BA. Comparatively MS medium was superior over LS medium for root differentiation.

Das et al. (2011) reported micropropagation of *S. rebaudiana* via shoot tip culture. MS medium containing 2 mg/l Kn was found to be most suitable for multiple shoot induction from shoot tip culture. By using this medium, more than 11 shoots were obtained from a single shoot tip within 35 days of culture. The roots were induced from shoots on MS basal medium, whereas it has a negative impact when it was fortified with IAA and BA. They also performed peroxidase assay and ISSR fingerprinting to confirm the clonal fidelity of in vitro derived plantlets. This result showed that Kn plays a crucial role in shoot induction from shoot tip explants.

Hwang (2006) reported high-frequency multiple shoot induction from nodal segments of field-grown *S. rebaudiana* on four basal media containing various combinations and concentrations of auxins and cytokinins. The highest shoot induction (23.4 shoots per explant) was observed on MS medium fortified with 2 mg/l IAA and 0.5 mg/l Kn. The medium suitable for root induction was MS medium supplemented with 2 mg/l IBA. The rooted plants were transplanted to soil and acclimatized successfully with 98.4 % survival rate. Stevioside contents were analysed in the in vitro derived field-grown plants and were estimated to be 10.68 mg/g dry weight (DW) which was comparable to that of mother plants (12.01 mg/g DW).

Ahmed et al. (2007) reported in vitro shoot induction from nodal segments of *S. rebaudiana* through enhanced axillary branching. The nodal explants were inoculated on MS medium fortified with different combinations of BA and Kn. The highest axillary shoot proliferation (8.75 shoots per explant) was achieved on MS medium containing 1.5 mg/l BA and 0.5 mg/l Kn. For rooting of the various concentrations of three auxins employed (IBA, NAA and IAA), IAA at 0.1 mg/l was optimum producing the highest rooting percentage (97.66 %).

A method for micropropagation of *S. rebaudiana* through nodular stem sections had been reported by Rafiq et al. (2007). Optimum shooting response was observed on MS medium supplemented with 2.0 mg/l BA. The highest rooting of shoots was obtained on MS medium supplemented with 0.5 mg/l NAA. The survival rate of in vitro derived plants was 92 and 83 % during hardening and transfer to greenhouse, respectively. Various biochemical attributes including carbohydrates and proteins were analysed in the leaves and compared with leaf extract of Egyptian cultivar to assess its quality.

By using 2-year-old stem node segments, Alhady (2011) developed a protocol for micropropagation in *S. rebaudiana*. According to the author, cytokinin plays a crucial role in inducing shoot multiplication. The effect of BA individually or in combination with Kn at varying levels was investigated. The optimum shoot induction frequency was obtained on MS medium amended with BA. An increasing concentration of BA enhanced shoot multiplication. The highest shoot induction was observed on MS medium supplemented with 2.0 mg/l BA and 0.5 mg/l Kn. However, the presence of Kn in the medium promoted shoot elongation. Of the various auxins like IBA and NAA used for rooting, IBA was superior over NAA. The optimum

rooting response (100 %) was observed on MS medium containing 1.0 or 2.0 mg/l IBA. The authors concluded that the presence of cytokinins BA and Kn in the medium enhanced axillary shoot proliferation from stem node explants.

Uddin et al. (2006) reported callus induction from various explants like leaf, internode and node in *S. rebaudiana*. The explants were inoculated on MS medium supplemented with various concentrations (2.0, 3.0, 4.0 and 5.0 mg/l) of 2,4-D. The results showed that internodal segments initiated callus earlier than node and leaf. The maximum intensity of callusing was observed on MS medium supplemented with 3.0 mg/l 2,4-D.

In another report on callus induction and multiplication, Gupta et al. (2010) cultured nodal, leaf and root explants on MS medium supplemented with various levels of plant growth regulators like IBA, Kn, NAA and NAA in combination with 2,4-D. The highest response (100 %) was observed from leaf explants when cultured on MS medium supplemented with NAA and 2,4-D after 3 weeks, whereas with 2,4-D alone it induced only 10 % callusing. The morphological analysis of the calli derived from various explants showed variations. The calli formed from leaf and root explants were shiny green, while that of nodal explants was hard and brown. The investigators concluded that leaf explants could serve as the best planting material for callus production, and 0.75 mg/l NAA with 1 mg/l 2,4-D could produce maximum amount of callus within a short span of time.

Patel and Shah (2009) reported callus induction and shoot organogenesis in *S. rebaudiana*. For callus induction, nodal and leaf explants were cultured on MS medium supplemented with different concentrations of BA and NAA. Among the various combinations tried, 2.0 mg/l BA and 2.0 mg/l NAA produced the highest callus induction. The highest shoot organogenesis was also obtained on the same medium. On ¼ MS medium supplemented with 0.1 mg/l IBA, the regenerated shoots were rooted. The rooted shoots were hardened successfully with 63 % survival rate.

Callus induction from leaf explants and subsequent shoot organogenesis were reported in *S. rebaudiana* (Moktaduzzaman and Rahman 2009). Further, the analysis of somaclonal variation among regenerated plants was studied by RAPD analysis for the identification of possible somaclonal variants which are useful for quality control in plant tissue culture and in the introduction of new variants. The leaf segments were cultured on MS medium supplemented with various concentrations of NAA, BA and 2,4-D. They observed the highest callus induction frequency (91.67 %), fresh weight (621.7 mg) and dry weight (79.00 mg) on MS medium supplemented with 1.5 mg/l NAA and 1.0 mg/l BA. The multiplied calli showed best regeneration ability on MS medium fortified with different concentrations and combinations of BA and NAA. The highest shoot number (2.17) and the mean shoot length (3.22 cm) per culture were observed at 1.8 mg/l of BA with 0.12 mg/l of NAA. The rooting of in vitro derived shoots was obtained on IBA- and NAA-containing media. The highest number of roots and length of roots per culture were observed on 1.0 mg/l NAA. The in vitro derived plants were transferred to soil in 75 % soil and 25 % sand mixture, hardened and finally transferred to the field. Bands generated through RAPD analysis confirmed that some in vitro plants showed

100 % similarity, whereas others showed 71, 57 or 14 % similarity to the mother plants.

In another report on callus induction and shoot organogenesis in *S. rebaudiana*, Sairkar et al. (2009) cultured nodal segments on MS medium supplemented with 0.1 mg/l BA for shoot initiation. For callus induction, a combination of 2.0 mg/l 2,4-D and 1.0 mg/l Kn was sufficient to give best result. Higher regeneration ability of plantlets (3.8 plantlets/calli) was achieved on MS medium supplemented with 5.0 mg/l BA and 1.0 mg/l NAA. The optimum number of plantlets (83.2) was obtained on MS medium fortified with 3.5 mg/l BA at multiplication stage. The optimum rooting (11.1 roots/shoot) was recorded on ½ MS medium with 100 mg/l activated charcoal. The rooted plantlets were acclimatized in 1:1:1 ratio of sand/soil/vermicompost and successfully transferred to soil.

Anbazhagan et al. (2010) standardized a protocol for the mass propagation of *S. rebaudiana*. Shoot tip, nodal segment and leaf explants were cultured on MS medium supplemented with various concentrations of BA, Kn and IAA both individually and in combination for inducing direct shoots. The optimum shoot induction was obtained on MS medium containing BA and IAA at the concentrations of 1.0 and 0.5 mg/l, respectively. Among the various explants employed for shoot induction, shoot tip gave the highest response. Half-strength Nitsch (N6) medium containing 1.0 mg/l IAA produced the optimum rooting. The plantlets developed were transferred to pots and finally planted in the open field. About 82 % of acclimatized plants survived and established in natural field conditions.

Singh et al. (2011) successfully induced callus and shoot induction from leaf explants in *S. rebaudiana*. The optimum callusing was observed on MS medium supplemented with 1.0 mg/l 2,4-D and 1.0 mg/l Kn. The best shoot differentiation was obtained on MS medium supplemented with 0.5 mg/l BA and 0.1 mg/l NAA. The authors further studied the metabolic changes during differentiation in callus cultures and found that metabolites like starch, total soluble sugars and total phenols showed a decline, while total soluble proteins increased in callus culture.

In order to meet the growing demand, an efficient protocol for *S. rebaudiana* micropropagation has been developed by Thiyagarajan and Venkatachalam (2012). Nodal explants were cultured on MS supplemented with various concentrations of BA (0.5–3.0 mg/l) and Kn (0.5–3.0 mg/l) for multiple shoot induction. Similarly, in vitro derived buds were cultured on MS medium fortified with various concentrations of BA (0.5–3.0 mg/l) in combination with 0.5 mg/l IAA or IBA or NAA for shoot bud multiplication. The optimum frequency (94.50 %) of multiple shoot induction with the highest number of shoots (15.69 shoots/explant) was observed on MS medium with 1.0 mg/l BA. In vitro derived nodal bud explants when cultured on MS medium supplemented with BA (1.0 mg/l) produced a mean number of 12.3 shoots/explant after three subcultures on the same media composition. Elongated shoots were rooted on half-strength MS medium supplemented with different concentrations of NAA (0.1–0.5 mg/l) and/or MS medium with different concentrations (0.5–2.0 mg/l) of auxins (NAA, IAA and IBA). The optimum rooting (96 %) was observed on half-strength MS medium supplemented with 0.4 mg/l NAA. The plantlets were transplanted to plastic cups containing sand and soil (1:2) and subsequently established in the field.

9.11 *Artemisia* spp.

9.11.1 *Artemisia petrosa*

A. petrosa usually grow in rocky regions at an altitude above 2200 m. It usually appears on top of limestone slopes exposed to cold winds. Plants growing in shady areas flourish well and produce taller floral axis than more exposed ones. The phytotherapeutical properties of this plant are comparable to those of other *Artemisia* species. The phytochemical thujone [4-methyl-1-(1-methylethyl)bicyclo(3.1.0)-hexan-3-one] isolated from the flowers is an important drug. For isolating the drug, the flowers are cut at the beginning of the blooming season (from July to September), 5–8 cm above the ground (Bellomaria et al. 1981; Bicchi et al. 1982; Caligari and Hind 1996). Since *A. petrosa* is an endangered species, it is protected by an Italian regional law, and its survival is threatened due to indiscriminate collection for commercial purposes, mainly liqueur production by local people and tourists (Conti et al. 1992, 2005).

An efficient protocol for in vitro micropropagation of *A. petrosa* ssp. *eriantha* has been developed by Pace et al. (2004). The explants were collected from in vitro grown seedlings. The addition of $CaCO_3$ to the medium significantly influenced the rate of propagation. The best callusing was obtained on MS medium supplemented with 1.0 mg/l 2,4-D. Similarly, the maximum shoot induction was observed on MS medium augmented with 2.0 mg/l BA. Under this optimal condition, the shoot number has reached an average of 30–35 shoots/explant. After in vitro rooting and transplantation, the plants were reintroduction in natural conditions.

9.11.2 *Artemisia chamaemelifolia*

A. *chamaemelifolia* is a perennial, aromatic plant. This plant comes under the critically endangered category from the *Red Data Book* of Bulgaria. The effect of various BA concentrations (0.1–1.0 mg/l) on in vitro propagation was investigated by Hristova et al. (2013). Although an increase in concentration of BA enhanced the shoot induction, it reduced the shoot length. The shoots grew well on concentrations of 0.5, 0.6 and 0.7 mg/l BA. However, these BA concentrations enhanced abnormalities like vitrification and necrosis. More than 50 % of the explants induced a mean number of 10 new shoots per explant at concentrations of 0.2, 0.3 and 0.9 mg/l BA. The presence of BA prevented the root formation.

9.11.3 *Artemisia annua*

A. annua is an important annual medicinal herb native to China. This plant contains several phytochemicals, essential oils and aromatic wreaths. An important antimalarial agent artemisinin has been detected and isolated from *A. annua* which is effective against *Plasmodium falciparum* (Bailey and Bailey 1976; Bennett et al. 1982;

McVaugh 1984; Elhaq et al. 1991; Klayman 1993; Teixeira da Silva 2003). The characteristic feature of *A. annua* is that all the plant parts have extreme bitterness (Tripathi et al. 2000, 2001; Ferreira and Janck 2009). This plant has been found effective in the treatment of skin diseases. It is also well known as a successful non-selective herbicide such as glyphosate (Duke et al. 1987; Paniego and Giulietti 1994).

Gopinath et al. (2014) utilized axillary buds to induce in vitro multiple shoots in *A. annua*. The axillary buds were cultured on MS medium supplemented with different concentrations of BA (0.5–2.5 mg/l) alone or in combination with NAA (0.1–0.5 mg/l). The most suitable plant growth regulator combination for shoot induction was 1.5 mg/l BA and 0.5 mg/l NAA. On this medium an average number of 27.5 shoots/explant was obtained. The multiplied shoots were transferred to MS medium amended with various concentrations of IBA for rooting. The best rooting (10.75 roots/shoot) was noticed on medium supplemented with 1.0 mg/l of IBA. The well-rooted plantlets were transferred to soil, acclimatized and established in natural conditions with 85 % survival rate.

9.11.4 *Artemisia vulgaris*

As an alternative to seed propagation, an efficient micropropagation system and subsequent rooting were developed for the medicinal plant *A. vulgaris* (Sujatha and Kumari 2008). From nodal segments a maximum of 32.8 shoots were produced on MS medium supplemented with 1.0 mg/l 2iP. The role of various types and concentrations of carbohydrates was investigated for multiple shoot induction and found that 3 % sucrose concentration resulted in optimum response. Healthy plantlets were transplanted to garden soil/farmyard soil/sand (2:1:1) mixture for acclimatization, and further maturity was achieved under field conditions. In vitro nodal segments were isolated from shoot cultures and encapsulated in high-density sodium alginate solidified by 50 mM $CaCl_2$. Sodium alginate at a concentration of 2.0 % produced the best quality beads. When encapsulated nodal segments kept at 5 °C did not germinate in light or dark conditions. All encapsulated nodal segments stored at 5 °C survived 20 weeks. In fact, 85 % of encapsulated nodal segments survived refrigerated storage for 60 weeks. According to authors this method is considered as a cost-effective cold storage protocol for alginate-encapsulated nodal explants for the germplasm preservation of *A. vulgaris*.

9.12 *Leontopodium nivale*

L. nivale is an endemic species in Italy due to indiscriminate harvest (Pace et al. 2009). It grows in rock crevices and on gravel slopes at altitudes between 2000 and 2800 m. *L. nivale* is seen in various locations in Italy and in the Balkan region (Conti et al. 1992). The extracts of this plant are utilized in European folk medicine especially in the treatment of abdominal aches, cancer, diarrhoea, angina, dysentery,

bronchitis and fever in humans as well as livestock (Kiene 1992; Knechtl 1992; Pickl-Herck 1995; Wieser 1995). Despite its high medicinal value, it also showed high anti-inflammatory activity mainly due to the presence of β-sitosterol (Hook 1994). In addition to this phytosterol, other phytochemicals like tannins, flavonoids and phenylpropanoids, which are of interest as constituents of both drugs and cosmetics, were also isolated from this plant (Dobner et al. 2003; Dweck 2004). An effective micropropagation protocol was developed for *L. nivale* (Pace et al. 2009). The cultured cotyledon explants induced callus on MS medium supplemented with 1.0 mg/l 2,4-D. The callus was multiplied and maintained on MS medium supplemented with NAA 0.1 mg/l and BA 0.4 mg/l. The callus organogenesis was achieved using BA at concentrations ranging from 0.5 to 2.0 mg/l. The regenerated shoots were multiplied, rooted and transplanted to soil successfully.

9.13 *Saussurea obvallata*

S. obvallata is a perennial herb commonly called 'Brahma Kamal', growing wild in the Indian Himalayan Region (IHR) at an elevation ranging from 3800 to 4800 m (Kirtikar and Basu 1984). This species comes under rare and endangered category (Samant et al. 1998). This plant is characterized by its beautiful flowers and is considered as one of the most exploited wild flowers of the region. The flowers are collected primarily due to its ethno-religious purposes as they are regarded as being sacred and are offered to the local deities of holy shrines. *S. obvallata* flower is the state flower of Uttaranchal, India (Saklani and Rao 2000). Further, this plant is used by local people in the preparation of several traditional medicines and treatment of intestinal ailments, coughs, bone ache and urinary track problems (Negi et al. 1999). The antiseptic and healing properties of this plant are well known and are used for healing cuts and bruises (Kirtikar and Basu 1984).

Joshi and Dhar (2003) developed a micropropagation protocol for *S. obvallata*. Multiple shoots were successfully induced from epicotyl explants on MS medium augmented with 0.2 mg/l Kn and 0.05 mg/l NAA. A mean number of 5.0 shoots were obtained within 75 days of culture. The effect of subsequent subcultures on shoot formation was also investigated in this study. On half-strength MS medium amended with 0.5 mg/l IBA, 100 % shoots produced roots. The plantlets were transplanted to ex vitro conditions and 66.7 % of the plants had been eventually established in the field.

9.14 *Senecio candicans*

S. candicans is a rare and endemic climbing shrub distributed in the Western Ghats, India. The people in various parts of Nilgiris use the leaf decoction of this plant for the treatment of gastric ulcer, and the gastroprotective properties of this plant have also been reported (Hariprasath et al. 2012).

Hariprasath et al. (2015) developed a protocol for in vitro micropropagation of *S. candicans* from leaf explants and compared the antioxidant activities of aqueous

extracts of in vitro calli and in vivo leaves. The leaf explants cultured on MS medium augmented with various concentrations (0.5–2.0 mg/l) of auxins and cytokinins under dark conditions produced callus. The highest callus induction (87 %) was obtained on MS medium supplemented with 2.0 mg/l BA and 2.0 mg/l IAA. The callus was subcultured on MS medium supplemented with different growth regulators (BA, Kn, IAA, NAA) after 28 days for shoot organogenesis. The highest per cent response (81 %) and shoot length (4.28 cm) were observed on MS medium supplemented with 2.0 mg/l BA and 1.0 mg/l IAA. The optimum root induction (90 %) from shoots was noticed on half-strength MS medium supplemented with 3.0 mg/l NAA. The antioxidant activity of in vivo leaf was significantly high compared to the in vitro callus in all three antioxidant assays. A linear correlation between antioxidant activity and the total phenolic content was observed.

9.14.1 *Spilanthes acmella*

S. acmella is commonly called as 'antitoothache plant', of the family Asteraceae. It holds an important place in Indian and global scenario owing to its medicinal properties. *S. acmella* is a perennial flowering herb and widely distributed in tropics and subtropics. The recent increase in interest in this plant is mainly owing to the amazing properties of ingredients found in the plant, which has been widely used to treat stammering, stomatitis, mouth ailments, toothache, flu, cough, rabies, tuberculosis and throat complaints (Akah and Ekekwe 1995; Singh 1995; Storey and Salem 1997; Ramsewak et al. 1999). The anti-inflammatory, antiseptic, analgesic, antioxidant, antibacterial, antifungal, antimalarial and cytotoxic properties of *S. acmella* had been reported (Burkill 1966; Oliver-Bever 1986; Di Stasi et al. 1994; Jondiko 1986; Saritha et al. 2002; Rai et al. 2004; Wu et al. 2008; Prachayasittikul et al. 2009). This plant is an essential ingredient in beauty care cosmetics such as muscle relaxant to accelerate repair of functional wrinkles (Belfer 2007). Furthermore, it is famous for its larvicidal and insecticidal properties (Ramsewak et al. 1999; Saraf and Dixit 2002; Pandey et al. 2007).

Prasad and Seenayya (2000) reported the excellent antimicrobial properties of *S. acmella* against red halophilic cocci from salt-cured fish. The alkaloids present in this plant have the potential to act as an insecticide (Krishnaswamy et al. 1975; Castillo et al. 1984) and were found to be effective against *Aedes aegypti* (Jondiko 1986). The juice of *S. acmella* is used to grind medicinal pills and to prevent bleeding from the mouth and nose of patients bitten by viper (Ramsewak et al. 1999). The fresh leaves are used as vegetables and as an additive to salads. The leaves are combined with chillies to offset the burn (Pandey et al. 2007).

An effective micropropagation method for *S. acmella* has been developed by Kurian and Thomas (2015) via repeated subculture of nodal segments. MS medium augmented with various concentrations of BA (0.5–4.0 mg/l) or Kn (0.5–4.0 mg/l) has been employed for nodal segment culture. High multiplication rate was achieved by repeated subculture on MS medium supplemented with 2.0 mg/l BA. The optimum multiplication rate (100 % response with a mean number of 11.6 shoots/

explant) was achieved during the second subculture cycle after 40 days. The best medium for rooting was MS medium fortified with 2.0 mg/l IBA. Here, 95 % cultures responded with a mean number of 3.2 roots per shoot. The plantlets were transplanted to soil, hardened with 91 % success. No morphological or phenotypic variations were noticed among the transplanted plants (Fig. 9.2).

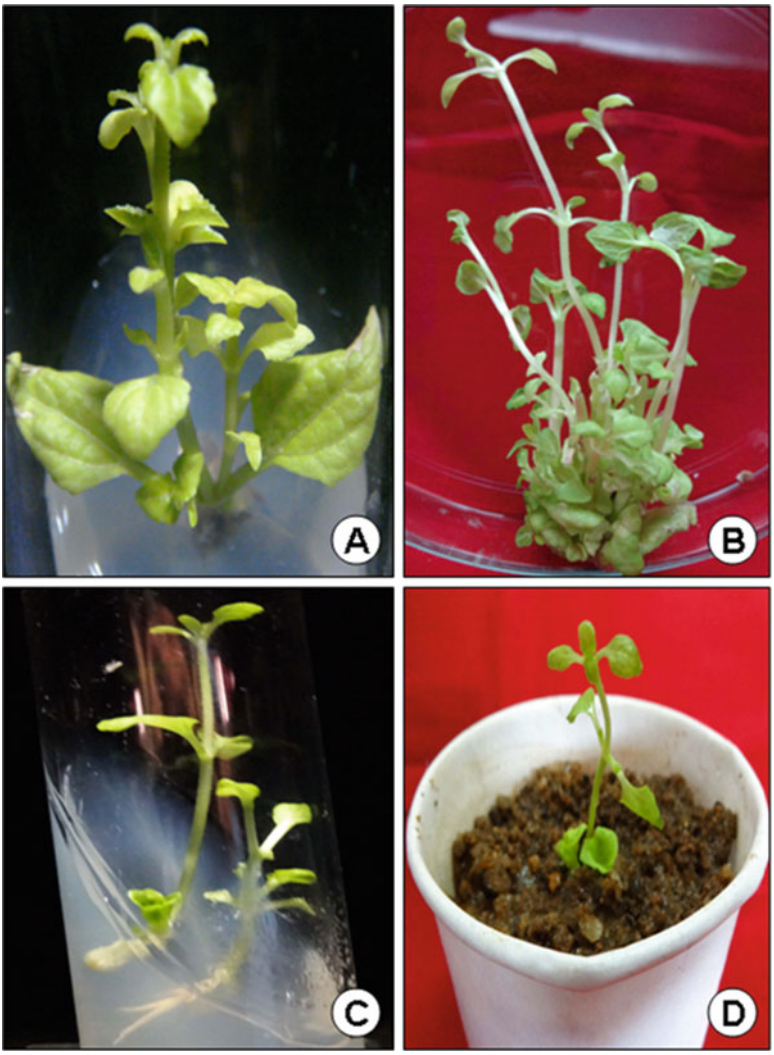

Fig. 9.2 Micropropagation of *Spilanthes acmella* (After Kurian and Thomas 2015). (**a**) Nodal segment culture on MS medium supplemented with 2.0 mg/l BA 20 days after culture. (**b**) Induction of multiple shoots on MS medium supplemented with 2.0 mg/l BA from nodal segments during second serial subculture after 40 days of culture. (**c**) Root induction from shoots on half-strength MS medium amended with 2 mg/l IBA 40 days after culture. (**d**) A 6-week-old acclimatized in vitro plant after transfer to soil

Singh and Chaturvedi (2010) standardized a micropropagation protocol and estimated the scopoletin, a phytoalexin in the leaves of *S. acmella*. The cultures initiated during the spring (January–April) season produced the highest shoot proliferation. MS medium amended with 1.15 mg/l BA resulted in 100 % bud break with maximum shoot proliferation. The in vitro developed single nodes were cultured on MS medium supplemented with 1.15 mg/l BA produced a 20.3-fold shoot multiplication rate. Half-strength MS medium promoted 100 % rooting of shoots. Further, the estimation of scopoletin in in vitro leaves (0.10 µg/g dry weight) by HPLC method is comparable to that of field-grown plants.

A method for the mass propagation of three species of *Spilanthes*, i.e. *S. acmella* L. var. *oleraceae*, *S. calva* and *S. paniculata*, for conservation purpose has been developed by Pandey et al. (2014) using seedling leaf explants. The leaf explants were cultured on MS medium fortified with a variety of plant growth regulators such as BA, Kn, 2iP, 2,4-D, NAA and IBA in various concentrations either alone or in combination. The best medium for shoot induction was MS supplemented with 1.15 mg/l BA for *S. calva*, with a mean number of 4.17 shoots per explant, whereas the optimum response for *S. acmella* var. *oleraceae* and *S. paniculata* was observed on MS medium supplemented with 0.2 mg/l NAA and 1.15 mg/l BA differentiating an average of 4.46 and 6.50 shoots per explant, respectively. The shoots were rooted on half-strength MS medium augmented with NAA or IBA. This method could be utilized for the ex situ conservation of these three *Spilanthes* species.

An effective micropropagation method for *S. calva* was developed via nodal segment culture (Razaq et al. 2013). *S. calva* is an important source of spilanthol, an antimalarial larvicidal compound. The nodal segments were cultured on MS medium amended with different cytokinins (BA, 2iP and Kn) for inducing multiple shoots. MS medium supplemented with 2.5 mg/l BA found optimum for shoot multiplication. On this medium, 91.6 % cultures responded with a mean number of 7.12 shoots per explant after 6 weeks. The maximum rooting of shoots was observed on MS medium augmented with 0.02 mg/l IBA. The in vitro micropropagated plants were acclimatized and transplanted to field, where 95 % plants survived. RAPD analysis confirmed the true-to-type nature of in vitro derived plants to field-grown mother plants.

9.14.2 *Spilanthes mauritiana*

S. mauritiana is an endangered East African medicinal herb (Watt and Brayer-Brandwijk 1962). It is used against stomach ache and diarrhoea (Kokwaro 1976). The local people in Cameroon employ this plant as a remedy for snakebite and in articular rheumatism (Dalziel 1937). It is used as a remedy for kidney stones and kidney infections in India (Dragendorff 1898).

A method for micropropagation of *S. mauritiana* using juvenile plant was standardized (Bais et al. 2002). The presence of 0.25 mg/ BA and 0.1 mg/l NAA in the medium yielded the highest shoot induction with minimum callusing. The optimum rooting was observed on MS medium amended with 0.2 mg/l IAA. The rooted shoots were transplanted to soil and acclimatized and kept in the greenhouse.

9.15 *Brachylaena huillensis*

Silver oak (*B. huillensis*) is a resourceful timber tree belonging to the family Asteraceae (Chonge 2002; WCMC 2008). This tree is native to Central, East and Southern Africa. Due to the high demand for its wood and associated products, this tree has been overexploited. The population of this plant is very low and is a threatened tree species (Ruffo and Malcondo 1990; IUCN 2008). The species of this tree is used for timber and carving artefacts (Mbuya et al. 1994), flooring blocks, sleepers, charcoal, essential oil, furniture and turnery (Mbuya et al. 1994; Cunningham 1998; Bryce and Chihongo 1999). Since the wood is highly durable, it is utilized as building poles, fence posts and transmission poles. It is also used as an ornamental plant. *B. huillensis* is also used for medicinal purposes for schistosomiasis and leaves are used for diabetes (Cunningham 1998).

Conventionally, *B. huillensis* propagates only through seeds and poor seed germination rate and viability are the limiting factors of conventional propagation. Developing an efficient micropropagation protocol is a viable option for large-scale propagation of this plant. Ndakidemi et al. (2014) investigated the role of antioxidant ascorbic acid in reducing lethal browning caused by the exudation of oxidized phenols during in vitro nodal segment culture of *B. huillensis*. Different concentrations of ascorbic acid (0, 50, 100, 150, 200 and 250 mg/l) were supplied along with basal woody plant medium (WPM; Lloyd and Mc Cown 1980) containing BA. They noted a reducing trend of browning with an increase in concentration of ascorbic acid. The optimum control was achieved by adding 200–250 mg/l of ascorbic acid in the WPM, augmented with BA.

9.16 *Rhaponticoides mykalea*

R. mykalea is an endemic plant of Turkey. This species has already been on the verge of extinction, and it is necessary to develop certain conservation measures including in vitro conservation to save this plant from extinction. Micropropagation via direct shoot induction from various explants and determination of secondary metabolites were studied in *R. mykalea* (Hayta et al. 2015). Seeds were germinated after damaging the seed coat, and embryos were excised and cultured on WPM, to achieve a maximum germination (40 %). The epicotyl and cotyledonary petiole were excised from germinating embryos and cultured on WPM containing 0.5 mg/l BA. The addition of 1.0 mg/l IBA along with 0.5 mg/l BA significantly improved the direct shoot induction, resulting in a mean number of 5.6 shoots per explant. A combination of 4.0 mg/l IBA with 990 μM putrescine produced the highest root induction from shoots (35.6 %). Plantlets with well-developed roots were transplanted to soil and acclimatized in a plant growth chamber. Acclimatized plants exhibited 100 % survival rate. The content of secondary metabolites in three tissue culture regenerated lines were also analysed by HPLC method. The analysis indicated that chlorogenic acid, quercetin and scutellarin are the chief secondary metabolites present in *R. mykalea*.

9.17 *Farfugium japonicum*

F. japonicum is native to streams and seashores of Japan. It grows to about 60 cm tall spreading by rhizomes. It is characterized by its yellow flowers borne in loose clusters in autumn and winter. The leaves of this plant are used as an antidote and are taken internally to treat fish poisoning and externally to treat lacquer poisoning (Duke and Ayensu 1984).

A method for direct or indirect somatic embryogenesis was developed for *F. japonicum* (Lee 2006). For somatic embryo induction, the leaf and petiole segment explants were cultured on MS or N_6 media (Chu et al. 1975) supplemented with 2,4-D alone or in combination with auxins and cytokinins. Yellowish embryogenic callus was induced from petiole explants within 7 weeks on MS medium augmented with 2,4-D. Comparatively, petiole explants produced better callusing than leaf explants. On MS medium supplemented with 2,4-D (0.4–22.5 mg/l), the frequency of embryogenic callus and number of somatic embryos per explant were significantly improved compared to other plant growth regulator combinations. However, on N_6 medium supplemented with 1.0 mg/l NAA and 2.0 mg/l Kn, direct induction of somatic embryos was obtained from petiole explants. Secondary somatic embryos were induced abundantly on the base of the primary somatic embryos when petiole segments with embryos were transferred to MS medium supplemented with 0.2 mg/l NAA and 0.45 mg/l Kn. The somatic embryos were converted into plantlets on MS basal medium. The transplantation success of the regenerated plants was 97 % after 1 month of transfer and was morphologically similar to the parental plant.

9.18 *Bellis perennis*

B. perennis (common daisy) is a medicinal plant utilized in the treatment of wounds, inflammation, common cold and rheumatism in traditional system of medicine (Hansel et al. 1992). An effective and quick regeneration system was standardized for *B. perennis* via indirect organogenesis (Karakas and Turker 2013). Leaf, pedicel and root explants from field-grown plants and leaf, petiole and root explants obtained from in vitro grown plantlets were used for in vitro regeneration experiments. The explants were cultured on MS medium supplemented with various plant growth regulator combinations. The field-grown plants responded better than in vitro grown plantlets. The optimum shoot induction was observed with pedicel explants on medium containing 0.5 mg/l TDZ and 0.5 mg/l IAA. The regenerated shoots were rooted on MS medium fortified with IAA (1.0 mg/l). Rooted plantlets were transferred to vermiculite for acclimatization. After 2 weeks, the plants were transferred to small plastic pots containing soil, and after 3 months the flowering of the in vitro derived plants was observed.

9.19 *Vernonia herbacea*

V. herbacea is a plant native to Brazil that gathers about 80 % of inulin-type fructans in the underground reserve organs, the rhizophores. It is employed in the study of fructan metabolism in relation to the effects of abiotic stresses such as low temperature (Asega et al. 2011), drought (Garcia et al. 2011), high CO_2 atmosphere concentration (Oliveira et al. 2010) and low nutrient availability (Cuzzuol et al. 2005). Trevisan et al. (2014) established an in vitro propagation protocol for *V. herbacea* using seeds and leaf disc explants. The stem nodes excised from 6-month-old germinating seeds were isolated and cultured on MS basal medium for shoot induction and rhizophore formation. Estimation of fructan content was estimated in leaves, stems, roots and rhizophores from in vitro and greenhouse plants. Fructan contents of aerial organs and roots from in vitro plants were higher, compared with greenhouse plants. The leaf disc cultured on MS medium augmented with IBA (0.05, 0.1 mg/l) produced roots. The leaf disc induced friable callus on MS medium fortified with IBA (0.5 mg/l), whereas BA (1.0 mg/l) induced compact callus. There was no shoot induction.

9.20 *Achillea occulta*

A. occulta is a threatened mountain species in Greece which has no other close relatives and its habitats are threatened by wild fires and overgrazing (Constantinidis and Kalpoutzakis 2009). A micropropagation protocol using shoot tip explants was standardized for *A. occulta* by Grigoriadou et al. (2011). Shoot tips from wild plants were used for initiating cultures on MS medium supplemented with 1.0 mg/l BA and 0.1 mg/l IBA. A combination of BA with NAA or IBA was also studied. The maximum response (3.5 microshoots/explant with 0.93 cm shoot height) was observed on MS medium supplemented with 1.1 mg/l BA and 0.5 mg/l IBA. The rooting of shoots was difficult and the optimum rooting was obtained on MS medium amended with 4.0 mg/l IBA. On this medium 12.5 % rooting percentage was achieved. The rooted young plantlets were transplanted to soil after acclimatization and produced flowers and seeds in the first year.

9.21 *Pentanema indicum*

P. indicum is an erect herb with hard woody roots and variable leaves. It is a female antifertility drug employed by the tribal people in Bihar state of India. This annual plant is distributed all over India, ascending up to an altitude of 1800 m. Several phytochemicals were isolated from this plant including sesquiterpene–vicolides, monoterpenediol–vicodiol and thymol esters (Vasanth et al. 1990; Mossa et al. 1997), cis–cis germacranolide (Sawaiker et al. 1998) and 4,5,6-trihydroxy-4-7-dimethoxy flavone (Krishnaveni et al. 1997). The phytochemical vicolide D isolated from this plant showed abortifacient and antifertility activities (Alam et al. 1989).

Antiviral activity of hexane soluble fraction of *P. indicum* against Ranikhet virus had been reported (Chowdhury et al. 1990).

Micropropagation and in vitro flowering of *P. indicum* had been studied by Sivanesan and Jeong (2007). For callus induction from leaf and stem explants, MS medium augmented with 2.0 mg/l BA and 1.0 mg/l IBA was optimum. The highest shoot organogenesis (19.0 shoots per explant) was observed on MS medium supplemented with 4.0 mg/l BA and 1.0 mg/l IAA after 5 weeks. When shoot tip and nodal explants were cultured on MS medium amended with BA and IAA, direct shoot induction was noticed. Shoot multiplication rate was enhanced by adding adenine sulphate (1.0 mg/l) to the regeneration medium. The highest rooting of shoots was obtained on MS medium fortified with 2.0 mg/l IBA. About 90 % of in vitro derived plants flowered on MS medium supplemented with 2.0 mg/l IBA. The plants derived from various explants were acclimatized and transplanted to soil with 96 % success.

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Part II

Tree Biotechnology

Plant Tissue Culture Approach for Cloning and Conservation of Some Important RET Medicinal Plants

10

A.K. Sharma, M. Sharma, M. Jain, K. Arora, S.K. Rai, and D.K. Purshottam

Abstract

In vitro protocol for rapid micropropagation/cloning in case of four medicinally important rare, endangered and threatened (RET) plants of Indo-Gangetic Plain, namely, *Clerodendrum serratum*, *Uraria picta*, *Operculina petaloidea* and *Embelia tsjeriam-cottam*, employing nodal stem segments of field-grown plant, was developed for the purpose of ex situ conservation. In all the four plants, sustained proliferation of shoots as well as 100 % induction of rooting in isolated shoots could be maintained even in long-term culture, and complete plantlets were produced, which grew luxuriantly and came to flowering under field conditions. The genetic fidelity of plants raised through tissue culture was assured by random amplified polymorphic DNA (RAPD) analysis in case of *C. serratum*, *U. picta* and *E. tsjeriam-cottam*. In *U. picta*, quantitative estimation of two isoflavonones, isolated from the roots of mother plant and from the tissue culture-raised plants, revealed no significant difference in their concentrations which further strengthened true-to-type nature of in vitro cloned plants. Another process was developed for rapid micropropagation of a medicinally important wild, endemic, primitive and endangered monoembryonic *Citrus* species, viz. *C. indica*, growing in thin population in Garo Hills in Northeastern Himalayan region of the country. Such in vitro developed processes may be utilized for the supply of enough raw materials to various pharmaceutical companies and for providing large number of cloned plants to re-establish them in their natural habitats for in situ conservation and sustainable utilization. In case of *Azadirachta indica*, a

A.K. Sharma (✉) • M. Sharma • M. Jain • S.K. Rai • D.K. Purshottam
Tissue Culture Laboratory, CSIR-National Botanical Research Institute, Lucknow 226001, UP, India
e-mail: ashok_nbri@hotmail.com

K. Arora
Botany Department, National P.G. College, 2, Rana Pratap Marg, Lucknow 226001, India

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medicinally important tree, a process for in vitro clonal multiplication of a 40-year-old mature tree through nodal stem segments was standardized. Further multiplication rate was augmented by inducing differentiation of multiple shoots from leaflet segments excised from in vitro raised proliferating shoots. In order to preserve the germplasm of highly heterozygous elite neem trees, an innovative method of long-term regenerative excised root culture was developed. The genetic fidelity of field-grown plants raised through nodal stem segments of a 40-year-old mature tree and through root segments, taken from long-term excised root cultures, was ascertained by RAPD markers as well as through chemical analysis in respect of azadirachtin content.

10.1 Introduction

The science of plant tissue culture means aseptic cultivation of plant protoplasts, cells, tissues, organs or complete plantlets in vitro in certain culture media and their incubation under controlled physical conditions, like light, temperature and humidity. To be precise, in plant tissue culture, one studies in vitro morphogenesis to understand the process of chemical regulation of growth and differentiation – both morphological and biochemical – with the aim of unravelling the underlying causal processes and mechanisms, the least understood aspect of development. In fact, morphogenesis is the science that treats the cause and origin of forms, i.e. inception and development of morphological characters, both normal and abnormal during growth and differentiation in the process of development of an individual. As such, for understanding the discipline of morphogenesis, knowledge of such other branches of science as morphology, anatomy, embryology, cytology, genetics, pathology, physiology, biochemistry, molecular biology, etc. is required.

The classic demonstration of hormonal regulation of organogenesis in tobacco pith tissue cultures by Skoog and Miller (1957) and demonstration of totipotency of carrot phloem cells by Steward et al. (1958) formed the basis of morphogenesis in tissue culture, which has actually given birth to the most useful and applied aspect of tissue culture, i.e. micropropagation. The first successful application of micropropagation was demonstrated by Morel (1964) in an important orchid, *Cymbidium*, using meristem culture. However, during the course of most of the further studies on multiplication of plants through tissue culture, the very basis of micropropagation, i.e. morphogenesis, has often been ignored, with the results that many a times are either not reproducible at all or to the same extent as they have been reported earlier. On the other hand, if the principles of morphogenesis are sufficiently taken into account in regeneration studies, the results are invaluable, which may lead to the development of methods of germplasm preservation resulting into establishment of 'tissue or gene banks'.

At CSIR-NBRI, plant tissue culture research was initiated on lower plants during the 1960s, which later on shifted to economically important higher plants belonging to the categories of ornamentals, medicinal plants and fruit and forest trees, mainly

towards developing regeneration and multiplication protocols for rapid production of cloned and clean stocks of intractable-to-multiply elite plants; creation of somaclonal variants; conservation of phytodiversity, including establishment of 'germplasm repositories' or 'gene banks'; and production of high-value active principles in case of some medicinal and herbal plants (Chaturvedi 1979; Chaturvedi and Sharma 2004).

This article highlights the main findings of tissue culture work accomplished mainly on four medicinally important RET plants of Indo-Gangetic Plain, namely, *Clerodendrum serratum* (Linn.) Moon (barangi; an endangered medicinal shrub), *Uraria picta* (Jacq.) DC (dabra; a rare medicinal herb), *Embelia tsjeriam-cottam* (baibidang; a vulnerable straggling shrub) and *Operculina petaloidea* (Choisy) Oost. (vidhara; a rare perennial climber), a wild endemic and endangered species of *Citrus*, viz. *C. indica* and a medicinal tree, *Azadirachta indica* A. Juss. (neem), primarily to develop in vitro protocols for cloning and germplasm preservation.

10.2 Materials and Methods

10.2.1 Collection of Plant Material

Twigs from fresh flush of growing shoots of RET plants, namely, *C. serratum* (Linn.) Moon, *U. picta* (Jacq.) DC, *E. tsjeriam-cottam* (Roem. & Schult.) DC and *O. petaloidea* (Choisy) Oost., were collected from plants growing in the herbal garden of Deendayal Research Institute, Chitrakoot, Madhya Pradesh. Fruits and twigs of *C. indica* Tanaka were collected from Garo Hills in Northeastern Himalaya, where as in case of *A. indica* A. Juss., twigs from fresh growing shoots were collected from a mature (40-year-old) elite tree growing in Lucknow.

10.2.2 Surface Sterilization of Explants

Aseptic cultures of aforesaid plants were raised using shoot tips/nodal stem segments having axillary buds and seeds (in *C. indica* only) as explants. Initially, explants were thoroughly washed for 30 min under running tap water through bacteria-free filter (zero-B), then treated for 10 min with 5 % (v/v) labolene solution (GlaxoSmithKline Pharmaceuticals Ltd., Mumbai, India) and rinsed with single distilled water. In case of neem, washed explants were pretreated for 2 h at 4 °C with sterilized chilled antioxidant solution, comprising four antioxidants, namely, polyvinylpyrrolidone (PVP), citric acid, ascorbic acid and glutathione reduced. Mercuric chloride (0.1 % $HgCl_2$) solution was used for surface sterilization of the pretreated explants for 10/15 min. Prior to surface sterilization, a quick dip of explants in ethanol (95 %, v/v) was given which is essential for wetting the explants. Finally, traces of disinfectant from surface-sterilized explants were removed by washing them four to five times with sterilized single distilled water. Explants were kept in sterilized antioxidant solution till inoculation for checking browning in case of neem only.

10.2.3 Proliferation of Shoots

Shoots regenerated from different explants were cultured in derived respective agarified nutrient medium containing growth hormones in particular concentrations/combinations for their proliferation.

10.2.4 Root Induction in Isolated Shoots

Shoots of about 3–4 cm in size were excised from the cultures of proliferating shoots of all the aforesaid plants and were cultured in respective rooting medium supplemented with standardized concentrations and/or combinations of auxins/phenolic acid.

10.2.5 Plantlet Production and Their Transplantation in Field

The in vitro raised plantlets were transplanted to pots containing a potting mix [mixture of soil and manure] after being acclimatized in an inorganic salt solution comprising half-strength Knop's solution (Knop 1865) fortified with trace elements and Na-Fe-EDTA solution of Murashige and Skoog's (1962) medium (MS medium) for about 30 days. The potted plants were grown in glasshouse for about 4–6 months, and finally the hardened in vitro raised plants were transferred to field in the proper season for better transplantation success.

10.2.6 Establishment of Excised Root Cultures

For establishing excised root cultures, roots measuring about 3 cm in length, excised from in vitro grown plantlets, were used as explants and cultured in White (1943) liquid medium in dark.

10.2.7 Regenerant Differentiation from Root Segments Taken from Excised Root Cultures

Initially, for inducing regenerant differentiation in root segments, taken from excised root cultures, standardized nutrient medium supplemented with cytokinin(s) was employed. After regenerant differentiation, shoots were cut and subcultured in the proliferation medium.

10.2.8 Significance and Importance of R&D

The in vitro processes developed for rapid multiplication of cloned plants of valuable RET medicinal plants, namely, *C. serratum*, *U. picta*, *O. petaloidea*, *E. tsjeriam-cottam* and *C. indica*, even in long-term culture, are efficient, simple and

reproducible. Further, such developed processes can be exploited for raising enough raw material for various pharmaceutical companies and also for providing large number of cloned plants of these plant species for the re-establishment in their natural habitats for in situ conservation and sustainable utilization.

Process developed for in vitro cloning of a 40-year-old mature neem tree has a great practical significance, as it may be utilized for rapid production of a large number of cloned plants of selected elite individuals taking into account the constraints in its conventional methods of propagation.

Cultures of proliferating shoots of the above-mentioned four RET plants of Indo-Gangetic Plain, *C. indica* and *A. indica*, kept regenerative in their respective balanced nutrient medium, in long-term culture, under normal culture room conditions maintaining genetic stability and maximum structural organization due to minimum stress in culture, which provides a moderately good system for germplasm preservation.

The innovative method of germplasm preservation of *A. indica* through regenerative excised root cultures is of immense importance, as it may lead to development of 'in vitro gene banks'/'germplasm repositories' of even highly heterozygous tree.

10.3 Results and Discussion

10.3.1 RET Plants of Indo-Gangetic Plain

10.3.1.1 *Clerodendrum serratum* (Linn.) Moon (An Endangered Shrub)

Overexploitation and unsustainable extraction of wild plants of *C. serratum* for medicine and trade coupled with low seed viability are the major causes for the constant decrease in its natural population in India. The leaf and root of this plant have great medicinal value. Root bark contains mainly sapogenins (Rangaswami and Sarangan 1969) and has been traditionally used in Ayurveda and Siddha systems of medicine for treatment of chronic bronchial asthma and other respiratory diseases, different types of fevers and skin infections. Apart from roots, leaves also contain important flavonoids and phenolic acids (Rastogi 1999). It is one of the ingredients of the ayurvedic drug, 'Kasadamana', an effective expectorant and antitussive remedy (CSIR 2001). Therefore, development of an in vitro protocol for rapid clonal production of *C. serratum* was warranted for providing consistent and uniform raw material for medicinal purposes as well as for its conservation.

For the first time, an in vitro process for rapid clonal propagation of *C. serratum* using nodal stem segments has been developed by Sharma et al. (2009). Nodal stem segments having axillary bud, taken from field-grown plants, showed bud-break within 15 days of culture on modified MS medium supplemented with 0.25 mg l^{-1} each of 6-benzylaminopurine (BAP) and indole-3-acetic acid (IAA) along with 15 mg l^{-1} adenine sulphate (AdS). Regenerated shoots could be multiplied further on the same agarified morphogenetic medium in the presence of 0.5 mg l^{-1} 2-chloroethyltrimethyl ammonium chloride (CCC) but supplemented with increased

concentration of AdS, i.e. 30 mg l^{-1}. The inoculum used for subculture was a group of five shoots, which produced on an average 4.98 new shoots per original shoot after 4 weeks of subculture. The shoots of about 3 cm were isolated from cultures of proliferating shoots and induced to root 100% by culturing them in half-strength MS medium supplemented with 1 mg l^{-1} indole-3-propionic acid (IPA). The in vitro rooted shoots – plantlets – grew luxuriantly in field after transplantation and came to flowering after about 10 months of transplantation. The genetic fidelity of these transplanted field-grown plants and their mother plant was assured by random amplified polymorphic DNA (RAPD) analysis (Fig. 10.1a–e). The protocol standardized holds good for in vitro cloning of *C. serratum*.

10.3.1.2 *Uraria picta* (Jacq.) DC (A Rare Medicinal Herb)

In India, *Uraria picta* is well known for its medicinal properties. The whole plant is medicinally important and is utilized by certain Adivasi and native tribes (Jain and Defilipps 1991). The fruits are effectively used against oral sores in children, and the roots are used against cough, chills and fever (Yusuf et al. 1994). Two isoflavonones isolated from *U. picta* have antimicrobial activity (Rahman et al. 2007). The species is rapidly attaining a status of rare due to ruthless extraction coupled with overexploitation by various pharmaceutical companies as well as local tribes for medicine and trade and also because of poor seed viability (Anand et al.1998). In the present scenario, there was an immediate need to develop in vitro methods for rapid clanal production of *U. picta* which may provide uniform raw material for medicinal purposes as well as for its re-establishment in natural habitat for in situ conservation and sustainable utilization.

For the first time, an efficient in vitro process for rapid propagation of cloned plants of *U. picta* using nodal stem segments was developed (Rai et al. 2010). Explants of nodal stem segments, taken from field-grown plants, showed bud-break followed by regeneration of shoots with restricted growth within 12 days on modified MS medium supplemented with 0.25 mg l^{-1} each of BAP and IAA and 25 mg l^{-1} AdS. Normal growth of shoots with good proliferation rate was achieved by reducing the concentrations of BAP and IAA to 0.1 mg l^{-1} each and incorporating 0.5 mg l^{-1} gibberellic acid (GA) in the medium, in which, on an average, 19.6 shoots per explant were produced. Further, during successive subcultures, increased concentrations of AdS (50 mg l^{-1}) and GA (2 mg l^{-1}) along with addition of 20 mg l^{-1} DL-tryptophan were found conducive to control the problem of necrosis of shoots. In this treatment, several 'crops' of shoots were obtained from a single culture by repeated subculturing of basal portion of stalk even in the long term. Hundred percent rooting was induced in the isolated shoots with the addition of 0.25 mg l^{-1} indole-3-butyric acid (IBA) in the culture medium. The in vitro raised plants after hardening in inorganic salt solution grew normally in soil and came to flowering. Genetic fidelity of the in vitro raised plants was assured by RAPD markers (Fig. 10.2a–e). Quantitative estimation of two isoflavonones in the root extracts of in vitro cloned plants and that of mother plant further confirmed true-to-type nature of plantlets having comparable concentrations.

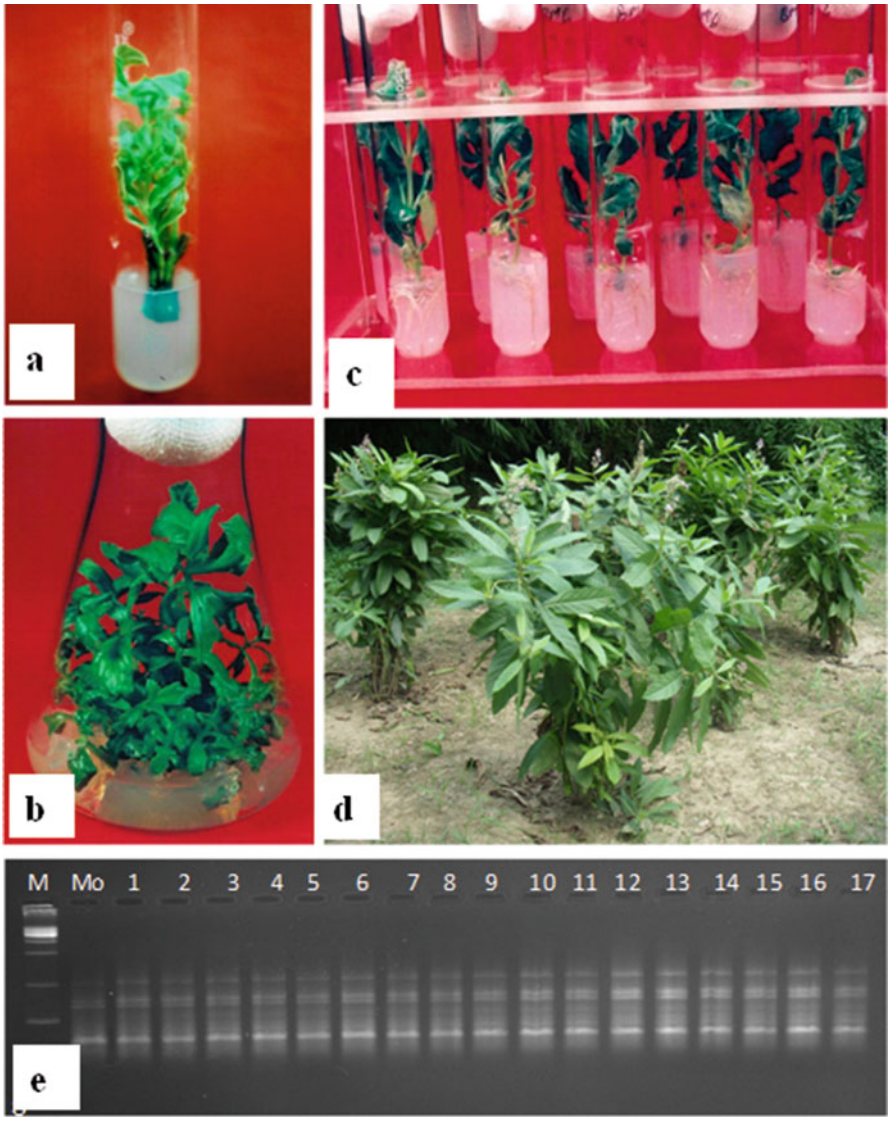

Fig. 10.1 (**a**–**e**) In vitro cloning of *Clerodendrum serratum*. (**a**) Bud-break resulting into shoot formation in nodal stem segment, taken from field-grown plant. (**b**) Proliferation of shoots. (**c**) Rooting of isolated shoots. (**d**) Transplantation of in vitro raised plants in field. (**e**) RAPD analysis of in vitro raised field-grown plants and mother plant; lane *M* 500-bp DNA size markers, lane *Mo* DNA from mother plant, lanes 1–17 DNA from randomly selected regenerated plantlets

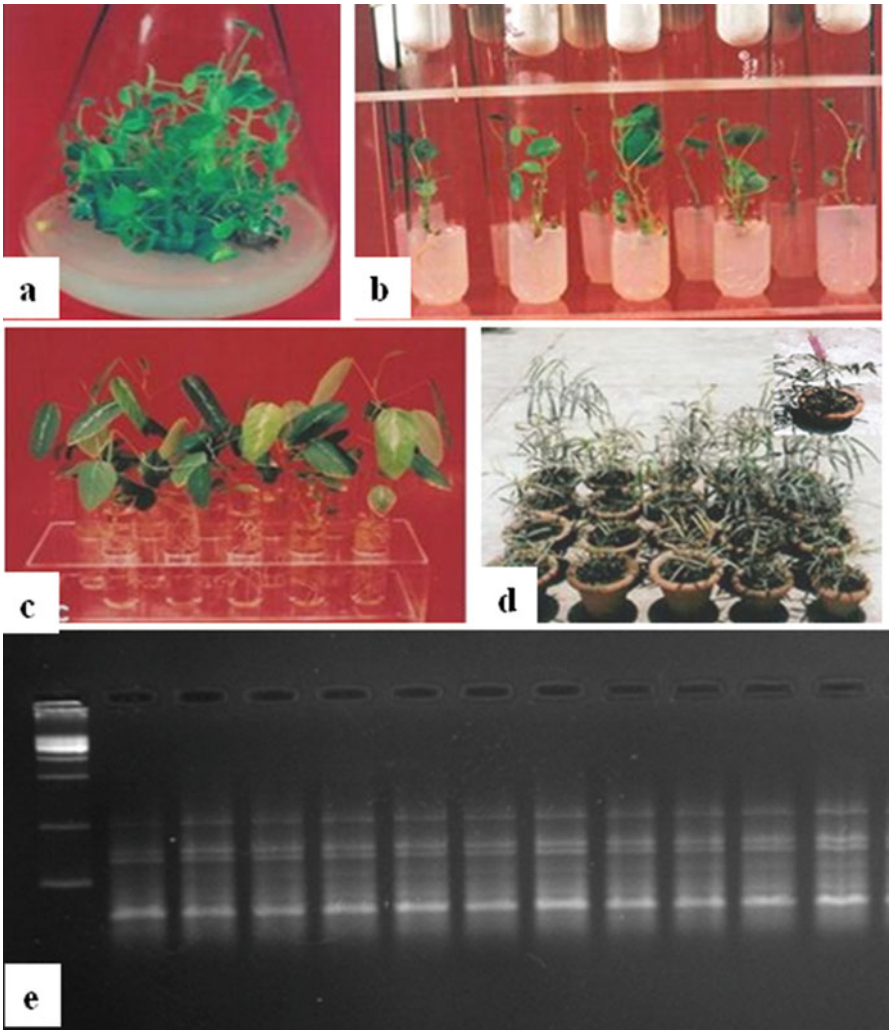

Fig. 10.2 (**a**–**e**) Cultures of *Uraria picta*. (**a**) Proliferating shoots as seen after 5 years of culture. (**b**) Rooting of isolated shoots. (**c**) Acclimatization of in vitro raised plantlets in liquid culture. (**d**) In vitro raised potted plants. (**e**) RAPD analysis of in vitro raised field-grown plants and mother plant; lane *M*, 500 bp DNA size markers, *Mo*, DNA from mother plant; lanes 1–8, DNA from randomly selected regenerated plantlets

10.3.1.3 *Operculina petaloidea* (Choisy) Oost, (A Rare Perennial Climber)

Operculina petaloidea is a medicinally important rare perennial climber, found in Madhya Pradesh. Its roots are used in the treatment of stomach disorders, gout and rheumatism. The ruthless extraction of plants by pharmaceutical companies and destruction of natural forests are the main reasons for its declining status, which

warrants development of in vitro strategies for rapid production of cloned plants of this species for re-establishment in its natural habitat for in situ conservation and sustainable utilization.

The aseptic cultures of *O. petaloidea* were established using shoot tips and nodal stem segments taken from field-grown plants. Mostly tender explants, particularly shoot tips, turned brown within a week, and only slightly thicker nodal explants showed bud-break after 15–20 days' incubation in the morphogenetic medium. For augmenting the rate of shoot proliferation, a modification of Schenk and Heldebrandt (1972) medium supplemented with BAP, IAA and AdS was found conducive, in which sustained proliferation of shoots with good branching and foliage development was achieved even in long-term culture. The isolated shoots could be induced to root 100 % in a low salt concentration medium fortified with an auxin (IBA). After proper acclimatization in an inorganic salt solution for 30 days, the plantlets raised in vitro were transferred to potted soil and subsequently to field, where they exhibited uniform luxuriant growth and came to flowering after about 6 months of transplantation (Fig. 10.3a–d).

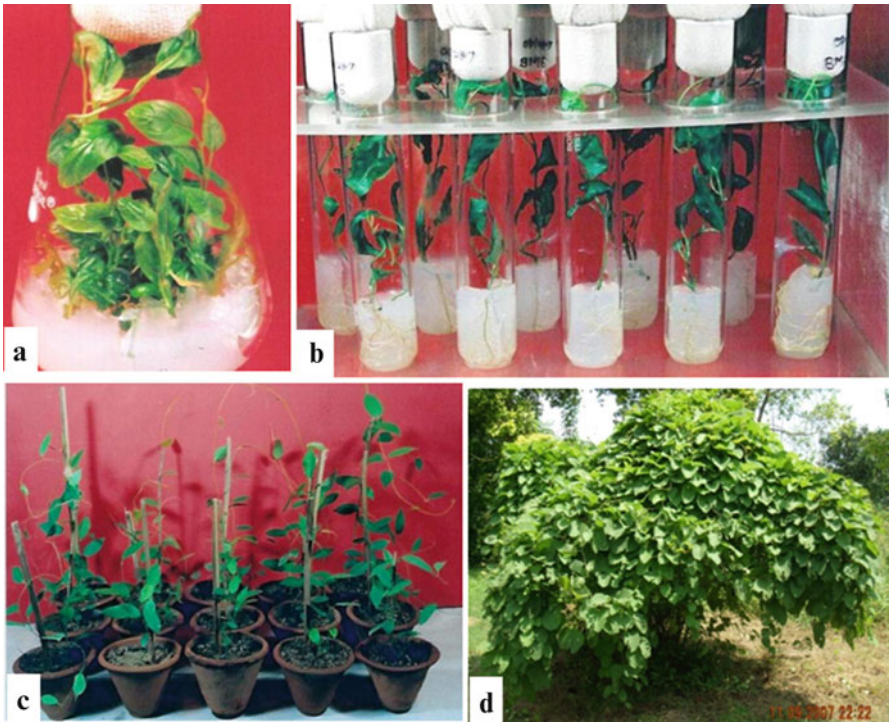

Fig. 10.3 (**a**–**d**) In vitro cloning of *Operculina petaloidea*. (**a**) Proliferation of shoots. (**b**) Rooting of isolated shoots. (**c**) Hardening of in vitro raised plants in liquid culture. (**d**) In vitro raised plants in field

10.3.1.4 *Embelia tsjeriam-cottam* (Roem. & Schult.) DC (A Vulnerable Straggling Shrub)

Embelia tsjeriam-cottam is popularly known for its medicinal properties as its seeds, root and bark have great medicinal value. Though this species was widely distributed, it is becoming critically endangered due to poor viability of seeds and lack of conventional methods of vegetative propagation. Hence, there was a great need to develop an efficient in vitro process for rapid micropropagation of *E. tsjeriam-cottam* to facilitate conservation and re-establishment in its natural habitat for sustained utilization.

The aseptic cultures of *E. tsjeriam-cottam* were raised through nodal stem segments of field-grown plants. However, the initial establishment of this plant was found to be a difficult proposition. For induction of growth in shoots regenerated from the nodal explants, a pulse treatment of GA for 20 days was found essential, and to induce multiplication in growing shoot, a different morphogenetic medium was derived, in which proliferation of shoots could be achieved. On an average, four to seven shoots were formed per explant of single node stem segment during initial two to three subcultures, but the number of shoots in subsequent subcultures increased up to 14. The isolated shoots of about 4 cm were excised from cultures of proliferating shoots and after root induction in the presence of 0.25 mg l^{-1} IBA developed into plantlets with good growth and leaf development. The in vitro raised plants were hardened directly in potted soil without passing through a phase of acclimatization in an inorganic salt solution and grew luxuriantly on transplantation to field. The genetic fidelity of in vitro raised field-grown plants was assured by RAPD analysis (Fig. 10.4a–f).

10.3.1.5 *Citrus indica* Tanaka

Citrus indica is a medicinally important endemic, primitive, endangered and monoembryonic wild *Citrus* species found in thin population size at Garo Hills in Northeastern Himalayan region of the country. Narrow distributional range, high habitat specificity, jhoom cultivation, deforestation, low natural regeneration and extraction of fruits from wild for local use are the main constraints associated with its in situ germplasm conservation and management. In addition, fruit setting percentage as well as the number of seeds per fruit is quite low. Generally, two to six seeds per fruit are formed that too are recalcitrant having very poor viability. Hence, there was an urgent need of intervention of in vitro strategies for developing methods for rapid multiplication of this important wild species of *Citrus* for rehabilitation in its natural habitat to promote in situ conservation.

Seeds extracted from ripe fruits of *C. indica* after removing the seed coat aseptically germinated 100 % in vitro and produced full-fledged seedlings within 8 days of culture in the modified White (1943) medium. Amongst various vegetative explants, nodal stem segments showed the maximum regenerative potentiality followed by shoot tips and leaf segments in a decreasing order, when cultured in morphogenetic medium containing various concentrations (0.1, 0.2 and 0.5 mg l^{-1}) of BAP, N^6-2-isopentenyladenine (2iP), kinetin (Kn) and zeatin (Z) in combination with 0.1 mg l^{-1} IAA and 15 mg l^{-1}AdS. Amongst different cytokinins used, BAP

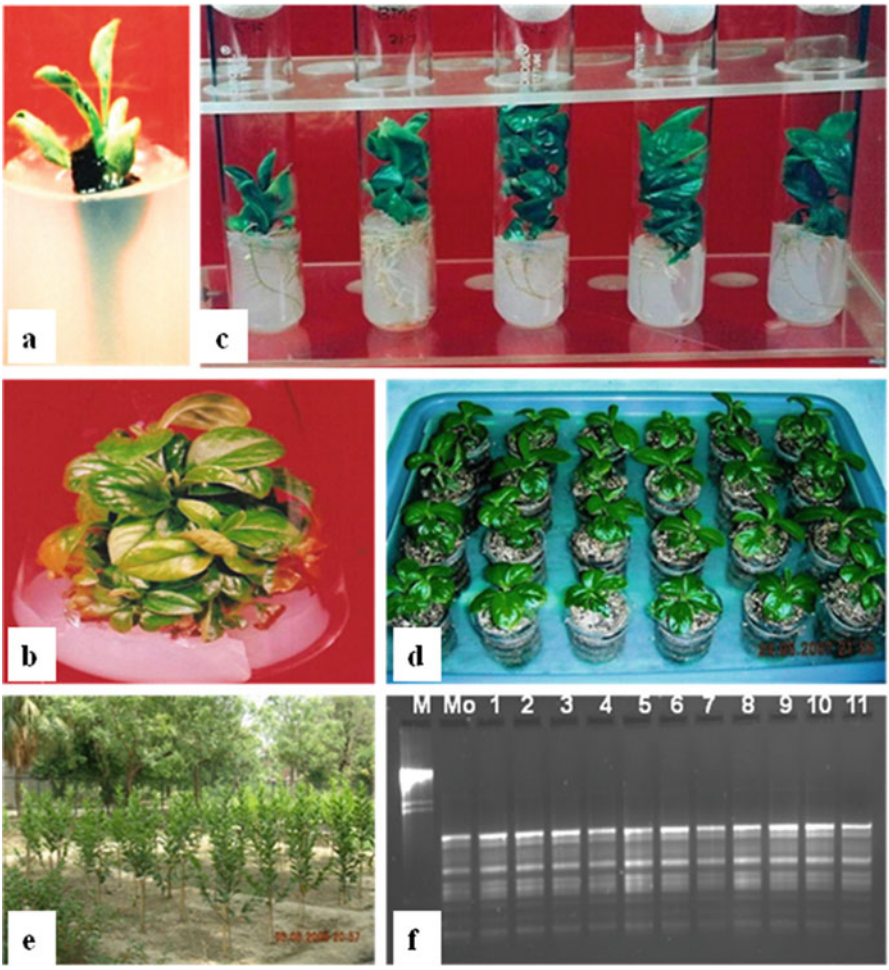

Fig. 10.4 (**a**–**f**) In vitro cloning of *Embelia tsjeriam-cottam*. (**a**) Bud-break resulting into shoot formation in nodal stem segment, taken from field-grown plant. (**b**) Proliferation of shoots. (**c**) Rooting of isolated shoots. (**d**) In vitro raised hardened plants in pots. (**e**) In vitro raised plants in field. (**f**) RAPD analysis of in vitro raised field-grown plants and mother plant; lane *M* Lambda DNA/Hind III digest size markers, lane *Mo* DNA from mother plant, lanes 1–11 DNA from randomly selected regenerated plantlets

was more effective than 2iP, kinetin (Kn) and zeatin (Z). The regenerated shoots did not proliferate further upon subculturing in the same morphogenetic medium. In order to enhance the rate of proliferation of shoots with normal growth, effect of different concentrations of GA and BAP along with 0.1 mg l^{-1} IAA and 15 mg l^{-1} AdS was assessed. In the optimum treatment, comprising 0.2 mg l^{-1} BAP, 0.1 mg l^{-1} IAA, 15 mg l^{-1} AdS and 0.1 mg l^{-1} GA, the shoot proliferation rate was increased to three times, i.e. 15 adventitious shoots per explant in place of 5 were regenerated with normal growth and good foliage. It is important to mention that the cultures of

proliferating shoots are being maintained for 8 years, providing continuous enormous supply of clonal planting material. It was also observed that during this long span of time, proliferating shoots did not lose the regenerative potentiality as also the shoots, excised from such cultures, and showed no decline in their rooting potentiality. Thus, a stock of these cultures comprised a 'tissue bank' – a means of in vitro germplasm preservation. The well-developed shoots, isolated from cultures of proliferating shoots, were induced to root in the presence of 0.25 mg l^{-1} IBA and 1 mg l^{-1} chlorogenic acid. The in vitro raised potted plants grew well under glasshouse conditions. The genetic fidelity of in vitro raised plants was assured by RAPD analysis (Fig. 10.5a–e).

10.3.1.6 *Azadirachta indica* A. Juss.

Azadirachta indica in nature is represented by a large number of genotypes differing in contents of their active principles, including two commercially most important constituents, that is, azadirachtin and neem oil (Ermel et al. 1987; Schmutterer 1995). The conventional methods of propagation of *A. indica*, both sexual and vegetative, are beset with constraints that restrict large-scale multiplication of selected trees and its commercial exploitation (Schmutterer 1995). The main limitations in sexual propagation are the recalcitrant nature of seed with short period of viability and high heterozygosity (Ezumah 1986; Sacande et al. 2001). In case of vegetative propagation by cuttings, availability of cuttings of right maturity, problems of rooting, favourable season of the year and the presence of endogenous infection within the material are the main constraints (Dogra and Thapliyal 1996). As a result, cloning as well as preservation of its diverse genotypes under field conditions is virtually impossible. All these limitations make it imperative to develop in vitro processes for cloning of mature neem trees of proven traits.

An efficient in vitro process for rapid clonal propagation of a mature (40-year-old) tree of *A. indica*, employing nodal stem segments, was developed (Arora et al. 2010). Season of collection and maturity of explants was found to have a direct influence on bud-break. Nodal stem segments collected during the month of April showed best response. Bud-break was maximized (78.6–81 %) when middle-order nodes (third or fourth node from apex) were taken. Amongst various cytokinins used, BAP at the concentration of 0.25 mg l^{-1} was found to be most effective in inducing multiple shoots, whereas growth and general condition of proliferating shoots were influenced by inorganic and organic constituents of the medium. On an average, 3.1 shoots per explant were regenerated in a modified MS medium supplemented with 0.25 mg l^{-1} BAP, 0.25 mg l^{-1} IAA and 15 mg l^{-1}AdS. The isolated shoots could be rooted in the presence of 0.5 mg l^{-1} IBA. Hundred percent root induction took place within 8–10 days of culture. The in vitro raised plantlets were successfully transplanted in potted soil, and finally they were grown under field conditions with 100 % survival. The genetic stability of in vitro raised field-grown plants was assured by RAPD markers (Fig. 10.6a–g). Furthermore, chemical analysis showed azadirachtin content of the in vitro cloned plants comparable to that of mother tree, which proved their chemical stability also.

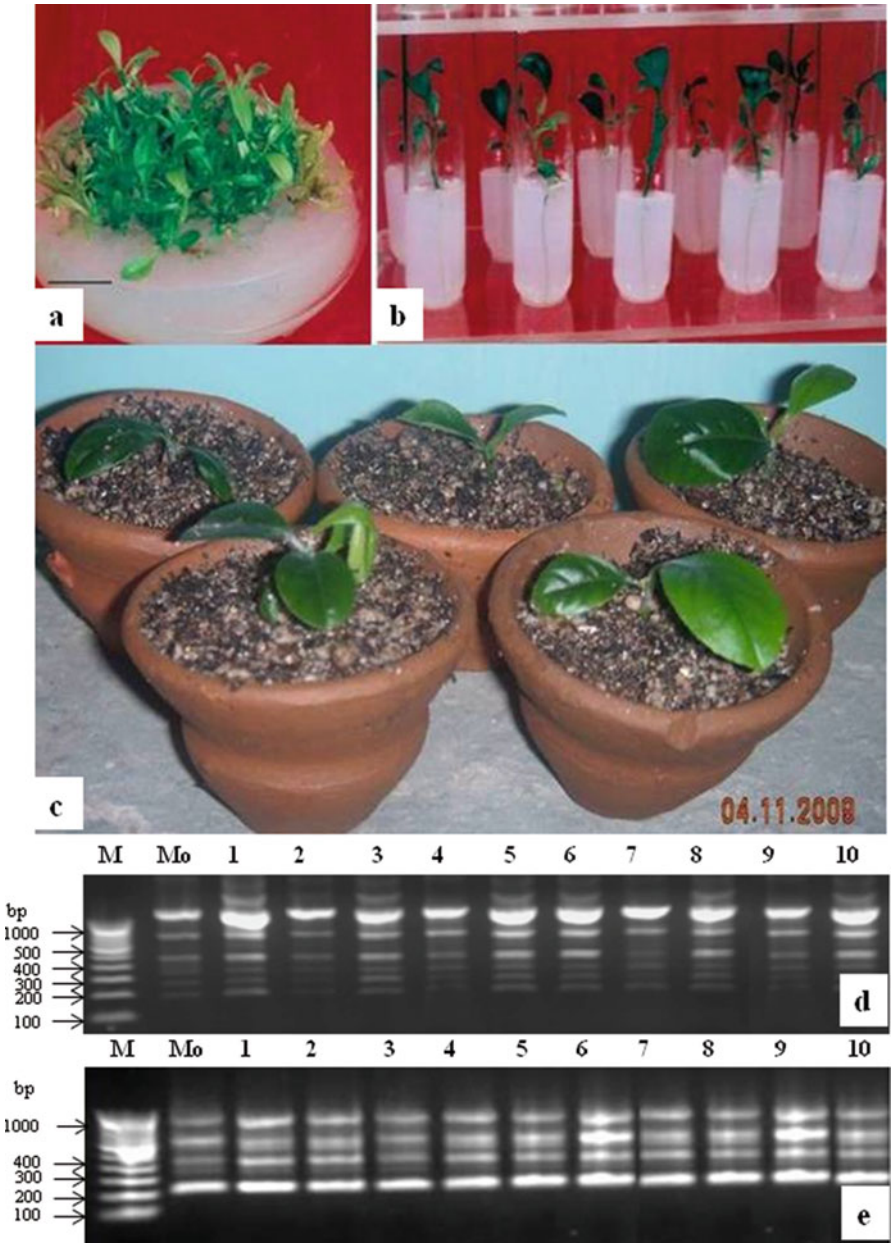

Fig. 10.5 (**a**–**e**) Clonal propagation of *Citrus indica*. (**a**) Sustained proliferation of shoots. (**b**) In vitro regenerated shoots showing uniform rooting. (**c**) In vitro raised potted plants. (**d**) RAPD profile of ten in vitro raised plants amplified with RAPD primer OPB 06. (**e**) RAPD profile of ten in vitro raised plants amplified with RAPD primer OPB 10

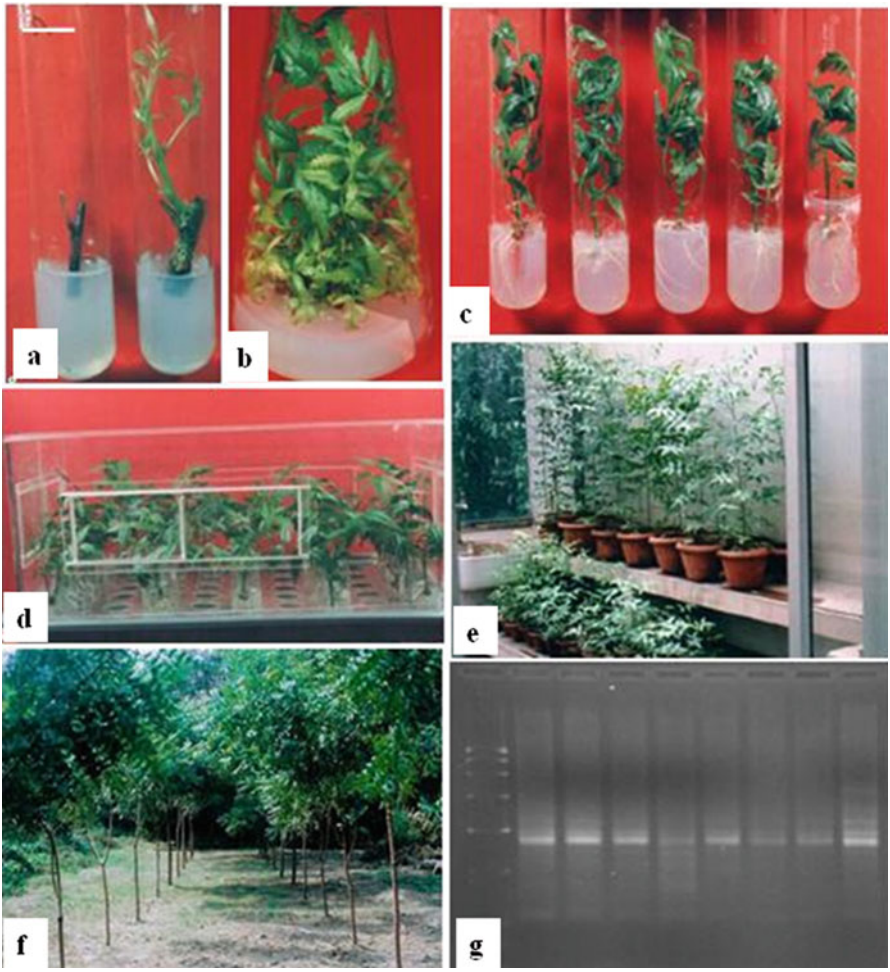

Fig. 10.6 (**a**–**g**) In vitro cloning of a 40-year-old tree of *Azadirachta indica*. (**a**) Difference in axillary bud-break in the nodal stem segment explants in respect of their relative position on a growing twig. Segment with first node, immediately below the shoot tip, turned brown without any bud-break (Lt.). Segment having fourth positioned node from shoot tip remained green with bud-break leading to regeneration of healthy, leafy shoot (Rt.). (**b**) Proliferation of shoots. (**c**) Rooting of isolated shoots. (**d**) Acclimatization of in vitro rooted shoots – plantlets – in inorganic salt solution. (**e**) A group of in vitro regenerated plants in potted soil. (**f**) Field cultivation of nodal stem segment-regenerated plants after 2 years of transplantation. (**g**) RAPD profile of field-grown plants of neem regenerated from nodal stem segments using primer OPU-20. Lanes from left are marker (*M*) containing low-range DNA ruler followed by lanes with DNA of the mother plant (*A*) and nodal stem segment-regenerated plants (*B-H*), respectively

In addition, a process with controlled pattern of regenerant differentiation from leaflet segments, excised from in vitro cultures of proliferating shoots of a mature (40-year-old) neem tree, leading to production of cloned plants was also developed (Arora et al. 2009). Depending upon the culture conditions, two types of morphogenetic structures, i.e. shoot buds (potential regenerants) and meristematic nodules (having very low potentiality of producing plantlets), were formed simultaneously. On subculture, the differentiated shoot buds developed into shoots, whereas the variation in response was observed in case of meristematic nodules which either budded into similar nodular structures or produced three kinds of organized structures: shoot buds, roots and a shoot and root on opposite poles resulting into plantlets that too are in varied proportions. Through this process, the rate of production of cloned plants of elite neem trees was augmented.

10.3.2 Germplasm Preservation of Neem Through Excised Root Cultures

The need for developing an efficient method for germplasm preservation of neem trees cannot be overemphasized as also the role of tissue culture to this effect, as being a highly heterozygous tree, the preservation of its diverse elite genotypes requiring different agroclimates under field conditions, is virtually impossible.

For the purpose of germplasm preservation of elite neem trees, an innovative process has been developed by inducing multiple shoot bud differentiation in segments of roots, taken from 5-year-old excised root cultures, established by employing explants of a 40-year-old tree (Arora et al. 2011). The root-regenerated shoot buds developed into vigorously growing normal green shoots leading to development of complete plants. The root-regenerated plants have been transferred to field with 100 % survival exhibiting luxuriant growth. Genetic fidelity of the field-grown plants has been ascertained by histological studies as well as by molecular analysis using RAPD markers. Histological studies revealed that the regenerants have been developed from the pericycle cells of the root, which are known to be genetically stable. Also, RAPD analysis of mother tree and its progenies showed monomorphic banding pattern of bands in RAPD profiles (Fig. 10.7a–g). The process developed is invaluable for establishing ‘gene banks’ of this tree of immense medicinal/insecticidal value by conserving its different genotypes growing world over in terms of regenerative excised root cultures.

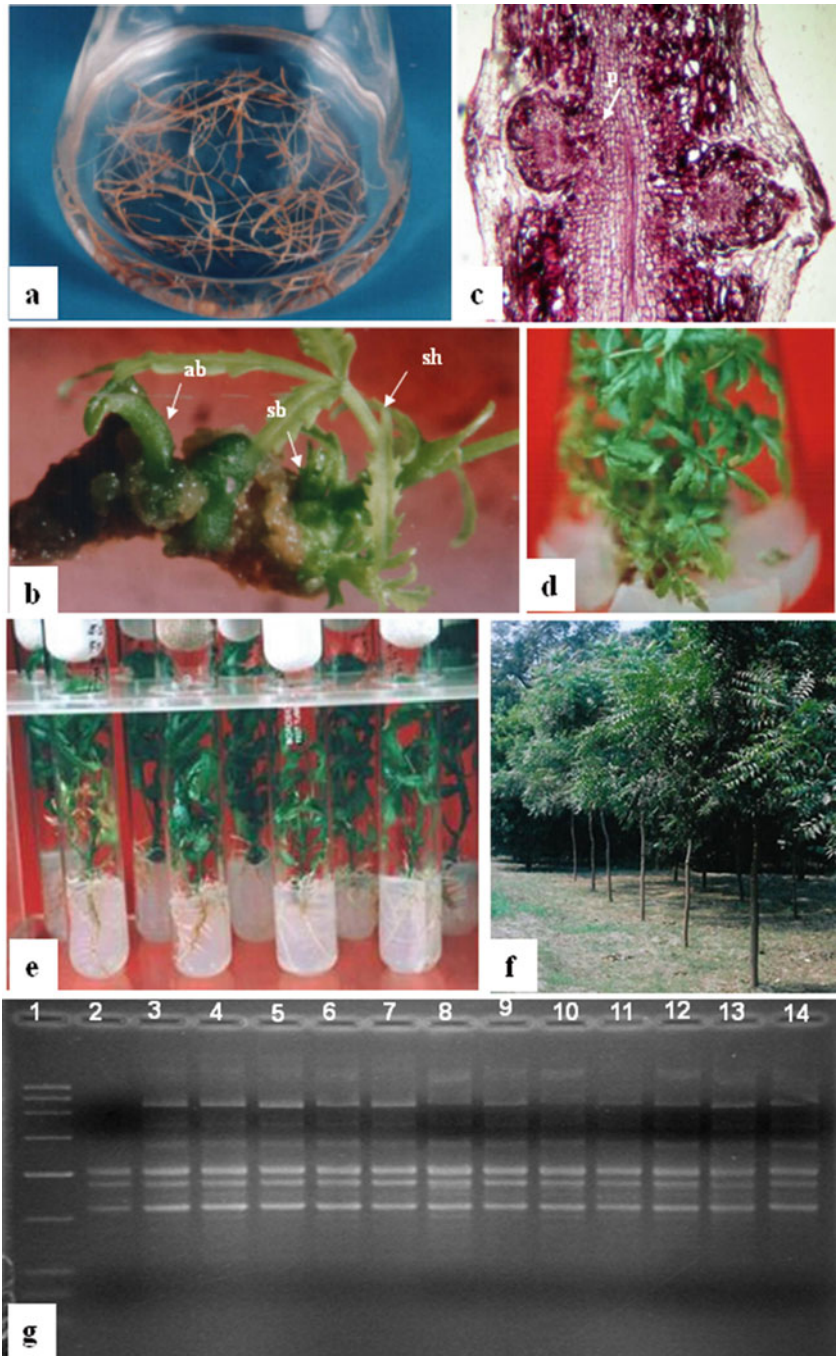

Fig. 10.7 (**a**–**g**) Excised root culture and germplasm preservation of *Azadirachta indica*. (**a**) Sustained growth of excised roots in prolonged culture. (**b**) Direct differentiation of more number of shoot buds (*sb*)/shoots (*sh*) and lesser number of aberrant regenerants (*ab*) in root segment taken from a 2-year-old excised root culture. (**c**) A magnified view of longitudinal section (LS) of

10.4 Conclusions Highlighting the Salient Achievements

1. For developing a model ex situ conservation programme for RET plant species of Indo-Gangetic Plain, efficient in vitro cloning protocols in case of *C. serratum*, *U. picta*, *O. petaloidea* and *Embelia tsjeriam-cottam* as well as for a wild, endemic, endangered and monoembryonic species of *Citrus*, viz. *C. indica*, were developed having immense significance.
2. The in vitro process developed for rapid clonal multiplication of a mature (40-year-old) tree of *A. indica* is of great practical importance, as most of the desirable characteristics appeared only at maturity. Besides in vitro cloning through nodal stem segments, augmentation of rate of clonal multiplication by inducing differentiation of multiple shoots from segments of leaflets deserves special mention in raising 'clonal neem orchards'.
3. Cultures of proliferating shoots of all the above-mentioned plants, maintaining sustained rate of multiplication as well as potential for root induction in isolated shoots, even in long-term culture, by incubating them in respective balanced nutrient medium through periodic subculturing, provide a moderately good system of germplasm preservation/ex situ conservation.
4. Development of an innovative method for germplasm preservation of neem through long-term regenerative excised root cultures is of immense practical value for establishing 'in vitro germplasm repositories' or 'gene banks' of elite neem trees requiring diverse agroclimates, which otherwise may not be possible through any conventional approach.

10.5 Prospects for Future Research

1. Under the mandate of CSIR-NBRI on conservation and sustainable utilization of wild and non-crop economic plants through conventional and biotechnological interventions, in vitro protocols developed for clonal propagation of *C. serratum*, *U. picta*, *O. petaloidea*, *Embelia tsjeriam-cottam* and *C. indica* may be exploited for large-scale production of cloned plants for raising enough raw material for various pharmaceutical companies and also facilitate rehabilitation of these plants in their natural habitats for in situ conservation and sustainable utilization.

Fig. 10.7 (continued) responded root explant showing endogenous differentiation of meristemoids from the pericycle (*p*) juxtaposed to the vascular tissue of explant beneath the cortical tissue. (**d**) Proliferation of shoots regenerated from root segments, taken from a 2-year-old excised root culture. (**e**) Rooting of isolated shoots obtained from cultures of proliferating shoots raised from segments of roots of a 2-year-old excised root culture. (**f**) In vitro raised plants, regenerated from root segments of a 2-year-old excised root cultures, as seen after 2 years of transplantation under field conditions. (**g**) RAPD profile of field-grown plants of neem regenerated from root segments, taken from a 2-year-old excised root culture obtained with primer OPF-04. Lanes from left are marker containing low-range DNA ruler (lane 1) followed by lanes with DNA of the mother plant (lane 2) and 12 root-regenerated plants (lanes 3–14), respectively

2. Heterozygous nature of neem coupled with very limited conventional methods of vegetative propagation makes cloning and preservation of its different genotypes under field conditions virtually impossible. In vitro protocols developed for cloning and germplasm preservation of mature neem tree may be utilized for large-scale production of cloned plants of proven qualities and for preservation of its diverse genotypes growing in varied agroclimates at one place in the form of their regenerative excised root cultures, an innovative approach, leading to development of 'gene repositories'/'gene banks', respectively.

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Biotechnological Approaches for the Improvement of *Eucalyptus*

11

Diwakar Aggarwal, M. Sudhakara Reddy, and Anil Kumar

Abstract

Forest trees have received relatively little attention as compared to crop species, even though biotechnological strategies could potentially have a greater impact on forestry and forest products. *Eucalyptus* is among the fastest-growing woody plants in the world and widely accepted for plantation forestry throughout the world due to its wide adaptability, extremely fast-growing nature, and most importantly excellent wood and fiber properties. Improvement of *Eucalyptus* by conventional breeding is constrained by long reproductive cycles, complex genetic characteristics, self-incompatibility, and a high degree of heterozygosity. Therefore, there is a requirement of developing faster methods of vegetative propagation for rapid cloning of superior germplasm and trait-based genetic improvement of selected clones for qualitative and quantitative tree improvement to suit the needs of end users. Over the last few years, considerable success has been achieved in the area of in vitro propagation as well as the genetic transformation of *Eucalyptus*. Furthermore, sequencing of the complete genome of *Eucalyptus* will strengthen various genomic approaches for the improvement of *Eucalyptus*. The present review presents a comprehensive account of the various in vitro propagation and genetic transformation techniques for the improvement of *Eucalyptus*.

Keywords

Micropropagation • Genetic transformation • Shoot organogenesis • *Agrobacterium tumefaciens* • Clonal fidelity

D. Aggarwal
Department of Biotechnology, Multani Mal Modi College, Patiala 147001, India

M.S. Reddy • A. Kumar (✉)
Department of Biotechnology, Thapar University,
TIFAC-CORE Building, Patiala 147004, India
e-mail: adatta@thapar.edu

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11.1 Introduction

Eucalyptus (family Myrtaceae) is among the most widely planted hardwoods in the world (Doughty 2000). These are generally long-lived, evergreen species (Ladiges et al. 2003). *Eucalyptus* is widely planted in the tropical and subtropical part of the world because of its superior growth, broader adaptability, and multipurpose wood properties. Native to Australia, *Eucalyptus* includes over 700 species (Brooker 2000), and these fast-growing trees were introduced into India, France, Chile, Brazil, South Africa, and Portugal in the first quarter of the nineteenth century (Doughty 2000) and were rapidly adopted for plantation forestry. The great economic interest in *Eucalyptus* species is due to the versatility of their wood (Eldridge et al. 1993). Its timber has applications for many different purposes such as pulp and paper production, electric poles, charcoal, timber, and furniture. *Eucalyptus* being a hardwood tree produces shorter fibers than softwoods like pines. Short fibers of this genus make it more reliable for use in paper and furniture industries due to its desirable surface characteristics, smoothness, brightness, and low tensile strength (Lal et al. 1993). The global area under its plantation is estimated at 20 million ha (GIT Forestry 2008), spreading over 37 countries and accounting for 16 % of worldwide forest area (FAO 2000). India has the largest area under *Eucalyptus* plantation (8 million ha), followed by Brazil (3 million ha) (Junghans et al. 2003). *Eucalyptus* plantations contribute 25 % of the total wood consumed in the developed countries and mainly used as a source of wood and fiber for pulp and paper industry (Zhou 2005). The average annual yield from ordinary seed-raised agroforestry plantations of *Eucalyptus* is in the range of 5–6 m^3 ha^{-1} $year^{-1}$ by the third year and 10–15 m^3 ha^{-1} $year^{-1}$ by the seventh year of plant growth (Lal et al. 1993). Some selected clones recorded much higher annual productivity ranging between 16 and 20 m^3 ha^{-1} $year^{-1}$ by the third year and 20 and 25 m^3 ha^{-1} $year^{-1}$ by the seventh year of the plantation (Lal et al. 1993). *Eucalyptus* is traditionally propagated by seeds, and interspecific hybrids are common in nature. In such genetically diverse stocks, trees with the better qualities, such as a straight clear bole, disease and pest resistance, drought tolerance, high productivity, fast growth, etc., occur at low frequencies. Due to extensive cross-pollination, seed progeny of superior trees fails to maintain their superior characteristics (Sankara Rao 1988). Thus, there is a felt need to develop faster methods of vegetative propagation of these promising elite clones. This will also be useful for undertaking the clonal forestry program.

Micropropagation is an attractive alternative to conventional vegetative propagation with the advantage of enhancing the rate of multiplication of valuable clones from limited explant material (Beck and Dunlop 2001). Various in vitro propagation techniques, such as axillary and adventitious shoot multiplication, shoot organogenesis, and somatic embryogenesis, are currently employed in plantation forestry programs for the large-scale multiplication of important tree species (Vengadesan and Pijut 2009; Tzfira et al. 1998; Haines and Martin 1997). Micropropagation exploits the regeneration potential of the selected tissue and is the preferred choice for the multiplication of difficult-to-root but economically or industrially important genotypes. It has been successfully used for rejuvenation and mass multiplication of

many tree species (Yasodha et al. 2004) including *Eucalyptus* (Aggarwal et al. 2012; Sharma and Ramamurthy 2000). Moreover, higher yield was reported from plantations raised from micropropagated plants as compared to seedling-derived plants (Khuspe et al. 1987). Thus, the potential impact of micropropagation on forest productivity, forest-based industry, and global timber supplies is now well realized (Yanchuk 2001).

In addition to micropropagation, there is a need to undertake the trait-specific improvement program for elite clones using various biotechnological tools. There have been rapid developments in the area of plant biotechnology in recent years; powerful tools that can enhance productivity and utilization are becoming available (Merkle and Dean 2000). Relevant methods include genetic manipulations to introduce exotic genes conferring resistance to biotic and abiotic stress including trait-specific genetic modifications and marker-assisted breeding program (Teasdale 1995). Improvement of plants through transgenic technology enables introduction/improvement of the specific trait(s) of interest in a selected genotype. The prerequisites for plant genetic transformation are (a) gene constructs carrying the polynucleotide sequences coding for desired proteins, (b) efficient methods to transform the explants, (c) procedures for selection of plant tissue harboring transgene, and (d) an efficient plant regeneration protocol form desired explants. In conventional breeding approach, the traits of interest have to reside within the same species. On the other hand, genetic transformation technology enables the scientists to transfer genes for selected traits across genera and kingdoms (Brunner et al. 2007). The transfer of selected genes is more important for tree species, as their improvement by the conventional breeding program is limited by long breeding cycles, high levels of heterozygosity, and incompatibility barriers (Machado et al. 1997). However, due to herbaceous nature and ease of genetic transformation and subsequent regeneration of transformed tissue till now, the major focus of genetic manipulations has been on crop species, and there are many reports of the successful introduction of foreign genes into crop plants (Girijashankar and Swathisree 2009). Due to the difficulties in regeneration and genetic transformation, forests trees still remain a challenge to genetic manipulations. During the last two decades, in vitro propagation and genetic transformation of tree species with the goal to modify wood quality for end use are important areas of research (Shani et al. 2004; Halpin and Boerjan 2003; Fenning and Gershezon 2002).

11.2 In Vitro Propagation Studies

Although most of the *Eucalyptus* species can be propagated vegetatively using traditional stem-cutting techniques, it has several constraints such as poor rooting of stem cuttings and graft incompatibility problems (Vengadesan and Pijut 2009; Bennett et al. 1994) and is further limited by the availability of propagules and season. Therefore, micropropagation is becoming increasingly popular for the establishment of clonal plantations because of the prospects of rapid cloning. The importance of in vitro clonal propagation of *Eucalyptus* is evident from the amount

of work carried out on the genus using various micropropagation techniques (Aggarwal et al. 2012, 2010; Dibax et al. 2010; Pinto et al. 2008; Prakash and Gurumuthi 2005; Sharma and Ramamurthy 2000; Termignoni et al. 1996; Subbaiah and Minocha 1990; Das and Mitra 1990; McComb and Bennett 1986). Success has been achieved in raising micropropagated plants from different species of *Eucalyptus* through various methods (Aggarwal et al. 2012; Dibax et al. 2005; Mullins et al. 1997; Chang et al. 1992; Subbaiah and Minocha 1990); this opened up the possibility for the large-scale clonal propagation of elite clones. Moreover, the establishment of a good regeneration system is fundamental for genetic transformation, which can only be achieved through in vitro propagation (Kumar et al. 2004).

11.2.1 Explants for Culture Establishment

For the establishment of aseptic cultures, various plant parts such as cotyledons, hypocotyls, and leaf fragments excised from in vitro-raised seedling have been used as explant (Prakash and Gurumuthi 2005; Sharma and Ramamurthy 2000; Termignoni et al. 1996; Subbaiah and Minocha 1990). In some cases, aseptic cultures were also initiated from zygotic embryos (Serrano et al. 1996). Besides taking explants from these in vitro-grown plantlets, explants such as coppiced shoots (Aggarwal et al. 2012; Burger 1987), scion shoots (Franclet and Boulay 1982; Gonocalves 1980), epicormic shoots (Ikemori 1987), and young, vigorously growing shoots from mature trees (Aggarwal et al. 2010, 2012; Sankara Rao 1988; Defossard et al. 1977) have also been used as explant materials. Disinfection of mature, field-grown material has proved difficult because of endogenous microbial contamination (Defossard et al. 1977). Age of material and season are important factors determining success in establishing aseptic cultures (Sharma and Ramamurthy 2000; Grewal et al. 1980). Sometimes it is impossible to disinfect mature, field-grown shoots without severely damaging the tissues. But the best sources of explants for the culture establishment are generally juvenile or rejuvenated tissues (Jones and Van Staden 1997). In general, it has been reported that the organogenic response from juvenile tissues is better (George 1996).

11.2.2 Shoot Multiplication and Elongation

There are many reports on micropropagation of *Eucalyptus*, including shoot organogenesis and somatic embryogenesis (Aggarwal et al. 2010, 2012; Dibax et al. 2010; Pinto et al. 2008; Mullins et al. 1997; Gupta et al. 1983). Gupta et al. (1983) have reported the protocol for micropropagation of *E. torelliana* and *E. camaldulensis* taking explants from 12- to 15-year-old trees. Multiple shoots were induced from nodal segments of *E. camaldulensis* on liquid MS (Murashige and Skoog 1962) medium supplemented with different concentrations of kinetin (Kn), 6-benzyl

adenine (BA), calcium pantothenate, and biotin and incubated at 15 °C with continuous illumination. In *E. torelliana*, culture in agitated liquid media alone was sufficient for induction of shoots. Das and Mitra (1990) were able to achieve shoot multiplication on MS medium supplemented with 0.5 μM NAA and 4.4 μM BA using shoot tips as explants collected after coppicing of mature trees of *E. tereticornis*. These authors reported that the addition of charcoal and gibberellic acid (GA_3) to the medium was beneficial for shoot multiplication. Sharma and Ramamurthy (2000) reported micropropagation of the elite clones of *E. tereticornis* using nodal segments taken from 4-year-old trees. It was reported that March-April months were the best periods for culture establishment as maximum explants showed shoot induction during this period. Shoot induction was successfully achieved on MS medium supplemented with 4.4 μM each of BA and NAA. Shoots were further elongated by lowering the concentration of both BA and NAA. The Phytagel was reported as a better gelling agent than agar for shoot elongation but caused hyperhydricity, which was controlled by increasing the concentration of calcium chloride to double. Glocke et al. (2006) reported micropropagation of ornamental *Eucalyptus* hybrid (*Eucalyptus erythronema* var. *erythronema* x *Eucalyptus stricklandii* cv. *'urrbrae gem'*) through enhanced axillary shoot proliferation. Shoot proliferation was higher on WPM and QL (Quoirin and Lepoivre 1977) media supplemented with BA, NAA, and GA_3 as compared to MS, B5, AP (Almehdli and Parfitt 1986), and TK (Tabachnik and Kester 1977) media supplemented with the same composition of plant growth regulators (PGRs). In this study, WPM and QL were shown to provide better shoot growth than MS, AP, B5, and TK media. GA_3 was required for shoot elongation. Pulse treatment with 20 μM IBA for 7 days induced roots and rooting frequency further improved by lowering WPM medium strength to half. Aggarwal et al. (2012) have investigated the several factors influencing micropropagation of a selected elite clone of *E. tereticornis* (Fig. 11.1). Among the different cytokinins tested, 6-benzyl adenine proved to be the most effective cytokinin for shoot multiplication and elongation. The initial size of the shoot clump (inoculum) also influenced shoot multiplication and elongation. The number of shoots proliferated per culture vessel was significantly higher (342 shoots per culture vessel) when larger shoot clumps (15–20 shoots) were inoculated, compared to smaller shoot clumps (4–5 shoots), which resulted in a reduced shoot proliferation rates (245 shoots per culture vessel). However, the number of elongated shoots (65 per culture vessel) and shoot length (5.23 cm) were higher in cultures which were inoculated with smaller shoot clumps in comparison to those cultures which were inoculated with larger shoot clumps (54 shoots per culture vessel with shoot length of 4.17 cm). The number of shoots proliferated and elongated was higher in cultures incubated under photosynthetically active radiation (PAR) compared to those incubated under cool fluorescent lights (CFLs). Osmotic potential of the sap and chlorophyll content of cultures incubated under PAR were also higher than those incubated under CFL.

Fig. 11.1 Micropropagation of elite plants of *E. tereticornis*. (**a**) Elite plants of *E. tereticornis* growing at Thapar Technology Campus. (**b**) Shoot multiplication on MS medium supplemented with 2.5 μM BA and 0.5 μM NAA. (**c**) Shoot elongation on MS medium supplemented with 0.1 μM BA and 0.5 μM NAA. (**d**) Microshoots rooted on one fourth-strength MS medium supplemented with 5.0 μM IBA. (**e**) Acclimatized plantlets under greenhouse conditions. (**f**) 1-year-old tissue culture-raised plants growing in the field

11.2.3 Rooting and Acclimatization

Rooting of microshoots is the first step during hardening of plantlets before preparation for transplanting to the field. Auxins are widely used for induction of roots in microshoots. For rooting of *Eucalyptus* microshoots also, auxins have been widely used (Aggarwal et al. 2012; Sharma and Ramamurthy 2000; Bennett et al. 1994;

Gupta et al. 1983). Out of the various auxins tested, the efficacy of IBA for induction of roots in microshoots of various plant species has been described in detail by Kato (1985) and proved more potent than other auxins like NAA and IAA. But the concentration and mode of application of IBA vary with different plant species (Jha and Sen 1992; Kato 1985). Further, lowering of nutrient salt concentration was also reported to be beneficial for rooting of microshoots in *Eucalyptus* (Aggarwal et al. 2012; Bennett et al. 1994). Gupta et al. (1983) were able to induce rooting in microshoots of *E. torelliana* by treatment with α-naphthalene acetic acid (NAA), whereas treatment with a mixture of indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), indole-3-propionic acid (IPA), and NAA in the dark for different time intervals was essential for induction of roots in microshoots of *E. camaldulensis*. After auxin treatment, transfer of shoots to a charcoal-containing medium and incubation under the light were beneficial for root induction. Rooting occurred within 15–20 days in 70 % of the shoots of *E. torelliana* treated with NAA for 48 h. On the other hand, 50 % of *E. camaldulensis* microshoots treated in the dark for 72 h with a mixture of auxins containing IBA, IPA, IAA, and NAA rooted within 20–25 days. Rooted plantlets thus obtained were successfully transferred to pots and field. Similarly, Das and Mitra (1990) reported that the key factor for root induction in *E. tereticornis* was incubation in the dark for a short period. Further, these authors have reported that during culture, genotypically different populations responded differently in spite of optimal growth conditions. Subbaiah and Minocha (1990) achieved 100 % rooting on mWPM supplemented with 2.4 μM IBA in the case of *E. tereticornis*. Further, Aggarwal et al. (2012) have reported that PAR light was more effective for efficient rooting than CFL in *E. tereticornis*. These authors reported the increase in rooting efficiency under PAR light which may be due to the involvement of blue light responding cryptochromes and red/far-red light responding phytochromes as reported by Lin (2002). Light quality has been shown to promote rooting efficiency in some plant species (Kumar et al. 2003; Rossi et al. 1993).

A major limitation in large-scale application of plant tissue culture technology is high mortality experienced by tissue culture-raised plants during or following laboratory to land transfer, mainly due to the extreme differences between the in vitro and ex vitro environment, and it was also reported that plants produced under in vitro conditions are reported to develop poor photosynthetic apparatus (Sharma et al. 1999; Brainerd and Fuchigami 1982; Kozai 1991). Several methods have been tried for acclimatization of tissue culture-raised plants for successful field establishment of *Eucalyptus* (Aggarwal et al. 2012; Girijashankar 2012; Machado et al. 1997; Macrae and Van Staden 1993, 1999). Aggarwal et al. (2012) reported that plants produced under PAR showed higher survival rates and subsequently more vigorous growth following transfer to soil, which commensurate with higher chlorophyll contents.

Further, they reported that inoculation of plantlets with bacterial isolates during acclimatization was found to be beneficial for the survival and subsequent growth of plants. Grijashankar (2012) reported the use of different soil compositions for successful acclimatization of the micropropagated plantlets of *E. camaldulensis*. Further, in order to enhance the survival rate, covering of micropropagated plantlets

with a transparent polythene cover proved beneficial. Macrae and Van Staden (1993) and Machando et al. (1997) highlighted the role of *A. rhizogenes* strains in inducing rooting in difficult-to-root plants.

11.2.4 Shoot Organogenesis and Somatic Embryogenesis

Subbaiah and Minocha (1990) were the first to report shoot organogenesis from various explants of *E. tereticornis*. They reported the regeneration of adventitious shoots from leaf and stem callus. Callus was induced from leaf or stem segments taken from seedlings on B5 medium (Gamborg et al. 1968) supplemented with 0.44 μM BA and 15–20 μM NAA in the dark. Multiple shoots were regenerated directly from hypocotyl segments of 4- to 6-week-old seedlings on B5 medium supplemented with 2.2 μM BA. Shoot regeneration protocol for *E. camaldulensis* using leaf explants taken from seedlings grown in culture has been reported (Mullins et al. 1997) on woody plant medium (Lloyd and McCown 1981) containing 1.0 g/l casein hydrolysate, 50 g/l sucrose, and 0.5 % (w/v) phytagar and supplemented with 16.1 μM NAA and 0.45 μM BA for the regeneration of shoots from leaf explants. Out of 24 clones used in the study, only 13 clones regenerated shoots. Subsequently, the same protocol was used for the successful shoot regeneration of other species like *E. microtheca*, *E. ochrophloia*, *E. grandis*, and *E. marginata*.

However, the frequency of regeneration varied from species to species and clone to clone within the same species. The major finding of this study was the emphasis on the need to develop clone-specific protocols.

Ho et al. (1998) were able to regenerate shoots from hypocotyl explants taken from aseptically grown 1-month-old seedlings of *E. camaldulensis*. The B5 medium supplemented with 100 ml/l coconut milk, 200 mg/l glutamine, and 100 mg/l casein hydrolysate was used as a basal medium in this study. Successful shoot organogenesis and multiplication of shoots were achieved on this modified B5 medium supplemented with 4.4 μM BA and 15 μM NAA via callus phase. Microshoots were successfully rooted on MS medium containing half-strength macronutrients and supplemented with 4.9 μM IBA.

Barrueto Cid et al. (1999) have achieved shoot organogenesis from *E. grandis* x *E. urophylla* using hypocotyls, cotyledons, cotyledonary nodes, and primary leaves as explants obtained from 14- to 50-day-old seedlings. These seedling-derived explants were cultured on modified MS medium, supplemented with 2.0 μM thidiazuron (TDZ). The callus obtained on TDZ-supplemented medium was transferred to modified MS medium supplemented with different concentrations of BA and NAA or zeatin. Shoots were induced from these calli at a high frequency on medium supplemented with 5.0 μM BA and 0.5 μM NAA. Shoot elongation was then achieved on medium supplemented with 1.0 μM BA, 0.5 μM NAA and 2.0 μM GA_3. Rooting was induced in 50 mm long microshoots cultured on medium containing 2.5 μM IBA followed by transfer to the basal medium for 30 days within 5–15 days. Plantlets were then successfully transplanted to the greenhouse conditions.

Prakash and Gurumurthi (2005) were able to induce indirect somatic embryogenesis from mature zygotic embryos of *E. tereticornis*. The basal MS and B5 media containing different concentrations of NAA, 2,4-dichlorophenoxyacetic acid (2,4-D), and BA were evaluated for callus induction and somatic embryogenesis. Higher frequency of both callus induction and somatic embryogenesis was observed on MS medium as compared to B5. The maximum frequency of friable callus (embryogenic callus) was obtained on MS medium supplemented with 10.74 μM NAA. When the callus was transferred to MS media containing various concentrations of BA, somatic embryos developed after 1–2 weeks with the highest frequency (54 %) on medium supplemented with 2.22 μM BA. The embryos were successfully germinated on basal MS medium.

Dibax et al. (2005) also reported shoot organogenesis from cotyledonary leaves of *E. camaldulensis* cultured on MS medium supplemented with various combinations of NAA and BA. The best shoot organogenesis frequency was observed on medium supplemented with 2.7 μM NAA and 4.44 μM BA. Inoculation of explants in the dark during the first 30 days increased percentage explants forming callus increased and reduced explant necrosis. Regeneration frequency from callus further increased to 54 % from 47 % when the basal medium was reduced to half strength. Shoot elongation was obtained on the modified basal medium by lowering the strength of ammonium nitrate and potassium nitrate to half. The addition of 0.2 % activated charcoal to above-modified basal medium induced roots in microshoots after 1 month of culture.

Pinto et al. (2008) have described the factors affecting maintenance, proliferation, and germination of somatic embryos in *E. globulus*. Somatic embryogenesis was recorded on MS medium supplemented with 15 μM NAA. Embryos were maintained on the same medium up to 2 years. The influence of basal medium (MS and B5), plant growth regulators (auxins and cytokinins), and light on induction of somatic embryos was studied. The MS medium without growth regulators was found to be more efficient for embryo formation and germination than the B5 medium. Reducing auxin levels increased the proliferation of globular somatic embryos. The addition of two cytokinins (BA and KIN) to the MS medium did not improve proliferation of globular secondary embryos.

Aggarwal et al. (2010) developed an efficient shoot organogenesis system from mature plants of selected elite clones of *E. tereticornis* (Fig. 11.2a, b). Cultures were established using nodal explants taken from freshly coppice shoots. Shoot organogenesis was achieved from leaf segments cultured on MS medium supplemented with 5.0 μM BA and 1.0 μM 2,4-dichlorophenoxyacetic acid (2,4-D). The addition of cefotaxime to the medium promoted shoot differentiation, whereas carbenicillin and cephalexin inhibited shoot differentiation.

Leaf maturity was also found to influence shoot regeneration; the fifth leaf (14–16 days old) from the top of microshoot was found to be the best for shoot organogenesis.

Dibax et al. (2010) have reported the regeneration of cotyledonary leaves from *E. camaldulensis*. The leaves were cultured on MS, WPM, and JADS medium

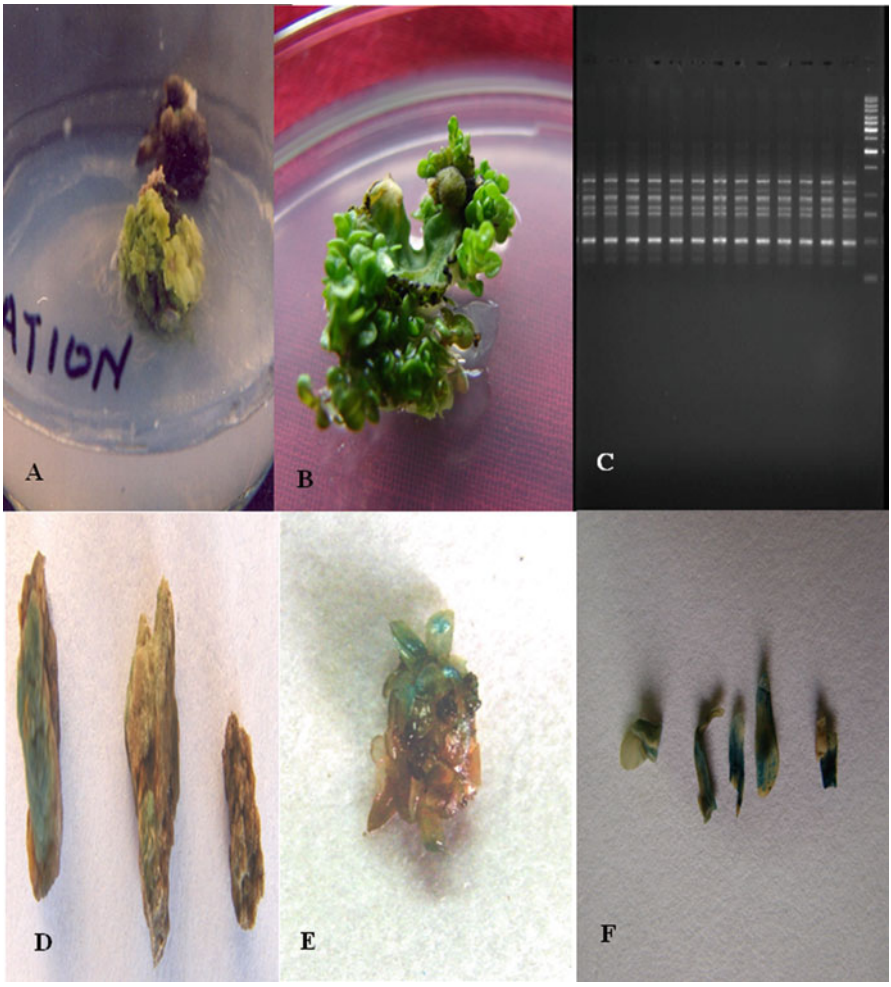

Fig. 11.2 Shoot organogenesis, Clonal Fidelity and *Agrobacterium* mediated genetic transformation of *E. tereticornis*. (**a**—**b**) Shoot organogenesis from leaf explants of *E. tereticornis* on MS medium supplemented with 5.0 μMBA and 1.0 μM 2,4-D; (**c**) Gel picture showing RAPD profile (Clonal fidelity) of mother plant and micropropagated plants of E. tereticornis. (**d**). Transformed *E. tereticornis* call us showing GUS activity. (**e**—**f**) Transformed *E. tereticornis* shoots showing Stable GUS activity after eight cycles of subculture

supplemented with 2.7 μM NAA and 4.44 μM BA. They have also reported the anatomy of the tissue during various stages of shoot regeneration.

Recently, Oberschelp et al. (2015) have reported in vitro organogenesis of *E. dunnii* from cotyledons and hypocotyl explants. Several combinations of 6-benzylaminopurine (BAP) plus α-naphthalene acetic acid (NAA) or indole-3-acetic acid (IAA) were added to a specifically developed basal medium. They have

also studied the effect of the hypocotyl segment's position (distal and proximal) on bud and callus regeneration. Histological analyses suggested that pluripotent cells give rise to buds and shoots.

It has been documented that shoot regeneration or somatic embryogenesis is difficult from mature plants as compared to juvenile tissue (Liu and Pijut 2008). However, it is important to develop direct regeneration protocol, which will help in taking up genetic manipulation work. Moreover, many *Eucalyptus* species are still considered recalcitrant to tissue culture and genetic engineering. Further, reports exist on the variable regeneration ability of the different species and also among clones of the same species (Mullins et al. 1997). Therefore, there is a felt need to develop clone-specific micropropagation and regeneration protocols (Table 11.1).

11.3 Clonal Fidelity of In Vitro-Propagated Plants

Clonal fidelity is one of the most important aspects of micropropagation industry. One of the problems encountered with the in vitro cultures is a generation of somaclonal variation arising as a direct consequence of in vitro culture of plant cells, tissues, or organs (Kumar et al. 2010; Rani et al. 1995; Schoofs 1992; Swartz 1990). The extent of somaclonal variation depends upon the media concentration and length of culture. Regeneration through adventitious shoot organogenesis is more prone to genetic variations as compared to axillary branching (Shenoy and Vasil 1992). Further, the extent of instability depends upon the mode of regeneration, whether it is direct organogenesis from explants or indirect organogenesis through callus phase (Rani et al. 1995). Moreover, the selection of the explant source is also known to influence the genetic variations in regenerated plants (Kawiak and Lojkowska 2004).

Molecular markers suitable for generating DNA profiles have proved to be an effective tool in assessing the genetic stability of regenerated plants (Martins et al. 2004). These markers are not influenced by environmental factors and generate reliable, reproducible results (Li et al. 2011). DNA-based markers most frequently in use include restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD, Williams et al. 1990), and inter-simple sequence repeat (ISSR, Zietjiewicz et al. 1994). The RAPD and ISSR markers have proven to be efficient in establishing clonal fidelity of regenerated plants. Both RAPD and ISSR markers have been successfully applied to detect the genetic similarities or dissimilarities in micropropagated material in many studies (Martin et al. 2006; Carvalho et al. 2004; Martins et al. 2004; Ramage et al. 2004; Sanchez et al. 2003). There are many reports highlighting the use of a combination of two markers amplifying different regions of the genome to study the genetic uniformity of in vitro-propagated plantlets (Lattoo et al. 2006; Martin et al. 2006; Ray et al. 2006; Dhiman and Singh 2003; Palombi and Damiano 2002).

Table 11.1 Summary of some of the in vitro propagation studies on *Eucalyptus* species

Sr. No	Species	Explant used	Morphogenic response	Media composition	Reference
1.	*E. saligna*	Cotyledonary nodes	Shoot multiplication	MS+NAA+ BAP	Lopes da Silva et al. (2015)
2.	*E. dunnii*	Cotyledons, hypocotyls	Shoot organogenesis	JADS+BAP+ IAA/NAA	Oberschelp et al. (2015)
3.	*E. tereticornis*	Coppiced shoots from mature plants	Shoot multiplication	MS+ BA+NAA	Aggarwal et al. (2012)
4.	*E. camaldulensis*	Nodal cuttings from 1.5-year-old tree	Direct organogenesis	MS+ BA+NAA	Girijashanker (2012)
5.	*E. tereticornis*	In vitro leaves	Shoot organogenesis	MS+ BA+2,4-D	Aggarwal et al. (2010)
6.	*E. urophylla*	Hypocotyl	Callus/regeneration	Modified MS+IAA+PBU	Huang et al. (2010)
7.	*E. camaldulensis*	Zygotic embryo and cotyledons	Callus/somatic embryogenesis	MS+NAA	Prakash and Gurumurthi (2009)
8.	*E. globulus*	Seedlings	Somatic embryogenesis	MS+NAA	Pinto et al. (2008)
9.	*E. erythronema* x *E. stricklandii*	Seedlings	Shoot multiplication	WPM+BA+ NAA+ GA3	Glocke et al. (2006)
10.	*E. phylacis*	Shoot explants	Callus/shoot regeneration	MS+TDZ+Zeatin+GA3+IAA	Bunn et al. (2005)
11.	*E. camaldulensis*	Cotyledons	Shoot regeneration	MS+NAA+BAP	Dibax et al. (2005)
12.	*E. tereticornis*	Zygotic embryo	Somatic embryogenesis/shoot regeneration	MS/B5+BAP+NAA/2,4-D	Prakash and Gurumurthi (2005)
13.	*E. nitens*	Seedlings	Shoot multiplication	½ MS+BA+NAA	Gomes and Canhoto (2003)
14.	*E. camaldulensis*	Nodes	Shoot regeneration	MS+BAP+IBA	Arezki et al. (2001)
15.	*E. globulus*	Cotyledons, hypocotyls	Bud induction/somatic embryogenesis	MS+TDZ+ 2,4-D/NAA	Nugent et al. (2001a)

16.	*E. tereticornis*	Nodal segments from 4-year-old trees	Shoot multiplication	MS+BA+IAA	Sharma and Ramamurthy (2000)
17.	*E. grandis* x *E. urophylla*	Cotyledons, hypocotyls	Callus/shoot regeneration	MS+ TDZ/BAP+NAA/IAA	Barrueto Cid et al. (1999)
18.	*E. globulus*	Hypocotyls	Shoot regeneration	MS+NAA+BA/TDZ	Azmi et al. (1997)
19.	*E. camaldulensis*	Seedlings	Shoot regeneration	WPM+NAA+BA	Mullins et al. (1997)
20.	*E. grandis*	Nodes from a 5-year-old plant	Shoot multiplication	MS+BAP+NAA	Sita and Rani (1985)
21.	*E. grandis*	Leaf explants	Callus/regeneration	KG+ BAP/zeatin+NAA	Laine and David (1994)
22.	*E. tereticornis*	Seedlings	Shoot regeneration	WPM/B5+BA+NAA	Subbaiah and Minocha (1990)
23.	*E. tereticornis*	Twigs from 8- to 10-year-old plants	Shoot multiplication	Modified MS+NAA+ IBA	Das and Mitra (1990)
24.	*E. tereticornis*	Nodal explants	Shoot multiplication	MS+ NAA+IAA/IBA	Sankara Rao (1988)
25.	*E. camaldulensis*/*E. citriodora*	Shoots	Callus/shoot regeneration/ somatic embryogenesis	MS/WPM+ BAP+ NAA	Murlidharan and Masceherens (1987)

MS Murashige and Skoog medium (Murashige and Skoog 1962), *BAP-6*-benzylaminopurine, *BA* 6-benzyl adenine, *IAA* indole-3-acetic acid, *IBA* indole-3-butyric acid, *NAA* α-naphthalene acetic acid, *TDZ* 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea, *Kn* kinetin (N^6-furfuryladenine), *2,4-D* 2,4-dichlorophenoxy acetic acid, *GA3* gibberellic acid, *WPM* woody plant medium (Lloyd and McCown 1981), *JADS* JADS medium (Correia 1993; Correia et al. 1995), *KG* KG medium (Laine and David 1994), *B5* B5 medium (Gamborg et al. 1968

A perusal of literature shows that no serious attempt has been made so far in this direction although thousands of tissue culture-derived *Eucalyptus* plants have been produced for reforestation programs and other economic benefits (Anonymous 1994; Keil and Griffin 1994; Rockwood and Warrag 1994; McComb and Bennett 1986). There are few reports on a genetic analysis of *Eucalyptus*, using RAPD and ISSR markers to establish clonal fidelity of in vitro-raised plants (Aggarwal et al. 2010, 2012; Rani and Raina 1998) (Fig. 11.2c). Tripathi et al. (2006) have reported the use of these markers to distinguish *Eucalyptus* plants raised through tissue culture.

11.4 Genetic Transformation Studies

The basic approach of genetic modification in plants involves the modification of its genomic DNA by incorporation of new genes. Various transformation techniques have been developed to assist the transfer of DNA into recipient plant cells (Hansen and Durham 2000). These transformation techniques involve the insertion of DNA fragment containing one or more genes into a chromosome/genome of an organism. In plants, it can be mediated either by a biological agent such as *Agrobacterium tumefaciens* (a common gram-negative soil bacterium that has the ability to transfer DNA fragment to host) (Tzfira and Citovsky 2006; Gelvin 2003) or by a direct gene delivery system (such as gene gun, electroporation, microinjection, lasers, polyethylene glycol (PEG), silicon carbide fibers) that utilizes physical, electrical, or chemical means to deliver gene of interest to a target cell (Torney et al. 2007; Weir et al. 1998; Nehra et al. 1994).

The transfer of selected genes through transgenic technology is especially important for *Eucalyptus*, as its improvement by conventional breeding approach is limited by long breeding cycles, high levels of heterozygosity, and incompatibility barriers (Machado et al. 1997). Literature review indicates that various methods tried for delivering foreign DNA into *Eucalyptus* are electroporation, biolistic gun, and *Agrobacterium*-mediated transformations (Girijashankar 2011). But only a few studies report successful development of genetic transformation protocol in *Eucalyptus* (Aggarwal et al. 2011; Tournier et al. 2003; Ho et al. 1998). These studies mainly focused on the development of genetic transformation protocol using juvenile tissues of seed origin (Prakash and Gurumurthi 2009; Tournier et al. 2003; Ho et al. 1998).

11.4.1 Biolistic Gun-Based Genetic Transformation

The first attempt to optimize biological and physical parameters for particle gun-mediated genetic transformation of *E. globulus* was carried by Rochange et al. (1995). Cultured zygotic embryos were used as the starting material for genetic transformation. Based on transient GUS expression assay, these authors observed

that both gunpowder apparatus and compressed-helium system exhibited similar transformation efficiency and reported that 6-day-old cultured embryos are the best explants for genetic transformation. These authors also highlighted the role of high osmotic potential before the bombardment and were able to obtain up to 130 GUS expression events per embryo with a good distribution all over the tissue (Fig. 11.2).

Serrano et al. (1996) were the first to report successful regeneration from *E. globulus* following biolistic transformation of zygotic embryos. Histochemical GUS assay was used to confirm the presence of transgene, and molecular analyses based on polymerase chain reaction and Southern blot were used to confirm the integration of the target DNA into the nuclear genome. The biolistic transformation was also carried on hypocotyls and cotyledons of *E. grandis* × *E. urophylla* hybrids (Alcantara et al. 2011; Sartoretto et al. 2002). All the abovementioned reports were able to obtain GUS-expressing callus, but regeneration of transformed shoots from such callus could not be achieved. However, shoots were regenerated successfully in the control plants that were used for the biolistic-based transformation but without a bombardment of the tissue. Unfortunately, there are no reports of biolistic gun-based transformation work on *Eucalyptus* where special efforts have been made for optimization of parameters for the successful genetic transformation.

11.4.2 *Agrobacterium*-Mediated Genetic Transformation

So far, *Agrobacterium*-mediated transformation is the most successful method for genetic transformation of *Eucalyptus*. According to Mullins et al. (1997), this indirect gene transfer approach is preferred over biolistic-mediated direct genetic transformation as it is known to reduce the insertion of multiple copies of the transgene, which can lead to a multicopy gene silencing. Machado et al. (1997) evaluated the susceptibility of *E. grandis* × *E. urophylla* hybrids to 12 wild strains of *A. tumefaciens* and 5 strains of *A. rhizogenes*. Different degrees of virulence have been recorded using these strains, indicating the possibility of transforming *Eucalyptus* and its hybrids using *Agrobacterium*. Tumors obtained after infection showed autonomous growth when cultured on plant growth regulator-free medium, and some tumors lead to the formation of shoots. This study suggested that *Agrobacterium* can be successfully used to transform *Eucalyptus* species. Further, Krimi et al. (2006) reported that *E. occidentalis* was more susceptible to *Agrobacterium* than *E. camaldulensis* and *E. cladocalyx*. The ability of *A. tumefaciens* to infect *Eucalyptus* was found to vary across species and genotypes, thus suggesting the need to develop clone-specific genetic transformation protocol.

Literature survey reveals that *E. camaldulensis* was a favorite species for *A. tumefaciens*-mediated genetic transformation (Chen et al. 2001; Ho et al. 1998; Azmi et al. 1997; Mullins et al. 1997; Kawazu et al. 2003). The choice of this species is due to its good regeneration potential following genetic transformations. There are reports on the genetic transformation of other *Eucalyptus* species like *E.*

globulus, *E. gunnii*, and *E. tereticornis* (Aggarwal et al. 2011, 2015; Torre et al. 2014; Serrano et al. 1996; Nugent et al. 2001a, b; Chriqui et al. 1992).

Mullins et al. (1997) have reported the genetic transformation protocol for *E. camaldulensis* using cotyledonary leaves as explants. Five disarmed strains of *A. tumefaciens* (A6, LBA4404, GV3111, AGLI, and GV3850) containing the same binary vector were used in the study. These authors also studied the transformation efficiencies of different clones belonging to different species of *Eucalyptus*. Although they were able to obtain transformed tissues from five clones of *E. camaldulensis*, the regeneration of transformed plants was achieved from one clone only. This report also highlighted the differences in transformation efficiencies among clones. Ho et al. (1998) were perhaps the first to report the recovery of transgenic *Eucalyptus* plants using *A. tumefaciens*. Hypocotyl segments obtained from 1-month-old seedlings of *E. camaldulensis* were used as explant materials. The transformation was accomplished by using *A. tumefaciens* (CIB542 derived from EHA101 containing binary vector pBI121). Harcourt et al. (2000) developed the insect- and herbicide-resistant *E. camaldulensis* plants. These authors successfully transformed the seedling explants (cotyledons and hypocotyls) with *cry3A* gene from *Bacillus thruringiensis* and *bar* gene (conferring tolerance to herbicide glufosinate ammonium) using *A. tumefaciens* strain AGL-1. The integration of transgene in the genome of transformed plants was confirmed through PCR amplifications and Southern blot analysis and the expression of the transgene by Western blot analysis. Transgenic plants thus obtained showed resistance toward "chrysomelid beetles" and tolerance toward herbicide glufosinate ammonium.

Chen et al. (2001) were the first to report the production of transgenic *E. camaldulensis* plants carrying cinnamate 4-hydroxylase (C4H) gene from *Populus tremuloides*, a key enzyme involved in lignin biosynthesis. First, C4H gene was cloned in both sense and antisense orientation in binary vector pBI121 which was moved into *A. tumefaciens* strain CIB542 for the transformation of plants. PCR-based analysis of the genomic DNA performed to confirm the integration of the foreign gene. Both Tournier et al. (2003) and Valerio et al. (2003) have reported the transformation of *E. grandis* x *E. urophylla* and *E. camaldulensis*, respectively, with a construct carrying cinnamyl alcohol dehydrogenase (CAD) in antisense orientation. In both studies a strong inhibition of CAD activity was recorded. The CAD is a key enzyme involved in the lignin biosynthesis whose downregulation may result in lower lignin content in wood.

Sonication-assisted *Agrobacterium* transformation (SAAT) system was used for the development of transgenic plants of *E. grandis* × *E. urophylla* hybrid (Gonzalez et al. 2002). The report indicated the higher percentage of transient GUS expression when explants were sonicated for 30 s, and pre-sonication greatly enhanced the transformation efficiency of seedlings. Using this method, four stable transformants were generated and confirmed with Southern blotting. Prakash and Gurumurthi (2009) have reported *A. tumefaciens*-mediated genetic transformation of *E. tereticornis* using cotyledon and hypocotyl as explants. Pre-cultured explants were cocultured with *A. tumefaciens* strain LBA 4404 harboring binary vector pBI121.

Explants after infection were transferred to a selection cum regeneration medium containing 2.2 μM BA, 0.5 μM NAA, 40 mg/l kanamycin, and 300 mg/l cefotaxime.

Aggarwal et al. (2011) developed a procedure for *A. tumefaciens*-mediated T-DNA delivery into the elite clone(s) of *E. tereticornis* using leaf explants from microshoots (Fig. 11.2d–f). This is the only report where various parameters like *Agrobacterium* strain, pre-culturing of tissue, cocultivation period, bacterial density, method of injury, etc. influencing transformation efficiency were investigated. Varied transformation efficiency was reported among the different clones of *E. tereticornis*. Regeneration of transformed shoots was achieved on modified MS medium (potassium nitrate was replaced with 990 mg/l potassium sulfate and ammonium nitrate with 392 mg/l ammonium sulfate, and mesoinositol concentration was increased to 200 mg/l). Stable transformation was confirmed on the basis of GUS activity and PCR amplification of DNA fragments specific to *uidA* and *nptII* genes (Table 11.2).

Torre et al. (2014) have reported the genetic transformation of *E. globulus* with the *EgCCR* promoter instead of CaMV 35S promoter. Cinnamoyl-CoA reductase (CCR), a key enzyme of the lignin biosynthetic pathway, was shown to be preferentially expressed in vascular tissues. The *EgCCR* that transformed plantlets exhibited high GUS expression levels associated with the vascular tissues, opening the possibility of targeting vascular-associated traits such as lignin content or vascular pathogen resistance in adult elite plants of *Eucalyptus*. Recently, for the first time, Aggarwal et al. (2015) have reported the genetic transformation of an elite clone of *E. tereticornis* with Korrigan (*KOR*), an important gene involved in cellulose biosynthesis. Transgenic plants showed overexpression of the *PdeKOR*, and the maximum expression of the gene was observed in the tissues of stem compared to leaves and shoot tips.

The transformation of selected clones will prove to be a powerful tool for trait-specific modification of existing clones with desirable traits, such as insect and herbicide resistance, male sterility, and reduction of lignin content.

11.5 Conclusion

During the past two decades, considerable progress has been made for the improvement of *Eucalyptus* through biotechnological interventions. Optimized protocols for micropropagation have been developed, but unfortunately not used commercially may be due to the higher cost of micropropagation. A lot of progress has also been made toward the development of efficient and reliable shoot regeneration/organogenesis protocols for different species of *Eucalyptus*. Somatic embryogenesis has been achieved for different species of *Eucalyptus* and can be used for commercial scale production of elite clones of *Eucalyptus*. Despite the fact that transgenic technology holds a great promise for the improvement of *Eucalyptus*, still progress is slow which may be due to the recalcitrant nature of the plant, and

Table 11.2 Summary of some of the genetic transformation studies on *Eucalyptus*

Sr. No	Species	Explant used	Method used	Gene(s) transferred if any	Reference
1.	*E. tereticornis*	In vitro leaves	*Agrobacterium tumefaciens* (EHA 105)	Endo-β-1,4-glucanase (Korrigan)	Aggarwal et al. (2015)
2.	*E. globulus*	Shoots with at least two leaves and one axillary/apical bud	Sonication-assisted *A. tumefaciens* mediated (AGL-1)	EgCCR-*E. globulus* cinnamoyl CoA reductase promoter	Torre et al. (2014)
3.	*E. camaldulensis*	Cotyledons, hypocotyls	*A. tumefaciens* (LBA4404)	*UidA*, *nptll*	Ahad et al. (2014)
4.	*E. globulus*	Hypocotyls	*A. tumefaciens* (EHA 105)	Bacterial choline oxidase gene (codA)	Matsunaga et al. (2012)
5.	*E. saligna*	Shoot tip	*A. tumefaciens* (EHA 105)	P5CSF129A mutant gene	Lopes da silva et al. (2011)
6.	*E. tereticornis*	In vitro leaves	*Agrobacterium tumefaciens* (EHA 105)	*UidA* and *nptll*	Aggarwal et al. (2011)
7.	*E. urophylla* × *E. grandis*	Leaves from micro cuttings	*A. tumefaciens* (AGL1)	Egu*CBF*1a and Egu*CBF*1b	Navarro et al. (2011)
8.	*E. saligna*	Cotyledons	*A. tumefaciens* (EHA 105)	P5CSF129A gene	Dibax et al. (2010)
9.	*E. tereticornis*	Cotyledons, hypocotyls	*A. tumefaciens* (LBA4404)	*UidA* and *nptll*	Prakash and Gurumurthi (2009)
10.	*E. globulus*	Stems of a 2-year-old plant	*A. tumefaciens*	Eni TUB1 and Eni FLA1	Taylor et al. (2007)
11.	*E. globulus*	Stem of trees	*A. tumefaciens*	EnTUB1	Spokevicius et al. (2005)
12.	*E. urophylla*	Leaves, petioles, and stem internodes	*A. tumefaciens*	UidA, nptll, ALS	Cheng (2006)
13.	*E. camaldulensis*	Cotyledons, hypocotyls	*A. tumefaciens*	Cbd, cel1	Shani et al. (2003)
14.	*E. camaldulensis*	Cotyledons, hypocotyls	*A. tumefaciens*	*CodA* choline oxidase	Yamada-Watanabe et al. (2003)
15.	*E. camaldulensis*	Cotyledons, hypocotyls	*A. tumefaciens*	CAD antisense	Valerio et al. (2003)

(continued)

Table 11.2 (continued)

Sr. No	Species	Explant used	Method used	Gene(s) transferred if any	Reference
16.	*E. grandis x E. urophylla*	Seedling leaves	*A. tumefaciens* sonication and vacuum infiltration assisted	*CAD* antisense, *UidA*, *nptII*	Tournier et al. (2003)
17.	*E. grandis x E. urophylla*	Seedlings	*A. tumefaciens*, sonication assisted	*Lhcbl2*, *UidA*, *nptII*	Gonzalez et al. (2002)
18.	*E. grandis x E. urophylla*	Cotyledons, hypocotyl calli	Biolistic gun	*UidA*, *nptII*	Sartoretto et al. (2002)
19.	*E. camaldulensis*	Leaves	*A. tumefaciens*	*PtreC4H*, *UidA*, *nptII*	Chen et al. (2001)
20.	*E. camaldulensis*	Cotyledons, hypocotyls	*A. tumefaciens*	*cry3A*, *bar*	Harcourt et al. (2000)
21.	*E. camaldulensis*	Cotyledons, hypocotyls	*A. tumefaciens*	*UidA*	Ho et al. (1998)
22.	*E. grandis x E. urophylla*	Seedlings	*A. tumefaciens*, *A. rhizogenes*	*Ti-nos*, *Ri plasmid*	Machado et al. (1997)
23.	*E. globulus*	Seedlings	*A. tumefaciens*	*UidA*, *nptII*	Moralejo et al. (1998)
24.	*E. globulus*	Zygotic embryo	Biolistic gun	*CAD*	Serrano et al. (1996)
25.	*E. camaldulensis*	Cotyledons, hypocotyls	*A. tumefaciens*	*UidA*, *nptII*	Chen et al. (1996)
26.	*E. globulus*	Zygotic embryo, cotyledons, hypocotyls	Biolistic gun, particle in floe gum	*UidA*	Rochange et al. (1995)
27.	*E. citriodora*	Protoplast	Electroporation	*UidA*	Manders et al. (1992)
28.	*E. gunnii*	Protoplast	Electroporation	*UidA*	Teulieres et al. (1991)

there is a lack of large-scale cultivation of transgenic *Eucalyptus* plants till date. Among the different techniques known for gene transfer, *A. tumefaciens*-mediated genetic transformation has been the most successful. Nevertheless, a good amount of work has been conducted on the development of various in vitro propagation techniques and development of shoot organogenesis and genetic transformation protocols of various *Eucalyptus* species; still they are exploited to their full extent.

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Biotechnology of Tropical Tree Crops

12

Yan Hong, Somika Bhatnagar,
and Smitha Chandrasekharan

12.1 Introduction

12.1.1 Tropical Tree Crops

Tropical tree crops can be defined as trees planted in a tropical region for commercial purpose/scale. A plantation is a densely planted stand of trees, managed to maximize output of specific products – wood (for wood chips, paper manufacturing, furniture, and construction), fruits, nuts, secondary metabolites, biofuels, and services such as carbon sequestration, watershed protection, and holding back desertification. Tropical plantations are found between 23.5° south latitude and 23.5° north latitude at elevations below 1000 m and divided into four major regions (Neotropical, Afrotropical, Indomalayan tropical, and Australian tropical) (Duery and Vlosky 2006):

(a) *The Neotropical* – The Amazon river basin covers 40 % of the South American natural forests. Four-fifths of the Amazon forest is still intact and healthy. Other areas have been subjected to heavy logging and converted into plantations. Main plantation trees are *Swietenia macrophylla* (mahogany), *Hevea brasiliensis* (rubber), *Eucalyptus grandis* (eucalyptus), and *Elaeis oleifera* (American oil palm).
(b) *The Afrotropical* – Most of the tropical rainforests of Africa exist in the Congo (Zaire) river basin. Due to frequent and continuing dry spells, these forests are

Y. Hong (✉)
School of Biological Sciences, Nanyang Technological University,
60 Nanyang Drive, Singapore 637551, Singapore
e-mail: yhong@ntu.edu.sg

S. Bhatnagar • S. Chandrasekharan
Temasek Life Sciences Laboratory, 1 Research Link, Singapore 117604, Singapore
e-mail: somika@tll.org.sg; smitha@tll.org.sg

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fast converting into deserts. Plantation trees commonly found in this region are rubber, *Tectona grandis* (teak), *Acacia mangium* (acacia), *Dalbergia melanoxylon* (African blackwood), *Cola acuminata* (kola tree), *Theobroma cacao* (cocoa), and *Elaeis guineensis* (African oil palm).

(c) *The Indomalayan tropical* – This region includes Indonesia, the Malay Peninsula, India, and China. Southeast Asia's rainforests are some of the oldest in the world, and they have existed for over 100 Ma. There has been uncontrolled deforestation and the virgin forest is reducing at an alarming rate. Main plantation trees are oil palm, mahogany, rubber, acacia, teak, *Dalbergia sissoo* (rosewood), *Gmelina arborea* (gmelina), *Paraserianthes falcataria* (sengon), and *Cocos nucifera* (coconut).

(d) *The Australian tropical* – Most of this rainforest is located in Papua New Guinea and a part in the Northeast of Australia. It is pretty much unaffected and untouched and is slowly expanding. Main plantation trees in this region are eucalyptus, acacia, *Santalum spicatum* (sandalwood), and *Paulownia tomentosa (*princess tree).

12.1.2 Demands for Tropical Tree Crops

With the increase in population, globalization, and economic development, the demand for tree products has been constantly increasing. In the last 50 years, forestry has changed from being a foraging operation to becoming what is increasingly a cropping operation. It was postulated that if the global plantation forest were to increase at 2.4 % per annum between 2010 and 2050, plantation forests could replace natural forests as the source for timber and fiber (WWF and IIASA 2012). Good progress has been made toward that direction. In 2013, 49 % of the world wood production came from plantation forest, and this contribution would increase to 69 % by 2050. Among the tropical subregions, contributions from plantation forest in South America, Central America, and Southeast Asia in 2013 were 77 %, 34 %, and 39 %, and these numbers would increase to 86 %, 63 %, and 62 % by 2050 (d'Annunzio et al. 2015).

12.1.3 Need for Sustainable Plantations

To meet the high demand for tropical tree crops, forest plantations in the tropics are expanding rapidly. Planting fast-growing and short-rotation trees is the first step to accelerate plantations and reduce pressure on natural stands. With the application of biotechnology, the cost of plantation forestry can be lowered and thus provide financial incentive for the industry to continue its shift away from higher-cost natural forestry. This will also help to preserve biodiversity, to protect native species, and to reduce rate of deforestation. Plantations help in sequestering carbon and releasing oxygen. It is equally important to select species which can grow on marginal land so that the agricultural land is not converted into plantations. With the rapidly

changing climate, the trees are also required to withstand harsh environmental conditions such as severe cold, drought, flood, salinity, etc. and grow sustainably without additional pressure on the soil and water resources. Thus economics and environmental concerns are the two major driving forces toward sustainable tree plantation (Sedjo 1999).

12.1.4 Conventional Tree Propagation: Methods and Their Limitations

(a) *Propagation by seeds* – Often the seeds are produced via open pollination; hence, they are heterozygous in nature and do not contain the true-to-type characteristics of mother plants. Seed production is a seasonal process, usually once or twice a year. In many species the seeds remain viable only for few months, and the germination percentage in nursery is often low. Improvement strategy is to select superior trees in the seed orchard and only harvest seeds from these trees. This takes time as trees have long juvenile phase. Most tropical tree species reach the sexual maturity and produce substantial quantities of seeds at around 3–6 years of age.

(b) *Propagation by stem cuttings* – Stem cuttings have been used to propagate true-to-type clones of elite trees, but they have limited success and face the technical challenges of rejuvenation and rooting. Mature tree material is mostly difficult to root or often produces poor-quality roots. Successful rooting depends on environmental factors and physiological state of the stem cuttings. The percentage of the stem cuttings surviving the time period between pruning, transportation, and final transplantation is often low.

(c) *Propagation by grafting* – The success of grafting depends on internal factors like scion/rootstock compatibility, nutrient and water content, activity of the cambium, as well as environmental factors like soil moisture and atmospheric temperature.

(d) *Difficulty in breeding tree species* – Domestication of forest trees based on controlled crosses and field trials started in the 1950s, and many breeding programs are only in their infancy. Breeding efforts have been largely confined to the *Pinus*, *Picea*, and *Eucalyptus* genera. Their speed of reproduction (as long as 20 years for one reproduction cycle for spruce) is one major limiting factor to tree domestication. Controlled pollination is difficult to conduct and sometimes fails to produce seeds. It also requires large parcel of land and other resources to screen for the most desirable plants for multiple years. Hybrids are often sterile and fail to produce seeds, and vegetative propagation techniques are needed for producing enough seedlings for plantation. Growth, disease resistance, stem form, and wood quality have been the major traits improved by tree breeders (Isik et al. 2015).

12.2 Tree Biotechnology: An Overview

Tree biotechnology is about making use of biotechnological and molecular tools for improvement of tree physiology and genetics. It consists of four components: micropropagation from preexisting meristems, regeneration through *de novo* meristems, genetic engineering, and DNA marker-assisted breeding. Micropropagation and regeneration are two tissue culture techniques that have been extensively exploited for the conservation of germplasm (including cryopreservation, synthetic seeds, and genebanks), creation of new varieties (such as somaclonal variants, haploids, mutagenized, and somatic hybrids), and mass propagation of trees like gmelina, acacia, sengon, and mulberry for plantation. Tissue culture is also used for the production of secondary metabolites (e.g., agarofuran in agarwood, *Aquilaria malaccensis*), industrial enzymes, and nutritional and therapeutic compounds (e.g., eugenol in clove, *Syzygium aromaticum*). With the advent of recombinant DNA technology (genetic modification, or GM), it is possible to transfer genes across the species barrier. GM provides precision and allows a wider range of outcomes compared to traditional breeding where selection is based on phenotypic variations within genetically compatible individuals. Recent advances in forest tree molecular biology, including gene discovery; functional characterization through various molecular tools like transcriptomics, epigenomics, proteomics, and metabolomics; and whole-genome sequencing, all have contributed to genetic engineering of tropical tree like rubber. It becomes an important avenue to accelerate the domestication of forest trees with the main advantage of adding commercially important traits to elite clones, exemplified by GM *Jatropha curcas* (jatropha) with high oleic acid, high oil content, and insect tolerance. Other traits under development include pathogen tolerance, herbicide tolerance, enhanced root development, flowering control, lignin modifications, latex improvement, and phytoremediation of environmental pollutants. On the other hand, genetic mapping and association between genetic markers and traits expedite breeding through markers-assisted breeding, which reduces time, space, and other resources in progeny screening. The financial incentives for the utilization of biotechnology in tropical tree crops appear to be strong. When the yields can be increased or/and costs can be reduced, net benefits of tree biotechnology can be achieved.

12.3 Micropropagation

Micropropagation can be defined as an *in vitro* clonal propagation technique of using the preexisting meristems in shoot tips or nodal explants to propagate the mother plants via tissue culture. In tropical timber trees, it is the fastest way to achieve desirable clones of superior trees in large numbers; seedlings are true to type to mother plants and pathogen-free.

Micropropagation can be divided into four stages – establishment, multiplication, rooting, and acclimatization:

(a) *Establishment* – Nodal explants and shoot tips are collected and sterilized with surface-sterilizing reagents, and *in vitro* cultures are established which are free of pathogens. Commonly used surface-sterilizing reagents are ethanol, mercuric chloride, hydrogen peroxide, benomyl, bavistin, carbendazim, PPM (Plant Preservative Mixture), and sodium hypochlorite. In an aseptic environment and following the aseptic techniques, the sterilized explants are cultured on suitable plant growth basal media like Murashige and Skoog (MS) medium (Murashige and Skoog 1962), Woody Plant Medium (WPM) (McCown and Lloyd 1981), and Gamborg B5 medium (B5) (Gamborg et al. 1976). These basal media are supplemented with auxins and cytokinins to promote new shoot formation and propagation either on the same medium or different ones.

(b) *Multiplication* – Shoots obtained from a single explant are separated into individual shoots that can be either cultured in the same propagation medium for continuous production of large number of shoots or cultured on a shoot elongation medium for shoot elongation before transfer onto a rooting medium.

(c) *Rooting* – Seedlings are transferred into the medium supplemented with auxin to promote root formation. In some trees like *Acacia senegal*, of which shoots show low direct root induction (25 %) in the medium, micrografting them on rootstocks of *in vitro* germinated seedlings can be used as an additional step to produce rooted plants (100 %) (Khalafalla and Daffalla 2008).

(d) *Acclimatization* – Rooted plantlets are gradually exposed to the open environment (acclimatization or hardening) before transfer into soil in nursery. It is a very critical step, and the success of survival rate in soil largely depends on how well the plants are acclimatized to physical (light, temperature, humidity, day length), chemical (mineral composition of soil), and biological (soil-microflora) environment. The transition from tissue culture bottles to soil in pots should be performed gradually by exposing the plants to lower humidity and higher temperature and light conditions on a daily basis, over a period of time (7–15 days). During the hardening stage in nursery, the plants can be supplied with antitranspirants (e.g., Folicote), growth retardant (e.g., Paclobutrazol), plant stress hormone (e.g., abscisic acid), fungicides (e.g., Captan), and useful microbes (e.g., bacterial endophytes and mycorrhiza) to increase survival percentage (Chandra et al. 2010).

Micropropagation protocols have been successfully developed for many tropical tree crops (see Table 12.1 for a summary).

Compared with other vegetative propagation methods, micropropagation can produce larger quantities of good-quality seedlings for each propagation cycle (higher propagation ratio), in small areas within a short span of time, irrespective of the weather and season. The micropropagated plants are disease-free, have good rooting system, and show uniform growth. They are true to type and possess all qualities of the selected mother plants. Sometimes *in vitro* propagation is combined with nursery cuttings to further increase propagation ratio and reduce costs.

Table 12.1 Tropical tree crop propagation/regeneration

Scientific name (common name)	Country of origin	Plantations in countries	Uses	Propagation method	References
Acacia mangium (acacia)	Australia, Papua New Guinea, Eastern Indonesia	Malaysia, Indonesia, Bangladesh, Philippines, Puerto Rico, Brazil	Wood, livestock feed	MP, OR, SE	Bon et al. (1998), Beck and Dunlop (2001), Xie and Hong (2001a, b, 2002), Monteuuis et al. (2013), Hong and Bhatanagar (2007), and Bhatnagar and Hong (2008)
Anthocephalus cadamba (jabon)	India, Australia, China, Indonesia, Malaysia, Papua New Guinea, Philippines, Singapore, Vietnam	India, Malaysia, Indonesia (Java), Costa Rica, Puerto Rico, Venezuela, South Africa, Suriname, Taiwan, China	Wood, industrial raw material	MP, SE	Kavitha et al. (2009) and Apurva and Thakur (2009)
Aquilaria agallocha (agarwood)	Northeast Asia	India, Iran, Bangladesh, Bhutan, Laos, Vietnam, Thailand, Malaysia, Indonesia	Industrial raw material	MP	Chung (2015) and Meng-Ling et al. (2005)
Azadirachta indica (neem)	India	India, Australia, Africa	Wood, industrial raw material, livestock feed	MP, OR, SE, AC	Akula et al. (2003), Arora et al. (2010), Biswas and Gupta (2007), Gautam et al. (1993), Houllou et al. (2015), Murthy and Saxena (1998), Rafiq and Dahot (2010), and Salvi et al. (2001)
Calophyllum brasiliense (Brazilian cedar)	Brazil	Central America, South America, Caribbean	Wood, industrial raw material, medicine	MP	Silveira (2014)

(continued)

Table 12.1 (continued)

Scientific name (common name)	Country of origin	Plantations in countries	Uses	Propagation method	References
Carica papaya (papaya)	Mexico, Central America	India, Brazil, Indonesia, Nigeria, Mexico	Fruit	MP, OR, PC, SE	Hossain et al. (1993), Vilasini et al. (2000), Bhattacharya et al. (2003), Anandan et al. (2011), and Chen and Chen (1992)
Cedrela odorata (American cedar)	Central America	Mexico, Argentina	Wood	MP, OR, SE	Peña-Ramírez et al. (2011), García-Gonzáles et al. (2011), Peña-Ramírez et al. (2010), Cameron (2010), and Pérez Flores et al. (2012)
Cinnamomum camphora (camphor)	South East and South central parts of China	Japan, Taiwan, Korea, Indo China, America	Industrial raw material, medicine	MP, OR, PC	Du and Bao (2005), Govinden Soulange et al. (2007), Babu et al. (2003), and Du et al. (2015)
Cinnamomum tamala (Indian bay leaf)	Bhutan, China, Nepal, India	Asia and Australia	Spice	MP, OR	Deb et al. (2013) and Madhabi et al. (2014)
Cinnamomum verum (cinnamon)	Malabar Coast of India, Bangladesh, Sri Lanka, Myanmar	Seychelles, Madagascar, Sri Lanka, India, China, Vietnam	Spice	MP	Govinden Soulange et al. (2007) and Mini et al. (1997)
Cocos nucifera (coconut)	India, Australia	Australia, Sri Lanka, India, Indonesia, Philippines, Maldives, USA, Middle East, Bermuda, Europe	Fruit, industrial raw material	AC, MP, SE	Perera et al. (2007), Verdeil et al. (1999), Chan et al. (1998), and Verdeil et al. (1994)
Coffea spp. (coffee)	Ethiopia	North Central and South America, Caribbean, Africa, Middle East, India, Indonesia, Vietnam	Industrial raw material	AC, SE	Etienne (2005), Berthouly and Etienne (1999), van Boxtel and Berthouly (1996), Gatica-Arias et al. (2008), Neuenschwander and Baumann (1992), and Ascanio and Arcía (1987)

(continued)

Table 12.1 (continued)

Scientific name (common name)	Country of origin	Plantations in countries	Uses	Propagation method	References
Cola spp. (kola tree)	West Africa	Jamaica, Brazil, Hawaii, Malaysia, India	Industrial raw material	MP	Fotso et al. (2002) and Dossa et al. (1994)
Cordia alliodora (timber of laurel)	Mexico	Central and South America, South Brazil, Mexico, North Argentina	Wood	OR	Londoño-Giraldo and Gutiérrez-López (2013)
Dalbergia melanoxylon (African blackwood)	Africa	Tanzania-Africa	Wood	MP	Kiondo et al. (2014) and Washa and Nyomora (2014)
Dalbergia sisoo (Indian rosewood)	India	India	Wood, livestock feed	MP, OR, SE	Vibha et al. (2014), Sahu et al. (2014), Ali et al. (2012), Chand and Singh (2004), Singh and Chand (2003), Pattnaik et al. (2000), and Pradhan et al. (1998)
Elaeis guineensis (oil palm)	Africa-Angola and Gambia	Malaysia, Indonesia, Africa	Industrial raw material	MP, OR, SE	Wooi (1990), Teixeira et al. (1993), Karun and Sajini (1996), Scherwinski-Pereira et al. (2010), Marbun et al. (2015), and Muniran et al. (2008)
Eucalyptus grandis (eucalyptus)	Australia	Throughout the tropical regions of the world	Wood, industrial raw material, medicine, livestock feed	MP, OR, PC, SE	MacRae and Van Staden (1990), Penchel and Kirby (1990), Warrag et al. (1990), Watt et al. (1991, 1999), Cid et al. (1999), Qiu et al. (2009), and Mycock and Watt (2012)

(continued)

Table 12.1 (continued)

Scientific name (common name)	Country of origin	Plantations in countries	Uses	Propagation method	References
Gmelina arborea (white teak)	India, Myanmar, Thailand, Laos, Cambodia, Vietnam,	India, Malaysia, Indonesia, Myanmar, Thailand, Laos, Cambodia, Vietnam, Philippines, Solomon Islands, Sierra Leone, Nigeria, Costa Rica, Brazil	Wood livestock feed	MP, OR, SE	Bhatnagar and Hong 2008, Kannan and Jasrai (1996), Naik et al. (2003), Valverde-Cerdas et al. (2004), Nakamura (2006), Behera et al. (2008), Sukartiningsih. et al. (2012), and Madke et al. (2014)
Hevea brasiliensis (rubber)	Amazon rainforest Bolivia, Brazil, Colombia, Peru, Venezuela	Brunei, Cambodia, China, Ethiopia, India, Indonesia, Laos, Liberia, Malaysia, Myanmar, Philippines, Singapore, Sri Lanka, Thailand, Uganda, Vietnam	Wood, industrial raw material	OR, SE, MP, AC	Cailloux et al. (1996), Carron et al. (1995), Hua et al. (2010), Ighere et al. (2011), Sirisom and Te-chato (2013), Sushamakumari et al. (1999), Veisseire et al. (1994), and Venkatachalam et al. (2007)
Jatropha curcas (jatropha)	Central America	All tropical and subtropical countries	Industrial raw material	MP, OR, SE	Sujatha and Mukta (1996), Sujatha et al. (2005), Datta et al. (2007), Purkayastha et al. (2010), Singh et al. (2010), and Nunes et al. (2013)

(continued)

Table 12.1 (continued)

Scientific name (common name)	Country of origin	Plantations in countries	Uses	Propagation method	References
Laurus nobilis (bay laurel)	Mediterranean Basin and Portugal	Europe and East Asia	Spice	SE, MP	Canhoto et al. (1999) and Chourfi et al. (2014)
Mangifera indica (mango)	India, Philippines	India, Pakistan, Africa, Brazil, Australia	Wood fruit	MP, SE, PC	Krishna and Singh (2007), Xiao et al. (2004), Pateña et al. (2002), and Ara et al. (2000)
Morus indica (mulberry)	China	Southern Europe, Middle East, Northern Africa, Indian subcontinent	Industrial raw material	MP, PC	Tewari et al. (1999), Bhatnagar et al. (2001, 2002), Kapur et al. (2003), Bhatnagar et al. (2004), Umate (2010), and Mehbooba Zaki (2011)
Musa spp. (banana)	India, South East Asia, Australia	All tropical countries	Fruit	MP, SE, PC, OR	Panis et al. (1993), Escalant et al. (1994), Assani et al. (2001), Khalil et al. (2002), Dahot (2007), Xiao et al. (2007), and Remakanthan et al. (2014)
Paraserianthes falcataria (sengon)	Moluccas, New Guinea	Fiji, Philippines, East Java, Indonesia, India, Australia, parts of America	Timber	MP	Bhatnagar and Hong (2008), Bon et al. (1998), Chujo et al. (2010), Hong and Bhatanagar (2007), Ravindran (1998), and Sasmitamihardja et al. (2005)

(continued)

Table 12.1 (continued)

Scientific name (common name)	Country of origin	Plantations in countries	Uses	Propagation method	References
Persea americana (avocado)	Mexico, Central America	Morocco, South Africa, Spain, Portugal, Colombia, Peru, India, Sri Lanka, Indonesia, Australia, New Zealand, USA, Ecuador, Rwanda	Fruit, industrial raw material	MP, SE	Barceló-Muñoz et al. (1999), Witjaksono et al. (1999), Perán-Quesada et al. (2004), Márquez-Martín et al. (2011), and Encina et al. (2014)
Paulownia fortunei (paulownia)	China, Laos, Vietnam	China, Korea, Japan, Australia	Wood, industrial raw material, livestock fodder	MP	Bergmann (1998), Bergmann and Whetten (1998), Venkateswarlu et al. (2001), Yadav et al. (2013), Clapa et al. (2014), and Shtereva et al. (2014)
Pongamia pinnata (Indian beech tree)	India	India, Myanmar	Industrial raw material	MP, OP, SE	Sugla et al. (2007), Sujatha and Hazra (2007), Shrivastava and Kant (2010), Sujatha (2011), Kesari et al. (2012), and Mahmood (2013)
Pinus merkusii (Sumatran pine)	Sumatra (Indonesia)	Indonesia, Malaysia, Philippines	Wood, industrial raw material	MP	Noerhadi and Wirjodarmodjo (1980)
Populus mexicana, *Populus euphratica* (poplar)	Mexico	Southwest Asia, Mexico	Wood	AC, OR, PC, SE	Michler and Bauer (1991), Baldursson et al. (1993), Gu et al. (1999), Ferreira et al. (2009), Li et al. (2013), and Chupeau et al. (1994)

(continued)

Table 12.1 (continued)

Scientific name (common name)	Country of origin	Plantations in countries	Uses	Propagation method	References
Samanea saman (rain tree)	South America and Central America	Southeast Asia	Wood, livestock feed	MP	Kasthurirengan et al. (2013)
Santalum album, *Santalum spicatum* (sandalwood)	India, Australia	India, Nepal, Bangladesh, Sri Lanka, Australia, Indonesia, and the Pacific Islands	Industrial raw material, medicine	MP, SE	Sankara Rao et al. (1996), Rugkhla and Jones (1998), Das et al. (2001), Sanjaya et al. (2006), Bele et al. (2012), and Singh et al. (2013a)
Shorea roxburghii, *Shorea robusta* (meranti)	Southeast Asia	Cambodia, Laos, Malaysia, Thailand, India, Myanmar, Vietnam	Wood	OR, MP	Scott et al. (1988), Nakamura (2006), and Singh et al. (2014)
Swietenia macrophylla (mahogany)	Tropical America	Fiji, Philippines, Africa	Wood	MP, SE	Astorga et al. (1996), Maruyama and Ishii (1999), Maruyama (2006), de Souza et al. (2007), and Pérez Flores et al. (2012)
Syzygium aromaticum (clove)	Maluku Islands in Indonesia	Indonesia, India	Spice	SE, MP, OR	Mathew and Hariharan (1990), Rema et al. (1997), and Nirmal Babu et al. (2015)
Tamarindus indica (tamarind)	Africa, India	Indonesia, Malaysia, Philippines, Myanmar, the Pacific Islands	Wood industrial raw material	OR, MP	Kopp and Nataraja (1990), Jaiwal and Gulati (1991), Sonia et al. (1998), and Mehta et al. (2000)

(continued)

Table 12.1 (continued)

Scientific name (common name)	Country of origin	Plantations in countries	Uses	Propagation method	References
Taxus spp. (yew tree)	Western, Central, and Southern Europe, Northwest Africa, Northern Iran, Southwest Asia	China, USA	Medicine	OR, MP	Chee (1994), Majada et al. (2000), Datta et al. (2006), Ewald (2007), and Abbasin et al. (2010)
Tectona grandis (teak)	Originated from Costa Rica, Indonesia	India, Indonesia, Myanmar, Philippines	Wood	MP, OR, SE	Baghel et al. (2008), De Gyves et al. (2007), Gangopadhyay et al. (2003), Kozgar and Shahzad (2012), Kushalkar and Sharon (1996), Sunitibala Devi et al. (1994), and Widiyanto et al. (2001)
Theobroma cacao (cocoa)	South America	Ivory Coast, Ghana, Indonesia, Nigeria, Brazil, Cameroon	Industrial raw material	MP, SE	Esan (1992), Li et al. (1998), Silva and Debergh (2001), Quainoo and Dwomo (2012), Traore et al. (2003), and Florez et al. (2015)

AC Anther culture, *OR* Organogenesis, *MP* Micropropagation, *PC* Protoplast cultures, *SE* Somatic embryogenesis

12.3.1 Selection Elite Plants for Propagation

(a) *Species selection* – The very critical first step for micropropagation is to select elite material to clone. Firstly, a tree species suitable for a particular climate and soil conditions is chosen. For wood harvest, fast-growing tree species with straight trunks, sparse thin branches, and good-quality wood are preferred. For fruits, trees producing higher number and larger size fruits at a younger age are desirable.
(b) *Accession evaluation* – Typically, a small number of trees of different accessions or progeny population derived from elite mother trees are planted in the target region for evaluation of suitability and matching with local environment, also providing mother plants for propagation. Accessions from as wide as possible sources are planted in the target region. Observation and evaluation are conducted over a period of time. Certain plot design like random block design is usually practiced to ensure a fair and nonbiased evaluation. For each trial, plants of different entries are grown under the same environmental and soil conditions and subjected to the same silviculture and plantation management practices.
(c) *Selection of best trees* – Elite trees are selected from these plantations or seed orchards based on their phenotypical traits such as growth rate, yield, quality, tolerance to abiotic stress, or resistance to pathogen attacks. These trees are selected as "mother plants" for propagation.

12.3.2 Technical Challenges in Micropropagation

In vitro shoot culture is greatly influenced by many internal and external factors. Optimal protocol is often plant species specific, even genotype specific.

(a) *Explant* – Apical shoots and axillary buds respond differently to *in vitro* culturing. Typically, apical shoot is preferred due to its younger age and ease of rooting, while axillary buds are preferred when a large number of explants are required to start the culture. In some tree species, axillary buds obtained from mature trees need to be rejuvenated in tissue culture before using them for multiplication purposes. For example, in acacia, the mature buds give rise to shoots with phyllodes. Only after a few rounds of subculture, shoots with pinnate leaves are obtained; this is an indication of rejuvenation.
(b) *Sterilization* – Nodal segments taken from mature trees are generally difficult to sterilize with high rate of contamination. The fresh growths and young shoots respond better to sterilization procedures. There is also seasonal variation in the percentage of contamination. Axillary buds collected in winter months are in dormant stage and have less chances of contamination. Explants collected during hot and humid weather have a flourishing microflora on them which is difficult to remove during sterilization procedure.

(c) *Media and hormone* – Optimization of the nutrient and hormone levels in the media is of the highest importance in developing a tissue culture protocol. The media composition makes significant differences in culture responses, including the percentage of explants producing shoots, growth of axillary shoots, rooting efficiency, and number and length of roots.

12.3.3 Research and Development Activities at Temasek Life Sciences Laboratory (www.tll.org.sg) and Bioforest (www.bio-forest.com) in Singapore

For the past few years, we have conducted research and development activities toward commercial production of eight important tropical tree crops, namely, gmelina, acacia, sengon, agarwood, clove, rubber, mulberry, and jatropha:

(a) *Gmelina* – Commonly named white teak is valued for good-quality wood for furniture, frames, and plywood industry. It is a popular choice for plantations across the tropics. A cost-effective and time-efficient propagation protocol from nodal explants of elite trees was developed. The micropropagation system leverages on the reuse of mother plant (up to six times over a period of 1 year). One *in vitro* established mother plant can produce 1,000,000 plants in 1 year. The plantlets obtained readily acclimatize with over 95 % success rate when transferred to nursery. These nursery plants can be further multiplied via a special stem-cutting technique into up to 16 plants within 4 months. Two- to three-month-old seedlings from nursery can be planted out in an open field with >95 % survival rate. At 5 years of age, the tissue culture trees of elite mother plants showed uniform and higher growth (in terms for height and diameter) than the seed-grown trees of same age and under the same soil and climate conditions. Similar protocols were developed for mass propagation of two highly sought-after tropical timber trees, sengon (for plywood) and acacia (for pulp and paper) (Bhatnagar and Hong 2008; Hong and Bhatanagar 2007).
(b) *Clove* – Dry buds are used as a whole spice, and its oil is widely popular as an essential oil with analgesic, antioxidant, and anti-inflammatory properties. Kretek, a widely smoked form of cigarettes in Indonesia is a blend of tobacco with clove buds. We have cloned and multiplied elite trees with desirable traits for plantations. Research is in progress on production of aromatic terpenes in tissue culture of clove (unpublished data).
(c) *Rubber* – It is of major economic importance because milky latex extracted from its tree trunk is the primary source of natural rubber. It is generally recalcitrant to micropropagation owning to high amount of latex content. We developed a special formulation with additives in hormonal media to overcome the bottle neck of latex exudes in the tissue culture and now can micropropagate rubber plants on a routine basis (unpublished data).

12.4 Regeneration

12.4.1 Organogenesis

Regeneration refers to the formation of new organs through *de novo* formed meristems. *In vitro* propagation by organogenesis involves induction of adventitious meristems with or without formation of callus from various somatic explants such as leaves, anthers, cotyledons, hypocotyls, ovaries, stem/internodes, and root segments. Many woody plant species are recalcitrant to adventitious generation with the formation of callus (McCown et al. 1991). For the purpose of propagation, direct shoot organogenesis is preferred than regeneration through callus because of less chances of somaclonal variation and genetic instability than propagation via callus. It is achieved by exposure to a pulse of higher concentration of cytokinin that promotes cell division. Direct shoot organogenesis is successful in many trees like *Leucaena leucocephala* (Sirisha et al. 2008), *Acacia mangium* (Shahinozzaman et al. 2013), *Pongamia pinnata*, and *Citrus jambhiri* (Saini et al. 2010). On the other hand, callus-mediated organogenesis is the preferred method for genetic transformation due to amenability to *Agrobacterium* infection and the ability to regenerate single cell-derived transgenic plants. The developmental fate of cultured callus cells to regenerate shoots and corresponding roots is highly dependent on the concentration and ratio of auxins and cytokinins present in the media. Among the various hormones, thidiazuron (TDZ) stands out as a potent growth regulator especially for tropical tree crops. TDZ has been proven to mimic both auxin and cytokinin responses. Low concentration of TDZ enhances micropropagation, and higher concentrations induce shoot organogenesis and somatic embryogenesis. It also helps in the hardening of transgenic plants. Application of TDZ has resulted in turning many woody plants more amenable to propagation and regeneration (Khurana et al. 2005). High activity and stability coupled with requirement in low concentration and for short-duration application makes TDZ the most potent growth regulator for woody plant tissue cultures.

Protocols for regeneration via organogenesis have been successfully developed for many tropical tree crops (see Table 12.1).

Agarwood – The wood of the Gods is highly priced and very unique, as upon microbial infection the tree produces a fragrant and resinous wood called as agarwood. Besides wood, the oil extracted from this wood is also very valuable in fragrance, cosmetic, and pharmaceutical industry. However, indiscriminate and excessive harvest of this highly sought-after tree has led to its listing as a critically endangered species under CITES. We have developed protocols of micropropagation (using shoot tips and nodal explants) and regeneration via direct organogenesis (using cotyledon and leaf explants) for three species: *A. crassna* (popular in Thailand, Laos, Cambodia, Australia, and Vietnam), *A. malaccensis* (Indonesia, Malaysia, and India), and *A. sinensis* (China). The protocols are very efficient and ready for large-scale commercial propagation of agarwood trees. Agarwood tree

plantation will not only save the endangered tree but also provide sustainable source for agarwood-derived high-valued products (unpublished data).

12.4.2 Somatic Embryogenesis

Somatic embryogenesis is an asexual form of plant propagation where an embryo or plant is derived from a single somatic cell. The potential of species/genotypes to form somatic embryos mainly depends on the genetic component. The developmental and physiological stage of the explant defines embryogenic competence at the cellular level (Fehér 2006). There are typically four stages of somatic embryogenesis:

(a) *Initiation* – *In vitro* germinated seed is the preferred source of zygotic embryo or cotyledon/hypocotyl explants. These explants are cultured on a nutritive medium rich in auxins for initiation of embryogenic callus, which later gives rise to meristematic cells.
(b) *Proliferation* – Upon periodical subcultures, the embryos grow prolifically. Globular, heart, and torpedo stages are seen progressively.
(c) *Maturation* – Cotyledonary somatic embryos appear in this stage. They are bipolar and not attached to the explant by vascular tissue.
(d) *Germination* – The somatic embryos germinate to develop root and shoot. At this stage they can also be preserved as artificial seeds.

The epidermal single-cell origin of somatic embryos makes somatic embryogenesis a good cloning technique, where an unlimited number of identical embryos can be produced for mass propagation. It also makes somatic embryogenesis suitable for genetic transformation.

There are several reports on regeneration of tropical tree crops using somatic embryogenesis (see Table 12.1 for a summary).

However application of somatic embryogenesis on industrial scale has been restricted by genetic effect (many species fail in obtaining embryogenic tissue), somaclonal variation, lack of reproducibility and lower efficiency of initial embryo production.

(a) *Rubber* – Four decades of work on rubber propagation at CIRAD, France, has resulted in the production of true-to-type rejuvenated plants via primary somatic embryogenesis technology. To reduce somaclonal variation due to long periods of maintenance and storage, an alternative system using a combination of embryogenic callus cryopreservation and indirect secondary somatic embryogenesis has been developed. It reduces the number of proliferation cycles and the length of time calli are exposed to hormones. *In vitro* regenerated plants of 11 rubber tree clones have been established in the rejuvenated budwood gardens, and trials are in progress on an 80 ha trial plot with Michelin (Montoro et al. 2012).

(b) *Oil palm* – The tree is the most important oil-producing crop in the world due to its highest productivity compared with other oil crops. Somatic embryogenesis was induced from meristematic tissues from six *in vitro*-grown clones of field-proven elite trees. Commercial-scale production was achieved through secondary somatic embryogenesis. The use of mixer to separate cells from embryogenic mass allowed the cell suspension culture system in bioreactor. Mass production of plantlets from the selected lines of cell cultures was achieved within 6 months by using the above system. Field test was conducted to verify the relationship between *in vitro* abnormalities and *ex vitro* growth (Kang et al. 2010). ASD's (Agricultural Services and Development, Costa Rica) tissue culture lab has produced about three million plantlets of 100 lines.

(c) *Coffee* – Since 1970s, various institutes have researched and reported somatic embryogenesis in coffee. Two technical innovations – mass production of somatic embryos in temporary immersion bioreactors and sowing them directly in nursery – made the technology economically feasible. The production capacity of *Coffea arabica* somatic embryos at ECOM plantation Central America reached around five million plants per year in 2011. It takes a total of 17 months in the lab and 8 months in nursery, before the plants can reach the field (Etienne et al. 2011). Using a similar technology at Nestle, millions of somatic embryos of *Coffea camephora* have been produced and planted in Thailand (Ducos et al. 2011).

12.4.3 Other Methods

(a) *Haploid production* – Haploids are plants with a haploid chromosome number. Of the three *in vitro* methods of haploid production (gynogenesis, androgenesis, and bulbosum method), androgenesis is considered the most efficient technique because of the presence of thousands of haploid cells per anther, as compared to gynogenesis where, at the most, seven cells per ovule are available. The last bulbosum method is applicable to a few cereals only. Homozygosity can be easily achieved through haploids in a single step by diploidization with colchicine, and this is especially useful for the highly heterozygous species. The applications of haploid production include development of homozygous diploid plants which can be high-yielding lines, chemical-induced pure elite mutant inbred lines, and transgenic line obtained via microinjection in pollens (Mishra et al. 2010). Haploids are of great importance in tree species where breeding is usually complicated and time consuming due to their long reproductive cycle, complex reproductive biology, and high degree of heterozygosity. Tree species with haploid and double-haploid plants developed include *Citrus*, *Malus domestica*, *Pyrus communis*, *Pyrus pyrifolia*, *Prunus persica*, *Prunus avium*, *Prunus domestica*, *Prunus armeniaca*, *Olea europaea*, mulberry, papaya, *Annona squamosa*, *Feijoa sellowiana*, *Opuntia ficus-indica*, and *Eriobotrya japonica* (Germanà 2009).

(b) *Triploid production* – In diploid plants, the endosperm is a triploid (i.e., having three sets of chromosomes) tissue as a result of double fertilization. *In vitro* culture of endosperm has been used for the production of triploids. Triploids are sterile and thus carry no risk of gene flow. Many of the triploid plants are found to be superior to their diploid counterparts in terms of yield and size of fruits. Triploids are useful in cases where seedless is desirable like in banana, apple, citrus, and papaya. Triploid plants may prove to be more useful for timber trees as generally they have more vigorous vegetative growth in comparison to their diploid counterparts. The first direct shoot formation from cultured mature endosperm was demonstrated by Johri and Bhojwani in *Exocarpos cupressiformis* (Johri and Bhojwani 1965). This led the way for more studies on endosperm-derived plantlets in several systems including *Pyrus communis*, coffee (Raghuramulu 1989), *Citrus* spp. (Gmitter et al. 1990), *Acacia nilotica* (Garg et al. 1996), *Mallotus philippensis* (Sehgal and Abbas 1996), mulberry (Thomas et al. 2000) and *Azadirachta indica* (Chaturvedi et al. 2003).

(c) *Somatic hybrids* – Somatic hybridization or transformation by manipulating protoplast holds potential for crop improvement. However, plant regeneration from protoplast is usually difficult but a prerequisite for the utilization of protoplast technology. Success in regeneration via protoplast culture was achieved for mulberry (Wei et al. 1994). Chupeau et al. (1994) reported recovery of transgenic trees after electroporation of poplar protoplast with pABD1, carrying the *nptII* gene for resistance to neomycin; pGH1, carrying a mutant acetolactate synthase gene, *als*, for resistance to sulfonylurea; and pGSFR781A, carrying a synthetic phosphinothricin acetyltransferase (*pat*) for resistance to phosphinothricin (Basta).

12.5 Tissue Culture for Production of Secondary Metabolites

In trees, secondary metabolites are accumulated at a certain age or maturity of the plant. They are produced in specialized differentiated tissue like resin ducts, laticifers, or secondary xylem. They accumulate at a slow rate and it is difficult to increase their production within tree. Many compounds are difficult to produce through chemical synthesis due to complex structure and high cost. Thus plant tissue culture offers an alternative method for production of secondary metabolites such as terpenes and flavonoids independent of climate, soil, and tree growth conditions. Suspension cell, callus, shoot, and hairy root cultures have been tried for production of bioactive compounds. Production of the anticancer compound paclitaxel via suspension cell culture is the most successful example. The largest player, Phyton (www.phytonbiotech.com), has the capacity of producing 500 kg annually. The company uses a unique plant cell fermentation technology to produce paclitaxel with suspension cell cultures of *Taxus baccata* (European yew). Some other examples are production of caffeine in suspension cultures of *Coffea dewevrei* (Sartor and Mazzafera 2000), anthraquinones in *Morinda citrifolia* (Deshmukh et al. 2011), azadirachtin-related limonoids in neem (Rafiq and Dahot 2010), agarwood

fragrance-related compounds in agarwood, and eugenol production in clove (unpublished data). The many plants tissue culture systems for secondary metabolite production were reviewed by Linden (2006). However, plant tissue culture systems face completion from *E. coli*, yeast, and endophytic fungal systems in production of useful compounds.

12.6 Cryopreservation

Cryopreservation is a method of long-term storage of plant cells or tissue in liquid nitrogen (−196 °C), keeping it viable, with no metabolic deterioration and no cell division, free of germs, and revivable upon thawing. A typical cryopreservation cycle consists of stages of selection of material, preparation of material, freezing, storage in liquid nitrogen, thawing, washing, reculturing, and finally regeneration of plantlets. Commonly used protocols include controlled rate cooling, vitrification, and encapsulation-dehydration, which vary mostly in preparation of material and freezing steps (Reed 2008). Cryopreservation requires less space and less labor than maintaining plants via tissue culture methods (Kaczmarczyk et al. 2012). However, each step in cryopreservation protocol has the potential to induce stress, and there is especially the risk of damage due to ice formation. The restoration of tissue function is also critical to successful cryoconservation. For trees of which seeds are not produced in sufficient numbers or are viable for a short period of time, shoot tips and axillary buds are the preferred material for cryopreservation as they contain the preexisting meristem which can directly develop into shoot upon rewarming. Other *in vitro* plant tissue for cryopreservation includes cell suspension, embryogenic callus, pollen, embryo axe, and somatic embryo. Some of the trees cryopreserved using vitrification method are *Artocarpus heterophyllus* (jackfruit, embryonic axis), citrus (embryonic axis, shoot tip, somatic embryo, callus), poplar (shoot tip), *Prunus* spp. (shoot tip) (Sakai and Engelmann 2007), and rubber (anther culture) (Zhou et al. 2014). Pritchard et al. (2014) summarized innovative approaches to the preservation of forest trees combining both *in situ* and *ex situ* approaches.

Another important method for cryopreservation is the encapsulation-dehydration method. Explants such as shoot tips and somatic embryos are encapsulated in alginate beads (containing mineral salts, organics, and sugar) to form synthetic seeds or artificial seeds. After revival, these can be used for direct germination. Artificial seeds have been developed for many tropical tree crops like acacia hybrids (axillary buds), papaya (somatic embryo), *Cedrela odorata* (shoot tip), *Citrus reticulate* (somatic embryo), coffee (shoot bud), oil palm (somatic embryo), *Eucalyptus citriodora* (somatic embryo), *Malus* species (various explants), *Mangifera indica* (somatic embryo), mulberry (axillary buds), sandalwood (somatic embryo), and cocoa (somatic embryo) with germination rate in soil ranging from 50 % to 100 % (Reddy et al. 2012). Artificial seeds stay viable for months at 4 °C.

Cryopreserved germplasm collections are stored at various cryobanks like IRD, France (oil palm); the National Bureau of Plant Genetic Resources (NBPGR), India (citrus, jackfruit, *Prunus amygdalus*, mango, banana, neem, mulberry, etc.); and the

National Citrus Repository, China (citrus), for conservation and future propagation.

12.7 Companies Involved in Tropical Tree Crop Biotechnology

The following is a non-exhaustive list of industry players in the area of tree crop plant biotechnology. All provide elite tissue culture clones of one or a few plantation tree species. Some companies liaise with plant tissue culture research institutes for assistance in developing protocols, while some emerged as spin-offs from public research institutes. Some even move into plantation by their own (Table 12.2).

12.8 Genomics

Advancements in the molecular biology of trees such as gene discovery, transcript profiling, genome sequencing, and genetic mapping have led to an increase in number of tree species being genetically engineered. Whole-genome sequencing allows better understanding of species-specific biology, provides clues on evolutionary events, identifies key genes associated with traits of interest, and generates other valuable genomic resources for the improvement of a tree species.

12.8.1 An Update of Tree Genome Sequencing and Gene Discovery

(a) *Poplar* – Plant genetics has gone through a revolution during the last 15 years. Started with the complete sequencing of *Arabidopsis* and rice, poplar (*Populus trichocarpa*) was the first tree whose genome has been sequenced completely (Tuskan et al. 2006) through integration of shotgun sequencing with genetic mapping. Poplar is taken as a model tree due to its fast growth, easy vegetative propagation, interspecific hybridization, amenability to tissue culture and genetic transformation, and a small genome (~500 Mb). The genome was originally sequenced to a coverage of 7.5× using shotgun Sanger sequencing. Based on the most recent version of the poplar genome (*Popular trichocarpa* v3.0 available on Phytozome), the main genome assembly is approximately 422.9 Mb arranged in 1446 scaffolds, Scaffold N50 (L50) = 19.5 Mb and Contig N50 (L50) = 552.8 Kb. 181 scaffolds are >50 Kb in size, representing approximately 97.3 % of the genome. There are 41,335 loci containing 73,013 protein-coding transcripts, a higher number than that for *Arabidopsis*. Most genes encoding biosynthesis enzymes for wood components (cellulose, xylan, glucomannan, and lignin) have been identified in poplar, and a few have been functionally characterized. Many tree crops had their genome sequenced after that. The key success factor was the use of next-generation sequencing (NGS) tech-

Table 12.2 Companies involved in tropical tree crop biotechnology

Tree species	Commercial tissue culture/plantation company	Location	Plantation regions/export countries	Webpage
Acacia mangium	Aditya Biotech	India	India	http://adityabiotech.tradeindia.com/
	Bioforest Pte Ltd	Singapore, Indonesia	Indonesia	http://www.bio-forest.com
	Clonal Solutions Australia Pty. Ltd	Australia	Australia	http://www.clonal-solutions.com.au
	Samling	Malaysia	New Zealand Malaysia	http://www.samling.com/itp.php
Aquilaria spp.	Asia Plantation Capital	Singapore	Thailand, Malaysia	https://www.asiaplantationcapital.com/
	Wescorp Agarwood	Australia	Australia	http://www.wescorp.com.au/agarwood.htm
Azadirachta indica	Aditya Biotech	India	India	http://adityabiotech.tradeindia.com/
	Iribov SBW	Netherlands		http://www.iribov.com
Coffea robusta	Nestle R & D Centre Tours	France	Thailand	http://www.nestle.com/randd/environmental-sustainability/plant-science
Elaeis guinensis	New Britain Oil Palm Oil Ltd	Singapore	Papua New Guinea, Solomon Islands	http://www.nbpol.com.pg/
	Glenealy Plantations SDN Bhd	Malaysia	Malaysia, Indonesia, Papua New Guinea	http://www.glenealy.com.my/
	Agricultural Services and Development Costa Rica	Costa Rica	Costa Rica, Ecuador, Nicaragua, Guatemala, Colombia, Thailand	http://www.asd-cr.com
	TSH Resources Berhad	Malaysia	Malaysia	http://www.tsh.com.my/

Eucalyptus grandis	Arborgen	USA, Brazil, Australia	USA, Brazil	http://www.arborgen.us/
	Birla Lao Pulp and Plantations Company Ltd	Lao	Laos	http://www.adityabirla.com/businesses/Profile/Birla-Lao-Pulp
	Clonal Solutions Australia Pty. Ltd	Australia	Australia	http://www.clonal-solutions.com.au
	Iribov SBW	Netherlands		http://www.iribov.com
	Galiltec Beyond Technologies	Honduras	Honduras, Guatemala, USA	http://www.galiltecbeyondtechnologies.com/
	Samling	Malaysia	New Zealand, Malaysia	http://www.samling.com/itp.php
	The Forest Company	Guernsey	Brazil, Columbia	http://theforestcompany.se/about-us
Gmelina arborea	Bioforest Pte Ltd	Singapore, Indonesia	Indonesia	http://www.bio-forest.com
	Samling	Malaysia	New Zealand, Malaysia	http://www.samling.com/itp.php
Jatropha curcas	Aditya Biotech	India	India	http://adityabiotech.tradeindia.com/
	Iribov SBW	Netherlands		http://www.iribov.com
	Joil Pte Ltd	Singapore	India, Indonesia, Africa	http://www.joil.com.sg
Khaya senegalensis	Clonal Solutions Australia Pty. Ltd	Australia		http://www.clonal-solutions.com.au
Musa spp.	Aditya Biotech	India	India	http://adityabiotech.tradeindia.com/
	Galiltec Beyond Technologies	Honduras	Honduras, Guatemala, USA	http://www.galiltecbeyondtechnologies.com/
	Clonal Solutions Australia Pty. Ltd	Australia		http://www.clonal-solutions.com.au

(continued)

Table 12.2 (continued)

Tree species	Commercial tissue culture/plantation company	Location	Plantation regions/export countries	Webpage
Paulownia spp.	Environmental Technologies Foundation Inc.	USA	SE Asia, S. America, Africa, USA	http://www.etf-inc.com
Paraserianthes falcataria	Bioforest Pte. Ltd	Singapore, Indonesia	Indonesia	http://www.bio-forest.com
	Samling	Malaysia	New Zealand, Malaysia	http://www.samling.com/itp.php
Pongamia pinnata	BioEnergy Plantations	Australia	Australia	http://www.ysgbiotech.com/
	Clonal Solutions Australia Pty. Ltd	Australia	Australia	http://www.clonal-solutions.com.au
Santalum album	Wescorp Sandalwood Pty Ltd	Australia	Australia	http://www.wescorp.com.au/sandalwood.htm
	Aditya Biotech	India	India	http://adityabiotech.tradeindia.com/
	AGBiotek	India	India	http://www.agbiotek.com
	Clonal Solutions Australia Pty. Ltd	Australia	Australia	http://www.clonal-solutions.com.au
Tectona grandis	Galiltec Beyond Technologies	Honduras	Honduras, Guatemala, USA	http://www.galiltecbeyondtechnologies.com/
	Clonal Solutions Australia Pty. Ltd	Australia	Australia	http://www.clonal-solutions.com.au
	Iribov SBW	Netherlands		http://www.iribov.com
	YSG (Yayasan Sabah Group) Biotech Sdn Bhd	Tawau, Sabah, Malaysia	Malaysia, Australia, Brazil, South America, Africa	http://www.ysgbiotech.com/
Taxus spp.	Iribov SBW	Netherlands		http://www.iribov.com
	Yew Bio-Pharm Group	China	China	http://www.yewbiopharm.com/operations/yew-trees/

nologies (Metzker 2010), which rapidly generates massive amount of sequencing data at a fraction of the cost for the rice sequencing project that was accomplished with earlier technologies.

(b) *Amborella* – *Amborella trichopoda* holds great importance as this tropical tree endemic to New Caledonia (South Pacific) is the oldest flowering species on Earth. The *Amborella* genome sequencing was accomplished in 2013 by using NGS, fluorescence *in situ* hybridization, and whole-genome mapping (Project 2013). It provides evidence for the evolutionary processes that paved the way for more than 300,000 flowering plant species. An ancient genome duplication predating angiosperm diversification was identified. There were also new gene families, gene duplications, and floral protein-protein interactions first appeared in the ancestral angiosperm. Transposable elements in *Amborella* are ancient and highly divergent, with no recent transposon radiations. It is an exceptional reference for inferring features of the first flowering plants.

(c) *Jatropha* – A combination of conventional Sanger method and the new-generation multiplex methods was used to sequence the genome of this promising oil-bearing tree crop (Sato et al. 2011). The total length of the non-redundant sequences thus obtained was 285.9 million bp consisting of 120,586 contigs and 29,831 singlets. They accounted for ~95 % of the gene-containing regions. A total of 40,929 complete and partial structures of protein-encoding genes have been deduced. Comparison with genes of other plant species indicated that 1529 (4 %) of the putative protein-encoding genes are specific to the Euphorbiaceae family. A high degree of microsynteny was observed with the genome of castor bean, another Euphorbiaceae family member. Polymorphism analysis using microsatellite markers developed from the genomic sequence data obtained was performed with 12 jatropha lines collected from various parts of the world to estimate their genetic diversity.

(d) *Oil palm* – It is the most productive oil-bearing crop. Although it is planted on only 5 % of the total world vegetable oil acreage, palm oil accounts for 33 % of vegetable oil and 45 % of edible oil globally. Its 1.8-gigabase (Gb) genome sequence was reported (Singh et al. 2013b). A total of 1.535 Gb of assembled sequence and transcriptome data from 30 tissue types were used to predict at least 34,802 genes, including oil biosynthesis genes and homologues of WRINKLED1 (WRI1) and other transcriptional regulators, which are highly expressed in the kernel.

(e) *Rubber* – Rahman et al. (2013) reported the draft genome sequence of rubber tree, the major commercial source of natural rubber. The assembly spans ~1.1 Gb of the estimated 2.15 Gb haploid genome. Overall, ~78 % of the genome was identified as repetitive DNA. Gene prediction shows 68,955 gene models, of which 12.7 % are unique to rubber. Most of the key genes associated with rubber biosynthesis, rubber wood formation, disease resistance, and allergenicity have been identified.

(f) *Agarwood* – The first draft genome of agarwood and a putative pathway for cucurbitacin E and I, the two compounds with known medicinal value, is

derived from *in vitro* agarwood cultures (Chen et al. 2014). *In vitro* materials were used to perform this study due to the lengthy growth period of resinous material in tree as well as to avoid contamination from microorganisms. Importantly, this propagation process is applicable to plant factories for large-scale production in the future. DNA and RNA data were utilized to annotate many genes and protein functions in the draft genome. The changes for cucurbitacin E and I were shown to be consistent with known responses of *A. agallocha* to biotic stress, and expression of a set of homologous genes in *Arabidopsis thaliana* related to cucurbitacin biosynthesis was validated through qRT-PCR.

Genomes of many tropical fruit trees have been sequenced such as those of papaya (Ming et al. 2008) and cocoa (Argout et al. 2011). The Global Musa Genomics Consortium published the first genomic sequence for a triploid tropical crop banana (D'Hont et al. 2012). Recently, a tropical oil tree crop jujube was also fully sequenced (Liu et al. 2014). Macadamia nut genome was recently fully sequenced (Nock et al. 2014) as well. A draft genome assembly was developed for the double-haploid coffee accession (Denoeud et al. 2014).

12.8.2 Implications of Whole-Genome Sequencing to Tree Crop Improvement

With the availability of genome sequences of tree species of both gymnosperms and angiosperms, the identification of a suite of wood-associated genes, and also the good process in function characterization of many of these genes, now we have better tools and a much deeper understanding of the molecular mechanism for wood development. Firstly, hormonal signaling plays critical roles in vascular cambium cell proliferation, and a peptide-receptor-transcription factor regulatory mechanism is proposed to be involved in the maintenance of vascular cambium activity. The differentiation of vascular cambium into xylem mother cells is regulated by coordination between plant hormones and HD-ZIP III transcription factors. A transcription network involving secondary wall NAC and MYB master switches and their downstream transcription factors would activate secondary wall biosynthesis genes during wood formation (Ye and Zhong 2015).

Secondly, genome analysis of tree species has expanded our knowledge on particular gene families that may confirm unique biological features of the species. In coffee, an expansion in the number of N-methyltransferase genes that might involve in caffeine biosynthesis was observed. In date palm and jatropha, the late embryogenesis-abundant genes are higher in numbers, which may relate to their role in conferring resistance to arid conditions. On the other hand, comparative analysis identifies synteny among species, the conservation of gene order over long stretches of chromosomal regions. The analysis of the number of synteny segments is useful for the detection of whole-genome duplication (WGD).

Thirdly, high-quality genome assemblies greatly facilitate the complete inventory of DNA variations in a species, including point mutations, copy number

variations (CNVs), insertion and deletions (INDELs), and epigenetic variations like DNA methylation. Comparison between cultivated varieties and wild species can create linkage between the genotype and phenotype for a wide range of traits.

Nowadays there are many public resources for plant genomic sequences and other genome resources. One example is Gramene (http://gramene.org), a curated, open-source integrated data resource for comparative functional genomics in crops and model plant species. With the objective of facilitating the study of cross species comparison, Gramene currently hosts annotated whole genomes in over two dozen plant species and partial assemblies for almost a dozen wild rice species in the Ensemble browser, genetic and physical maps with genes, EST and QTL locations, genetic diversity data sets, structure-function analysis of proteins, plant pathway databases, and descriptions of phenotypic traits and mutations. It is an integrated web resource for visualizing and comparing plant genomes and biological pathways. Genome features with community-based gene annotations from primary sources are included, to which supplementary annotations, functional classification, and comparative phylogenomics analysis are added (Monaco et al. 2014).

12.9 Marker-Assisted Breeding

Breeding tree species is a long process. One breeding cycle for hardwood tree species typically takes 5–10 years. Information about genes or alleles associated with desirable traits is valuable for the possibility of shortening the breeding cycle or decreasing the size of progeny trial. If juvenile plants can be genotyped and accurately predicted on their future performance, attention and resources can be focused on a small numbers of selected trees. By doing so, breeding becomes more predictable and more efficient.

DNA markers have been developed for many forest species. Expressed sequence tags (ESTs) are mRNA sequences derived through single sequencing reactions on random clones from cDNA libraries. They are a good source for DNA markers. Recently, whole-genome sequencing also uncovers a wealth of new DNA markers, particularly simple sequence repeat (SSR, also known as microsatellite) markers and single-nucleotide polymorphisms (SNPs). SNPs are more numerous and they are more suitable for association genetics (Neale and Savolainen 2004).

High-density and genome-wide markers enable us to develop an understanding of the genetic control of key horticultural traits. After genotyping large sets of individuals from either germplasm collections or segregating populations, a high-density genetic mapping and genome-wide association study (GWAS) enable linkage creation between genetic loci and trait variability. Once a region linked to a trait is located, it is then possible to scan the genome assemblies within the region for positional candidate genes. Further studies need to be conducted to validate the relationship between candidate genes and trait variability. Putative function can be elucidated on the basis of sequence homology with genes from model systems such as *Arabidopsis*; the differential gene expression between individuals carrying the allele or not can be examined; knocking out or complementing the allele in

accessions that carry or do not carry the desired allele, respectively, will give the strongest evidence for a cause-effect relationship between a candidate gene and a trait. One good example is the location of a mutation coding for the pillar trait (br locus) in fruit tree peach (Dardick et al. 2013). Two DNA pools consisting of pillar and standard phenotype individuals from an F2 segregating population were sequenced using Illumina NGS. The resulting sequences were mapped to a reference genome, and SNPs linked to the br locus were identified at the distal end of chromosome 2. Further fine mapping using high-resolution melting SNP markers and a search for structural variants pointed toward Ppa10082 as the potential gene that might be mutated in the pillar phenotype. Further gene expression analysis and transformation in *Arabidopsis* indicated that Ppa10082, which encodes for a homologue of rice OsTAC1, is responsible for control of the pillar trait by the br locus.

Genetic markers associated with desirable traits can be useful for improving the efficiency of breeding through marker-assisted selection (MAS). Briefly, genetic markers associated with the trait of interest are used to select parents and seedlings carrying the desired allele. A remarkable example is the development and use of SNP markers for selecting disease-resistant seedlings in apple breeding populations (Jänsch et al. 2015). In this report, markers flanking eight major loci controlling apple scab, fire blight, and powdery mildew resistances were searched in the inbreeding founders carrying the resistance alleles as well as in susceptible cultivars possessing high fruit quality that are widely used as breeding parents. This enabled the authors to choose a set of markers that are highly efficient for MAS across a range of breeding germplasm to achieve both pathogen resistance and high fruit quality.

In addition to MAS, the availability of high-density genome-wide marker screening systems has enabled the implementation of a further new method for improving the efficiency of tree breeding: genomic selection (GS). It is a new approach for improving quantitative traits in large plant breeding populations that combines whole-genome molecular marker (high-density markers and high-throughput genotyping) data with phenotypic and pedigree data for genomic prediction. The first proof of concept genomic selection in tree was carried out for loblolly pine (Zapata-Valenzuela et al. 2012). The genomic breeding values of 149 cloned progenies from 13 crosses were estimated by fitting 3406 polymorphic SNP markers simultaneously. The accuracy of genomic estimated breeding values ranged from 0.61 to 0.83 for wood lignin and cellulose content and from 0.30 to 0.68 for height and volume traits.

12.10 Improvement of Tropical Tree Crops Through Genetic Transformation

12.10.1 Introduction to Genetic Transformation

Plant genetic transformation or genetic modification (GM) refers to the development of plants with genetic material altered in a way that is unlikely to occur naturally through cross-pollination. It is a process by which genes from nongenetic compatible sources like microbes, other plant species, or animals can be introduced

into selected plants for additional desirable traits. It has the advantages of breaking up the genetic barriers and the ability to introduce short and well-defined DNA sequence into a recipient organism. The prerequisites for plant transformation approach are as follows: (a) an efficient plant regeneration protocol (via either organogenesis or somatic embryogenesis) through which a single transformed cell can regenerate into a whole plant, (b) gene constructs carrying the gene(s) conferring for trait(s) of interest, (c) a reliable and efficient transformation technique to deliver the gene of interest into the nuclear genome of the host cell, and (d) a suitable agent to select regenerated plants derived from transformed cells. Success in genetic transformation started from model plants tobacco and *Arabidopsis* and field crops like rice, maize, and soybean and are gradually achieved in tree crops like poplar, eucalyptus, papaya, acacia, jatropha, and banana.

12.10.2 *Agrobacterium*-Mediated Transformation

The most successful technique used to transfer foreign DNA into plant cells employs the natural genetic engineer, *Agrobacterium tumefaciens* (*Agrobacterium*), a soil phytopathogenic bacterium causing crown gall diseases in many plants. Wild-type *Agrobacterium* contains a circular and double-stranded plasmid (the tumor-inducing or Ti plasmid) with one part of it (T-DNA or transfer DNA) transferred to the host cell nuclear genome. T-DNA has two border sequences (right and left borders) defining sequences to be translocated. To harness this natural genetic transformation, the two border sequences were cloned into a plasmid vector and the tumor causing auxin-, cytokinin-, and nopaline-/octopine-producing elements were deleted together with original border sequences from Ti plasmid, resulting in a disarmed *Agrobacterium*. A simple vector (binary vector) with border sequences is commonly used to clone expression cassettes of target genes and a selection marker within the border sequences, which will be translocated into host cell genome. In *Agrobacterium*-medicated genetic transformation, the vector with desirable gene cassettes is transformed into a disarmed *Agrobacterium*, which is used to infect plant cells (explant). Infected plant cells are allowed to regenerate into whole plants in the presence of a selection agent. *Agrobacterium*-medicated transformation has the advantage of integration into host genome a well-defined DNA fragment within the border sequences in single copy or low copy numbers, making it much easier for stable transgenic trait and regulatory approval.

12.10.3 Other Genetic Transformation Techniques

Particle bombardment is another technique for genetic transformation through which foreign DNAs coated onto particles are delivered into the host cell by compressed air or gunpowder projection. Foreign DNAs delivered have the chance to be integrated into the host genome. Compared to *Agrobacterium*-mediated transformation, particle bombardment is a convenient and easy method since it requires minimum pre- and post-bombardment manipulation. There are other techniques such as

microinjections, pollen tube-mediated transformation, and chloroplast transformation. They are used less often mainly due to integration of multiple copies into a target genome over multiple locations, which may affect the stability of a transgenic trait and complicate regulatory approval.

12.10.4 Technical Challenges in Tree Transformation

Generally speaking, developing GM perennial woody species is more challenging than for annual crops mainly due to the difficulty of regeneration, many being recalcitrant to *Agrobacterium* transformation, difficulty in establishing field trials, the long time needed for evaluation of transgenic trait, and possible transgenic instability during the long lifespan of forest trees.

12.11 The Road Map and Costs in Developing a Commercial GM Tree

Typically, transgenic trees are firstly grown in confinement in green houses followed by field trials under close monitoring. Data from these small-scale, multilocation trials under different environments is collected and analyzed over a period of time for assessment of stability of a GM trait. The environmental and human safety of the product is also analyzed before submission to regulatory bodies to assess and approve for growing these transgenic trees in plantations.

Harfouche et al. (2011) mapped the passage from basic research in lab to commercial plantation of a transgenic tree into five phases:

(a) Discovery and FTO (freedom to operate): to identify genes with potentially valuable traits in trees and identify the tree genotypes to transform, also to obtain freedom of operation through licensing agreements.
(b) Phase I Proof of concept: to generate transgenic plants, verify the desirable traits, select the best transgenic events, and also discuss with regulatory bodies to plan necessary studies to generate adequate data.
(c) Phase II Early development: to conduct preliminary field tests, on small scale, at different locations, and under different environmental conditions, to select successful candidates for commercial production, and to generate data for regulatory approval process.
(d) Phase III Advanced development: to demonstrate durability of the gene of interest and generate data for regulatory approval process.
(e) Phase IV Regulatory approval: to obtain regulatory approval and to commercial market launch.

It is estimated that a commercial GM tree event in the USA may take 10–13 years and cost 70–100 million USD to develop, with the bulk of the time (>60 %), and most costs (>85 %) occur in the last three phases (Harfouche et al. 2011).

12.12 Various Traits in Genetically Modified (GM) Trees

The many desirable traits include disease resistance, resistance to insects and pests, bioremediation of polluted water and land, and tolerance to abiotic stresses like drought and salinity. For timber trees, fast growth and improved wood quality are on the top of the wish list. So far, a lot of tree transformation work has been conducted in the temperate species of the genus *Populus*. Having a small genome and amenability to regeneration and genetic modification made poplar a model plant for tree genetic engineering. Knowledge and experience from this model tree species can quickly spread to other tree species.

12.12.1 Biomass

Increasing the tree growth rates and stem volumes and shortening their rotation times are of immediate and high importance to the wood industry as this would yield more biomass per unit area. In addition to primary uses for furniture, plywood, paper, and pulp, tropical wood trees are gaining popularity as feedstocks for "second-generation biofuels" like cellulosic ethanol. The biomass can be increased by approaches such as plant hormone gene modifications, delaying flowering time so that all the energy is utilized for vegetative growth, especially wood, and change in ploidy level, enhancing the rooting ability and increasing the nutrition levels (nitrogen and sulfur). Overexpression of a gibberellin 20-oxidase in hybrid aspen led to 20-fold higher active gibberellin levels and increase in dry shoot biomass by 64 % (Eriksson et al. 2000). The overexpression of poplar cellulase gene, *PaPopCel1*, increased the length and width of stems with larger and greener leaves in *Agrobacterium*-mediated transformed sengon (Hartati et al. 2008), a tropical legume timber tree species. It is a commendable achievement in extending our knowledge from temperate model tree species to a tropical timber species of significant commercial value.

12.12.2 Lignin Modifications

Lignin is a complex organic component of the cell walls which gives the tree strength by binding cellulose, hemicelluloses, and pectin together. Highly lignified wood is hard and very desirable for furniture but difficult to produce paper and cellulosic ethanol since lignin needs to be separated from the cellulose at a very high financial and environmental cost. It is desirable to genetically engineer trees for less amount of lignin for easy processing but sufficient enough to provide strength to the tree without affecting conduction of water. Strategies like antisense inhibition or RNAi inhibition to manipulate the key enzymes in lignin biosynthetic pathways have led to reduced production of lignin in some trees. Overexpression of ferulate-5-hydroxylase (F5H) in poplar resulted in less condensed lignin and improvements in lignin extractability and bleaching, while fiber quality remained the same (Huntley et al. 2003).

Cinnamyl alcohol dehydrogenase (CAD) is another key enzyme for lignin biosynthesis. Downregulation of this enzyme improved wood quality for chemical pulping, as demonstrated with wood harvested from 4-year-old field trials. Less chemical was used for a higher pulp yield (Pilate et al. 2002). Importantly, no adverse phenotypes on plant growth or health were noticed during the 4-year trial period. Co-transformation of poplar with two constructs was also successful in increasing of F5H expression while reducing of CAD at the same time. Together they resulted in reduced lignin, more cellulose, and less condensed lignin (Li et al. 2003). An alternative approach is to manipulate transcription factors. When a LIM domain transcription factor (*Ntlim1*) was downregulated in transgenic eucalyptus plant, expression of several lignin biosynthesis genes such as *PAL*, *C4H*, and 4-hydroxycinnamate CoA ligase (*4CL*) were all decreased (Kawaoka et al. 2006) concurrently with reduction of lignin content by 26 %. Genetically modified poplar trees with cinnamoyl-CoA reductase (CCR) gene downregulated were found to yield up to 161 % more ethanol after wood fermentation (Van Acker et al. 2014).

12.12.3 Tolerance to Environmental Stresses

Tropical timber trees face various types of environmental stresses like excessive heat, flooding, drought, salinity, and acidity of soil. Such stresses can significantly affect the tree at all stages of growth and cause severe loss of productivity. There are three general strategies to confer abiotic stress tolerance by genetic modification: (1) expression of genes that are involved in signaling and regulatory pathways, (2) expression of genes that encode proteins conferring stress tolerance, and (3) expression of enzymes for synthesis of functional and structural metabolites (Hong and Bu 2013). The overexpression of choline oxidase (codA) in *Eucalyptus camaldulensis* resulted in higher production of glycine betaine and increased salt stress tolerance (Yamada et al. 2003). When two cold-induced transcription factors were overexpressed in a cold-sensitive eucalyptus hybrid, the GM tree was found to be more cold tolerant with wax deposition on cuticle, with reduced leaf area, decreased cell size, retarded growth, and better water retention capacity (Navarro et al. 2011).

12.12.4 Resistance to Biotic Stress

Insects, pests, bacteria, fungus, and viral pathogens can affect the health of timber trees. In the hot and humid tropics, the disease can spread quickly and cause widespread damage. Spraying insecticides and fungicides can only control infection for a limited time only. Moreover, spraying over large plantations is hazardous to surrounding human, animals, soil, and water. Genetically engineered trees can produce self-protecting proteins/compounds continuously, thereby avoiding sensitivities to application timings. It is less expensive and possible to kill insects or pathogens that reside inside the wood or bark of the tree. Two strategies are used to modify plants for enhanced insect and pest resistance. The first is to upregulate innate resistance

traits (endogenous traits) such as increase synthesis of phenolics, and the second is to introduce new traits (exogenous traits) such as the production of *Bacillus thuringiensis* (hereafter Bt) toxin proteins. Bt poplar was the first stable transgenic tree crop reported (McCown et al. 1991), and it's also the first transgenic tree grown on commercial scale in China (Tian et al. 1993). Bacterial attacks are less frequent, but some genus like *Xanthomonas* can cause severe damage to trees. Shao et al. developed transgenic eucalyptus (*E. urophylla*) events showing overexpression of the antimicrobial peptide cecropin D increased resistance by 35 % against the bacterial wilt disease caused by the pathogen, *Pseudomonas solanacearum* (Shao et al. 2002). To address the concern of pathogens developing resistance against a single gene product, gene pyramiding or gene stacking can be used to combine multiple gene products.

12.12.5 Phytoremediation

Phytoremediation is the removal of environmental pollutants like heavy metals, pesticides, pharmaceuticals, petroleum compounds, polycyclic aromatic hydrocarbons from the industrial runoffs, municipal waste waters, and landfills using plants. There are various approaches. Pollutants in water and soil can be taken up inside the plant tissue (phytoextraction) or absorbed to the roots (rhizofiltration); pollutants inside plant tissues can be transformed by plant enzymes (phytotransformation) or can volatile into the atmosphere (phytovolatization); pollutants in soil can be degraded by microbes in the root zone (root zone bioremediation) or incorporated in soil material (phytostabilization) (Hong and Bu 2013). Because of the huge biomass, deep and widespread root network, and perennial nature, trees are better candidates than other plants. Transgenic trees have been created for enhanced biodegradation and phytoremediation of pollutants. Yellow poplar (*Liriodendron tulipifera*) was transformed with a mercuric reductase gene (*merA*) to covert toxic ionic mercury to much less toxic element mercury (Rugh et al. 1998). Another organomercurial lyase gene (*merB)* converts more toxic methylmercury (MeHg) to less toxic ionic mercury. It was found in *Arabidopsis* that combining both genes in the same plant could increase the ability to grow in the presence of organic mercury. When two genes were engineered to eastern cottonwood through retransformation *merB* into a GM tree already with *merA*, *in vitro*-grown merA/merB plants were highly resistant to phenylmercuric acetate and detoxified organic mercury compounds two to three times more rapidly than controls did (Lyyra et al. 2007). The availability of metal transporters, translocators, and chelators and the ability to express membrane proteins could further enhance mercury phytoremediation capabilities. Transgenic poplars overexpressing a bacterial gene encoding glutamylcysteine synthetase and elevated level of glutathione were found tolerant to zinc toxicity (Bittsanszky et al. 2005). One report described the development of transgenic poplars overexpressing a mammalian cytochrome P450, a family of enzymes commonly involved in the metabolism of toxic compounds. The transgenic plants showed enhanced metabolism of trichloroethylene and the ability of removing a

range of other toxic volatile organic pollutants including vinyl chloride, carbontetrachloride, chloroform, and benzene (Doty et al. 2007). Since timber trees are not used for human or animal consumption, hence chances of these chemicals reentering the food chain are minimal. These phytoremediating trees can provide beauty, do carbon sequestering, and check soil erosion at the same time.

12.13 Regulatory Frameworks for Transgenic Trees

Field testing and approval for commercial release for transgenic plants in the USA are governed mainly by three federal regulatory agencies: the FDA (Food and Drug Administration) is responsible for biotechnologically derived medical products, the USDA (United States Department of Agriculture) for transgenic plants, and the EPA (Environmental Protection Agency) for pesticidal plants and genetically engineered microbial pesticides. Under USDA, the Animal and Plant Health Inspection Service (USDA APHIS) plays the key role in overseeing field tests and eventual deregulation of GM plants. The fundamental principle for US regulatory framework is "substantial equivalence": if a GM plant is substantially equivalent with its non-GM counterpart, it can be deregulated and allowed to market. In comparison, EU has a much more strict and complicated system. On top of the needs to address concerns on toxicity, allergenicity, and possible impact on the environment, there are also the requirements for labeling and traceability for GM products. Regulatory frameworks in other countries are generally between US and EU regulatory frameworks. A common feature for all regulatory frameworks is the need for approval before a GM event is released into open environment for trial plantation, through which field data are collected and submitted for the final approval for commercial release.

12.14 GM Tree Commercial Plantation and Field Trials

(a) *Poplars* – One of the first reported field trials with genetically modified forest trees was established in Belgium in 1988, and the GM trait evaluated was herbicide tolerance in poplar. From 1988 to 2002, there had been more than 200 reported trials, involving at least 15 forest species, and the majority of them were carried out in the USA (64 %). More than 50 % of the field trials were done with *Populus* species, and the main target traits were herbicide tolerance (31 %), marker genes (23 %), and insect resistance (14 %) (Valenzuela 2006). Another source estimated more than 700 field trials with GM trees (Walter et al. 2010) by 2010. Until today, there are only two commercial-scale productions of transgenic trees: The first forest tree species *Populus nigra* with the Bt gene was released in China in 2002 (Valenzuela 2006). The second is GM papaya that has been planted in Hawaii since 1998. The GM papaya was resistant to PRSV (papaya ring spot virus) (Gonsalves 2004). FuturaGene (www.futuragene.com) announced in April 2015 that it obtained regulatory approval to commercially deploy a yield-enhanced genetically modified eucalyptus variety in Brazil. This

is the first approval for commercial plantation of a GM tree after a long gap of 13 years since Bt poplar approval in 2002.

(b) *Eucalyptus* – *ArborGen* (www.arborgen.us) is another forest biotechnology player that has conducted nearly 900 field trials containing roughly a million trees. Its freeze-tolerant tropical eucalyptus product (AGEH427) is currently going through the government review process for deregulation in the USA. Other GM traits ArborGen develops include improved growth and wood quality, increased density and drought tolerance, as well as bioenergy possibilities, mostly achieved through collaboration with academic institutes including the BioEnergy Science Center (BESC), University of Georgia, and University of Florida.

(c) *Jatropha* – *JOil* is a Singapore-based company (www.joil.com.sg) specializing in the development of *Jatropha curcas* as a feedstock plant for biodiesel and bio-jet fuel. Through collaboration with its technology partner Temasek Life Sciences Laboratory, GM jatropha trees with insect tolerance, high oil content, high oleic acid, and virus tolerance were successfully developed (Ye et al. 2014; Qu et al. 2012; Kim et al. 2014; Gu et al. 2014). Recently, a GM jatropha tree with much lower phorbol ester toxin in seed was also achieved (Li et al. 2015). The company obtained permission to conduct a field trial on high-oleic acid jatropha, the first GM field trial for an oil production tree species.

Since the first GM plant trial in 1988, a very large amount of performance and safety data related to GM crops and trees have been gathered, and none of them reported any substantive harm to biodiversity, human health, or environment. One example is the field trials with GM poplars with modified lignin composition to access ecological impacts on the environment. Field trials in the UK found no differences between the GM trees and non-GM trees in growth and development, damage by insects, and levels of carbon, nitrogen, and microbial biomass (Hopkins et al. 2007). The two commercially successful GM trees also support that GM trees can be safe. Approximately 1.4 million Bt poplars have been planted in China. These GM trees successfully established and conferred resistance to insect attacks. The oldest trees were 15 years in 2010 and no harm to the environment was observed (Walter et al. 2010). In 2001, 3 years after the release of the GM papaya in Hawaii in 1998, the total production of papaya was 60% more than that in 1998 when PRSV devastated papaya plantations. Such increase of production was also contributed by non-GM papaya trees that were protected by GM papaya in the same area (Gonsalves 2004).

12.15 New Technology Development to Address the Concern on Transgene Escape

Tree genetic engineering provides an opportunity for sustainable production of forest products. However, the commercial plantation of GM trees is hindered by regulatory and social hurdles. Concerns have been raised about the safety of the genes

used, the potential impact of transgenic outcrossing with sexually compatible wild relatives, and the possible impact on non-target organism. Transgene flow remains the major concern. There are the intensive efforts on transgene containment strategies to address this gene flow concern for GM trees.

The many transgene containment technologies under development include:

(a) *Plastid transformation* – Instead of nuclear genome transformation. Since plastid genomes are inherited maternally, plastid transformation reduces the risk of gene flow. One example is the plastid transformation of poplar (Okumura et al. 2006).
(b) *Engineering flower sterility to prevent gene flow* – Flower-specific promoters have been used to drive a toxin to interfere flower development. One example is the use of PTLF::*barnase* to control flower in poplar (Wei et al. 2006). An alternative approach is to downregulate a gene critical to flower development such as *EgAGL1* and *EgAGL2*, two *AGAMOUS*-like genes in eucalyptus.
(c) *Remove transgene from pollen* – One example is the use of site-specific recombination systems to facilitate efficient excision of transgenes from pollen of hybrid aspen (Fladung and Becker 2010).
(d) *Cisgenesis approach* – Genetic modification of plants with cisgenes only. A cisgene is a natural gene coding for a desirable trait and from the crop plant itself or from a sexually compatible donor plant. The gene belongs to the conventional breeder's gene pool. The GM jatropha with higher oil content is one such example (Kim et al. 2014). In this report, the RNAi approach was used to clone gene fragments of the endogenous gene *JcSDP1* under its own promoter to silence *JcSDP1* transcription, resulting in higher oil content in seed. With the marker gene excised, this GM jatropha contains no foreign DNA.
(e) *Site-specific genome editing* – CRISPR/Cas9, a modification of the prokaryotic defense mechanism, becomes the preferred gene-editing technique for plant scientists. This system utilizes a single guide RNA to bind to its complementary sequence on the genome and recruit Cas9 enzyme for site-specific cleavage. In plants, the cleaved DNA ends are rejoined by NHEJ process through which insertion/deletion events can occur and result in a frameshift or introducing a premature stop codon. There have been many reports of CRISPR/Cas9-mediated gene editing for trait improvement (Schaeffer and Nakata 2015). Recently, this genome editing tool was successfully used in poplar, demonstrating its utility in tree species (Fan et al. 2015). The precise genome editing of endogenous genes without introducing any foreign genes is expected to alleviate concern for transgene escape.

12.16 Summary

There is little doubt that tropical tree crops will play key roles in supplying sustainable wood, fiber, food and feed, medicine, and renewable energy. It is envisaged that tree plantations will contribute more to the total harvest in the coming years. Plant

biotechnology will enable and facilitate this trend by accelerating breeding for more productive elite varieties suitable for local climate through combining the use of traditional breeding with genetic analysis to select best plants in a cost- and time-effective manner, thanks to our better understanding of plant genomes and development of tools for high-throughput genetic diversity analysis. *In vitro* propagation technologies, which are increasingly adopted by the industry, would mass produce high-quality pathogen-free plantation materials for deployment to the field. *In vitro* culture also helps preserve natural biodiversity and keep breeding materials on a long-term basis. While domestication of trees and traditional breeding are gaining success in enhancing growth and productivity, genetic modification gains traction as an important tool to introduce novel and desirable traits that are not present in natural pools. There have been many exciting developments of environment-friendly wood for paper and pulp industry and bioenergy industry through cellulose/lignin biosynthesis control; trees that can resist environmental stresses or sequester/decompose environmental contaminants and oil tree crop with less toxicity and high oil productivity. There are some safety issues of GM trees especially the long-term impact on the environment due to gene flow. Intensive technology development in this area is expected to address the concern soon. More efforts are needed to translate new discoveries in tree biotechnology effectively and economically into commercial plantations.

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Part III

Genetic Engineering

In Vitro Regeneration of Salt-Tolerant Plants

13

Remya Mohanraj

Abstract

An increase in global population and the demand for food production have steered the direction of research toward production of plants that can give better yields even with adverse environmental conditions. One such is the generation of salt-resistant plant varieties that would grow on saline soil and holds the possibility of irrigation with seawater. An upsurge in soil pollution accompanied by depleting water reserves warrants the production of salt-resistant crops as the next step in meeting the global demand for food.

Plant tissue culture not only offers the advantage of culturing a large number of cells in a small space but also provides options for genetic transformation and selection. The present review attempts to summarize the various developments in the area of plant tissue culture for regeneration of salt-resistant varieties.

13.1 Introduction

Naturally saline soil hinders the production of crops in many parts of the world. Salinity affects plant growth by influencing water absorption and important biochemical processes such as nitrogen assimilation and protein biosynthesis (Dubey 1994). Plant cells exposed to salinity are subject to damage by oxidative stress (Queiros et al. 2007). An increase in soil salinity decreases soil water osmotic potential leading to reduced uptake of water and thus water shortage in plants. It affects the integrity of plant metabolites. Augmentation of Na^+ and Cl^- ions reduces

R. Mohanraj (✉)
Department of Biology, Houston Community College,
1300 Holman, Houston, TX 77004, USA
e-mail: remyam@gmail.com

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the absorption of vital minerals and thereby imposes toxicity on plants (Tester and Devenport 2003; Karimia et al. 2009). Plant growth reduction by virtue of salt stress is also affiliated with decreased photosynthetic activities, like the electron transport (Greenway and Munns 1980). Other factors that inhibit photosynthesis under high salinity are reduction of chloroplast stromal volume and generation of reactive oxygen species (Price and Hendry 1991).

Salinity poses limitations for producing different crops in several countries, particularly in the arid and semiarid areas of the world (Zaman et al. 2005). Almost 25 % of the cultivable land all over the world has extreme quantities of salt, mostly NaCl (Sharry and Teixeira 2006). The constrained supply of good-quality water in many arid and semiarid regions of developing nations requires the adoption of saline water for irrigation, where available for crop production. Therefore, it is imperative to screen crop plant varieties for salt tolerance (Bekheet et al. 2006). Nevertheless, developing plants that can tolerate salt stress and yet provide desirable yield continues to pose a challenge for agricultural scientists across the globe. Studies on genetics have revealed that salinity tolerance in plants is a quantitative trait influenced by the operation of multiple genes. This has made it hard to attain salt tolerance in plants through traditional methods (Foolad and Lin 1997).

Cell and tissue culture techniques are evolving as possible advances toward the regeneration of plants with elevated tolerance to biotic and abiotic stresses, especially salt stress. In the recent past, various studies have been focused on the production of plants with enhanced salt tolerance through selection of salt-tolerant cells from tissue culture. Cells and tissues in culture could be extremely useful in selecting salt-tolerant plants and in studying physiological and biochemical basis of salinity tolerance (Chen et al. 1980; Umiel et al. 1980; Benderradji et al. 2012). Understanding physiological and biochemical basis of salt conflict in in vitro cultures will provide means for the development of plants with enhanced resistance to salt stress (Niknam et al. 2004). Tissue culture systems allow us to impose rigorous nutritional and environmental controls on the experimental system and allow the selection of highly salt-tolerant cell lines from populations that are otherwise vulnerable to stress. Also, the regenerated plantlets are clones of the original starting material. This simplifies subsequent testing (Dix 1985).

This chapter attempts to highlight the various approaches in the production of salt-tolerant varieties using in vitro techniques. The current trends and future prospects for the same have also been discussed.

13.2 Mechanisms Underlying Salt Tolerance

During the past few years, scientists have endeavored to understand the robust mechanisms and to identify the metabolic process critical in plants that tolerate salt stress. Institution of ion equilibrium has been found to be critical for plant survival

under salt stress settings. Cellular response to such conditions includes rising Na^+ discharge and Na^+ accumulation in the vacuole. Along with adaptation to Na^+ toxicity, the plants should devise a mechanism to uptake K^+ since it is an important nutrient. Hence, mechanisms that transport K^+ preferentially over Na^+ could act as imperative determinants of salt tolerance (Rodriguez-Navarro 2000).

A notable metabolic response to salt stress is the bioproduction of osmolytes that have the potential to mediate osmotic adjustment and protect subcellular structures and oxidative damage by their free radical scavenging capacity (Hare et al. 1998). Also, there is an increase in the accumulation of abscisic acid (ABA) (Taylor et al. 2000) which could boost the free cytoplasmic Ca^{+2} (Wu et al. 1997). Studies on RD20 gene encoding for a Ca-binding protein for which ABA and salt stress are inducers propose a relationship between salt stress, accumulation of ABA, and pathways that signal Ca (Borsani et al. 2003).

Recognition of cellular processes and gene expression that is troubled by salt stress could be useful in determining mechanisms of salt tolerance. Since salt stress-regulated genes play a significant role during tolerance, studying such genes has paved way for a clear understanding of the complex mechanisms involved in conferring salt tolerance to higher plants (Hasegawa et al. 2000). Genes could be identified through selection of varieties followed by characterization.

13.3 In Vitro Approaches for the Production of Salt-Tolerant Varieties

13.3.1 General Methodology

Numerous attempts have been made to apply in vitro techniques for the development of salt-tolerant plants. In general, the following steps are followed:

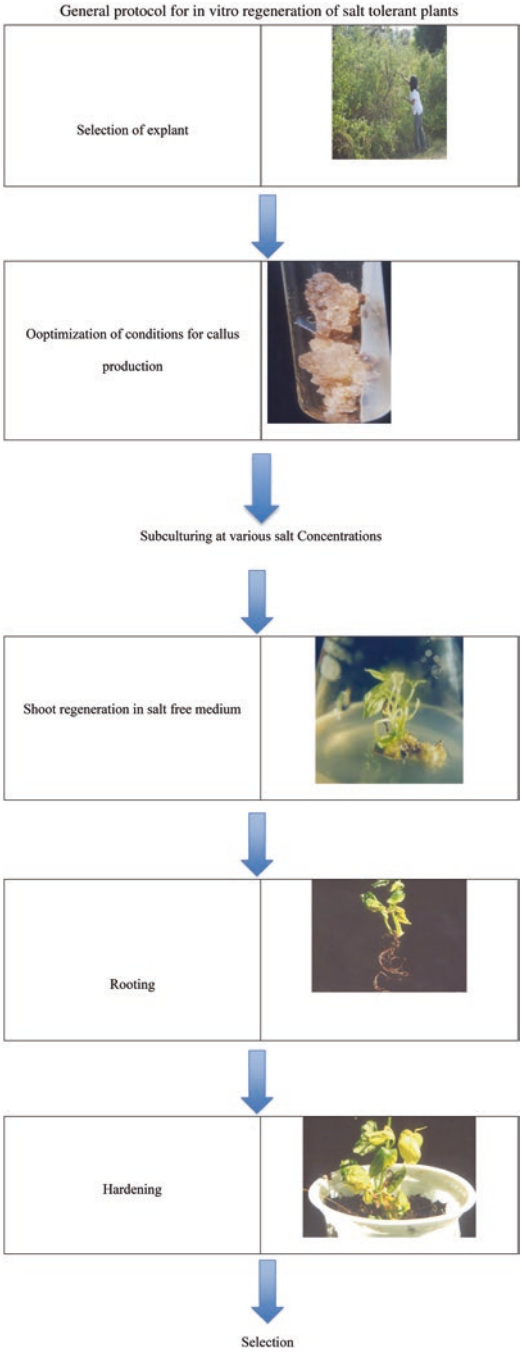
Explants for callus regeneration are selected either from naturally growing or in vitro developed seedlings. The explants after surface sterilization are plated on MS medium supplemented with different combinations of growth regulators so as to find out the optimal concentration of growth regulators necessary for callus regeneration.

The calli obtained from the previous step are subcultured (repeatedly) on callus growth medium supplemented with various concentrations of salt mixture (Ibrahim and El-Kobbia 1986).

Salt tolerance ratio is calculated as follows Bekheet et al. (2006):

$$\text{Salt tolerance ratio} = \frac{\text{Fresh weight on salt medium}}{\text{Fresh weight on salt free medium}}$$

Calli that are able to regenerate under salinity conditions are transferred to shoot regeneration medium without salts. The regenerated shoots are subcultured on rooting medium. The rooted plantlets are then subjected to hardening.


General protocol for in vitro regeneration of salt tolerant plants
Selection of explant
Ooptimization of conditions for callus production
Subculturing at various salt Concentrations
Shoot regeneration in salt free medium
Rooting
Hardening
Selection

The effect of various salt concentrations on the growth and chemical composition of onion tissue cultures followed by in vitro selection for salt tolerance was studied by Bekheet et al. (2006). A protocol for in vitro selection of salt-tolerant somaclonal variations from cell suspension cultures of calli of triploid Bermuda grass was developed by Shayun Lu et al. (2007). Salt-tolerant lines of *Nicotiana tabacum* were developed in vitro by Rout et al. (2008). Shanthi et al. (2010) conducted an experiment to study the performance of rice genotypes (Pokkali, CSR 10, TRY 1, and TRY2) for salt tolerance under in vitro condition. Srinath Rao and Prabhavathi Patil (2012) developed salt-tolerant varieties of *Vigna radiata*.

13.3.2 Selection of Salt-Tolerant Varieties

It is important to verify if the salt-tolerant trait is transferred to the progeny via seeds. Therefore, seeds from the regenerated plantlets should be allowed to germinate and the seedlings tested for salt tolerance. Selection of salt-resistant cell lines has relied upon their capacity to grow on otherwise inhibitory levels of NaCl (Dix 1985; Tal 1983). It has been reported that exposing the cells directly to sublethal salt concentrations is an efficient way to raise salt-tolerant varieties (Sumaryati et al. 1992).

Isozyme analysis and SDS-PAGE profiles could indicate if the progenies derived from tolerant sources are tolerant to salt. Also, specific plant genes are overexpressed during salt stress. Screening for the presence of these genes could be an efficient method for the selection of stable variants. Bouharmont et al. (1993) devised a method for in vitro selection of salt-tolerant varieties at the cellular level. Selected cell lines were characterized by their ability to maintain a normal content of K^+ and Ca^{++} in spite of increasing concentrations of Na^+ in the medium.

Genotypic assessment of regenerants could be performed using RAPD fingerprinting. DNA is extracted from regenerants and PCR reactions are conducted. Random primers are used for the amplification, and products are resolved on an agarose gel and stained with ethidium bromide.

13.3.3 Transgenic Salt-Tolerant Plants

Genetic engineering offers tools for the molecular manipulation of genes that could confer salt tolerance. One of the methods is the identification of genes that are involved in processes that are critical for salt tolerance (osmolyte synthesis, ion homeostasis, etc.). Another way is the identification of genes whose expression is regulated by salt stress. Yet another relies on the identification of salt tolerance determinants based on functionality (Borsani et al. 2003).

In essence, in order to improve salt tolerance of crop plants, it is imperative that alterations be brought about at various cellular, physiological, and metabolic mechanisms that are controlled by expression of specific genes. These genes could encode proteins implicated in Na+ sequestration (H+-ATPase, NHX-type

transporters) (Zhang and Blumwald 2001), synthesis of specific osmolytes (proline, glycine betaine, polyols), detoxification of toxic compounds (reactive oxygen species-scavenging enzymes), signal perception and regulating factors, and other unknown functions (Yancy et al. 1982; McCue and Hanson 1990).

Genes necessary for imparting salt tolerance have been isolated from organisms ranging from prokaryotes to halophytes to glycophytes. These genes fall into five groups, namely, synthesis of osmolytes, protection of cell integrity, oxidative stress, ion homeostasis, and transcription factors. Some of these genes are betA (choline dehydrogenase), BADH (betaine dehydrogenase), CodA (choline oxidase), COX (choline oxidase), TUR1 (inositol synthase), MtlD (mannitol synthesis), ProDH (proline dehydrogenase), HVA1 (LEA protein), and GS (glutamine synthase) (Borsani et al. 2003).

In one of the very first successful experiments using genetic engineering for raising salt-tolerant lines, Tarczynski et al. (1993) introduced an *E. coli* gene (mtlD) into tobacco. Overexpression of a gene coding for P5CS from mung bean plants in transgenic tobacco resulted in the accumulation of proline up to 18-fold over control plants. This resulted in enhanced biomass under conditions of salt stress (Kishor et al. 1995). Transgenic tobacco plants harboring *E. coli* betA gene that codes for a choline dehydrogenase were more tolerant to salt conditions when compared to the wild type (Lilius et al. 1996). Transformation of *A. thaliana* with codA gene led to the accumulation of glycine betaine and enhanced its tolerance to salt stress (Hayashi et al. 1997). An *Arthrobacter pascens* gene encoding COX enzyme was used to generate transgenic plants in *Arabidopsis* sp., *Brassica napus*, and tobacco (Huang et al. 2000). Transgenic tobacco plants that expressed BADH gene accumulated a higher amount of glycine betaine in cytosol and chloroplasts and exhibited increased tolerance to production under salinity stress (Holmstrom et al. 2000). Improved salt tolerance was obtained in transgenic tobacco by expressing a yeast invertase gene in apoplast (Fukushima et al. 2001).

Recently, gamma ray-induced in vitro mutagenesis and selection for salt (NaCl) tolerance were investigated in sugarcane (*Saccharum officinarum* L.) by Nikam et al. (2015). Embryogenic calli that were irradiated (10–80 Gy) were subjected to in vitro selection by exposure to different concentrations of NaCl (0, 50, 100, 150, 200, and 250 mmol L^{-1}). It could therefore be inferred that radiation-induced mutagenesis is an effective way to enhance genetic variation. However, success of in vitro mutagenesis programs will depend on evaluation of mutant clones under field conditions to confirm their performance for the selected trait of interest.

13.4 Future Prospects

In spite of the fact that salt-tolerant lines could be easily selected, and successful regeneration of whole plants has been achieved, there have been only a few reports on the determination of salt tolerance of the regenerants or verification of its sexual inheritance. A survey of literature reveals that the main reasons behind the limited success of the in vitro approach include difference in the tolerance mechanisms

operating in vitro and in vivo, paucity in distinguishing adapted cells from true mutants, multigenic nature of salt tolerance, and inadequacy during selection of regeneration capacity (Tal 1993). This calls for approaches directed at combining the molecular, physiological, and metabolic aspects of abiotic stress tolerance which could narrow down the differences between short- and long-term effects of the genes and their products and between the expression of genes and the entire plant phenotype under stress (Bhatnagar-Mathur et al. 2008). Though transgenic technology will assuredly continue to aid in the pursuit of molecular mechanisms that directionalize tolerance, the intricacy of the attributes suggests that engineering such tolerance into sensitive species might take a little longer (Flowers 2004).

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Plant Tissue Culture for In Vitro Mutagenesis, Large-Scale Propagation, and Genetic Transformation

14

Pratibha Misra and Syed Saema

Abstract

An ever-increasing demand of uniform plants of commercially valuable plant species needs their clonal propagation on a large scale using different strategies of tissue culture. We have reported a large number of plants which were propagated in vitro using different plant parts. A number of problems were solved before large-scale propagation of these crops, like shoot organogenesis, multiplication of shoots, rooting, acclimatization and hardening, control of shoot browning/necrosis and defoliation, etc. Besides, popularization of temperate climate crops, e.g., Asiatic hybrid lilies and gerbera, to subtropical climate of Lucknow, was achieved using tissue culture. New varieties of chrysanthemum differing in color and shape of the florets were developed using in vitro mutagenesis.

Transformation protocols using *Agrobacterium tumefaciens* have been optimized in *Jatropha curcas*—a plant important for biofuel and *Withania somnifera*—an important Indian medicinal plant using leaf segments (LS). *W. somnifera* has some medicinally important sterol glycosyltransferases (SGT) which are the enzymes that glycosylate sterols and play an important role in providing tolerance to the plant against biotic and abiotic stresses. These genes were differentially expressed in different organs of the plant and also in response to biotic and abiotic stress. Functional characterization of *WsSGT* gene was done by its overexpression in homologous and heterologous expression systems of *N. tabacum* and *A. thaliana*, whereas for suppression/gene silencing, RNAi and artificial miRNA technologies were used. The transgenic plants showed improved germination and tolerance to salt, heat, and cold stress when compared to WT plants. Increased enzyme activity and sterol glycosides could be demonstrated

P. Misra (✉) • S. Saema
CSIR, National Botanical Research Institute,
Lucknow 226001, India
e-mail: pratibhaflora@yahoo.com; pratibhamisra@nbri.res.in

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through biochemical analysis. Stress-responsive elements were observed when the promoter of *WsSGTL1* gene of *W. somnifera* was cloned and sequenced.

Keywords
In vitro culture • In vitro mutagenesis • Genetic transformation • Functional characterization

14.1 Introduction

Plant tissue culture is employed for rapid multiplication of stock plant material to produce a large number of uniform clonal plants. The micropropagated plants have a number of advantages over the usual conventional system of plant propagation, such as being clonal in nature and disease-free plants. Micropropagation is advantageous in producing rooted plantlets ready for transplantation and to grow. This saves the time of the grower when seeds or cuttings are difficult to grow and time consuming. The multiplication of plants through tissue culture is far more beneficial to the traditional system through which seeds are produced in uneconomical amounts or sometimes plants are sterile and no longer produce viable seeds or when seeds can't be stored. The majority of the orchids can only be grown from seeds using micropropagation strategies. A large number of planting material could be produced per square meter and the propagules can be stored longer and in a smaller area. Moreover, aerial root tips and leaf tips of orchids could also be used as explants for the establishment of plant material. However, a practical evaluation exhibits that only few complete plants have been regenerated; still fewer have simply been grown in the soil, while their micropropagation on a large scale has hardly ever been possible.

Since most of the ornamental crops are highly heterozygous, their seed progeny is not true-to-type. Conventionally, to produce genetically similar plants, vegetative propagation is being done. But this could not meet the market demand due to slower rate of propagation. Therefore, most of the modern floriculturists follow the micropropagation techniques for large-scale propagation of ornamentals. A large number of ornamental crops are being commercially propagated through micropropagation. Rainbow cauliflowers rich in anthocyanins (purple colored), beta carotenes (orange colored), and chlorophylls (green colored) have been developed by European plant company Syngenta (http://www.dailymail.co.uk/news/article-514799/The-orange-purple-green-cauliflowers-scientists-claim-healthier-you.html) through breeding. Tissue culture could be the only option to propagate them on a large scale so as to fulfill their market demand.

Tissue culture and molecular researches are always receiving a rousing welcome by the blooming floriculture industry. Crop improvement through in vitro mutagenesis develops many new flower color/shape of mutants in ornamental floriculture. Intervention of molecular research in ornamental plants and their potential of mass propagation provides the sought after value addition and real product differentiation. This deliverable capability of molecular research has opened new and lucrative

vistas in floriculture. Thanks to GM longer vase life and lasting fragrance add value; while color and chimera variance continue to create its niche in the global floriculture trade, the resultant benefits and profits in commercial floriculture attracts the molecular researches. Till now, only genetically modified carnation has reached up to the market, while others are in their early days of molecular research. In conventional vegetative propagation of ornamentals, rooted cuttings are given treatment of physical (gamma rays) and/or chemical mutagens before planting. In these plants, mutation seems as chimeras which remained the crucial bottleneck for mutation breeding. The chimeric tissue has the mutated cells which are present along with the normal cells. During further cell division, the mutated cells compete with the encircling normal cells for their survival and this is called as diplontic selection. The surviving mutated cells are expressed in plants. The dimensions of the mutated sector can vary from a small slender streak on a petal to a complete flower head and from a portion on a branch to entire branch. The isolation of mutated tissue in pure form is possible when a portion of a branch or a complete branch is mutated. Nevertheless, a small sector of a mutated branch or flower cannot be isolated using the available conventional propagation tactics. Consequently, many new flower colors/shapes of the mutants or spontaneously developed mutants induced by mutagens are lost because of the lack of microtechniques for the management of such chimeric tissues either in vivo or in vitro. The protocols for in vitro regeneration of many flowers are now well established and available. Adventitious shoot regeneration from floret explants of chrysanthemum is also available. However, in all the cases, shoots were produced from floret-derived callus, and there is invariably a lack of somatic/genetic homogeneity due to prolonged callus phase. In our laboratory, tissue culture techniques using different doses of gamma radiation have been optimized to regenerate shoots from stem internodes, stem nodes, shoot tips, and ray florets for in vitro management of chimera and in vitro mutagenesis. Chimera isolation is practically very useful not only for chrysanthemum but also for breeding of other ornamentals. Chimera isolation and establishment of solid mutant is a two-step process—first, in vivo mutagen treatment and, second, in vitro regeneration of viable plants from mutated sectors and to apply RAPD analysis for any genetic polymorphism among mutants and their parents. The present technique opens new vistas for isolating new flower color/shape of ornamental cultivars by means of retrieval of mutated cells.

Tissue culture knowledge is very important for the success of genetic transformation, because many times a well-defined regeneration system has been reported to be recalcitrant for regeneration during genetic transformation (Pandey et al. 2010). During the last three decades, momentous developments in plant transformation technologies have taken place by releasing a large number of transgenic crop plants for commercial production. *A. tumefaciens*, a gram-negative bacterium, allows the introduction of genetic information into cells. This has revolutionized the field, improving cultivars by expressing traits that would not be possible to obtain by classical methods. Nowadays, various physical, chemical, biological, and *in planta* methods are routinely used for transformation. *Agrobacterium* and virus-mediated transformations are mainly used under biological method of transformation.

Researches in plant transformation are mostly going on the problems related to stable integration and reliable DNA expression after integration rather than DNA introduction into plant cells. Different biotic and abiotic resistance, e.g., disease resistance, insect resistance, frost resistance, herbicide resistance, etc., are being developed using transformation technology. Basmati rice with carotene has been developed and named as golden rice which contains increased levels of pro-vitamin A with 37 mg/g of carotenoid, of which 84 % is β-carotene (Ye et al. 2000). This is under trial. Suntory Flowers developed blue color rose by introduction of genes responsible for delphidine pigments to ordinary roses where this pigment was lacking naturally. For the induction of anthocyanins, two genes from snapdragon flowers had been transferred to tomato plants. In comparison to previous research, more accumulation of anthocyanins in tomatoes has been reported for metabolic engineering in both the peel and flesh of the fruit. The fruits were dark purple in color. Eady et al. (2004) from New Zealand and his collaborators in Japan are trying to bring tearless onion in the market. Delivery of corn-based edible vaccines is being prepared in the form of transgenic corn kernels and corn snacks. In tomato, ripening control genes have been introduced which will be visualized by the color of the tomato. Besides this malarial antigens are also being transferred in tomato. A genetically stable expression of functional miraculin protein, a new class of alternative sweetener which is not only sweet but also has an unusual property to turn sour taste (like lemon) into sweet, has been accumulated in high levels in tomato (Sun et al. 2007; Hiwasa-Tanase et al. 2011). Gene silencing (RNAi-technology) has extensively been used to develop virus resistance in plants. Transgenic papaya has been developed by using this technology for resistance to papaya ring spot virus. In tobacco, resistance to *Phytophthora nicotianae* could be established via gene silencing.

In the present chapter, a review is being presented for the use of plant tissue culture for the development of variations through in vitro mutagenesis, large-scale multiplication, and propagation of crops and its application for genetic improvement of plants and functional studies of genes using genetic transformation.

14.2 In Vitro Mutagenesis

14.2.1 Chrysanthemum

For commercial floriculture, there is perpetual demand for new and novel varieties. Mutation breeding is a promising means for crop improvement, in addition to the development of many new flower color/shape of the mutants in ornamental floriculture. Conventionally, vegetatively propagated ornamentals, rooted cuttings, are being treated with gamma rays before planting. In these plants, mutation appears as chimeras, which remains the main bottleneck to isolate. In our laboratory, a protocol has been optimized for the management of such chimeras via direct shoot organogenesis from flower petals.

Total 17 small and mini varieties were cultured using ray florets after treatment with 500 and 1000 Gy gamma ray irradiation. Only ten varieties differentiated

shoots in the treated ray floret explants, which were grown, rooted, and transplanted in the field (Fig. 14.1a, b). In the field, after flowering, during the season only "Flirt," "Lalima," "Puja," "Sunil," and "Maghi white" showed variation in flower shape/color (Datta et al. 2005). In "Flirt" variety about three different combinations of red and yellow colors have been obtained, while in "Lalima" the original grayed red petals having yellow chimera, two pure yellow color mutants have been isolated differing in their spoon-shaped and flat petals (Fig. 14.1c; Misra et al. 2004). In "Maghi white," a tubular variety has been isolated from the spoon-shaped florets (Fig. 14.1d). In "Sunil" variety silver-colored flowers on the basal side of the florets (Fig. 14.1e) have been obtained while in "Puja," dark-colored flowers (Fig. 14.1f). A large flower variety of Chrysanthemum cv. "Madam E. Roger" has been commercially propagated on a large scale through tissue culture. In vitro mutagenesis could be obtained and a solid mutant of yellow flower color was obtained in the field after treatment with 1 Gy γ-radiation dose (Fig. 14.1g, h; Misra and Datta 2007). Data were recorded regarding their morphological characters.

In vitro mutagenesis via direct regeneration helped in the development of solid mutants without diplontic selection in less duration. The mutants obtained with

Fig. 14.1 In vitro mutagenesis in chrysanthemum cvs.; (**a**) shoot induction in ray florets; (**b**) multiplication of shoots; (**c**) Mutants of "Lalima" cv. showing different shapes and color of the florets; (**d**) "Maghi" mutant with tubular florets; (**e**) "Sunil" mutant with tubular silvery basal portion; (**f**) Mutant of "Puja" cv. with dark-colored flowers; (**g**, **h**) Mutant of "Madam E. Roger," a large flower variety with yellow flower

different type of variations were clonally propagated through tissue culture and sold to the consumers.

14.2.2 *Polianthes tuberosa* L. (Tuberose)

Tuberose is generally propagated through bulbs. The normal cv. has a very good rate of multiplication, but it has been observed that varieties developed through gamma radiation have very low rate of propagation. That is why gamma ray-induced chlorophyll-variegated mutants of tuberose, i.e., Rajat Rekha (having white streak along the midrib) and "Swarn Rekha" (having yellow streaks at the margins), originally developed in NBRI, had been clonally multiplied through axillary buds. These plants were having ornamental value even without flower or at their vegetative stage. To increase the rate of multiplication, tissue culture method was used, as the conventional rate of multiplication was very slow in both of these cultivars. The regenerated shoots were all true-to-mother type having similar streaks. There is very limited work done on micropropagation of tuberose. Little information is available on shoot regeneration from callus cultures of tuberose (Murlidhar and Mehta 1982; Waithaka 1986; Shen et al. 1991).

Leaf explants of tuberose were used for regeneration of shoots in the medium containing BAP, GA_3, or $AgNO_3$ either alone or in combination. After 4 weeks of culture initiation, small nodular structures were differentiated from the cut ends of the lower segments of the leaf in the combination of 1.0 mg/l BAP with 15 mg/l GA_3 or 5 mg/l $AgNO_3$, but the number of shoots was small (3 and 2, respectively, Fig. 14.2a, b), while the other combinations did not show any shoot formation. The nodular structures were yellowish green, rounded, shiny, and smooth in appearance (Figs. 14.2c). The maximum number of nodular structures was formed when all these growth regulators were used in combination.

The nodular structures ceased to differentiate further if they remained in the same high concentration of the growth regulators. Therefore, a lower concentration of BAP (0.05 mg/l) + abscisic acid (ABA, 0.1 mg/l) + GA_3 (5 mg/l) + IAA (0.5 mg/l) was used. In this treatment, these nodular structures started differentiating into shoot buds (Fig. 14.6d, e). Six to seven shoots differentiated from each segment in 4-week periods. Sanyal et al. (1998) reported shoot organogenesis from leaf callus in ordinary tuberose, whereas clonal propagation of *P. tuberosa* var. "Rajat Rekha" has been reported by Datta et al. (2002) where shoot buds were regenerated in two steps directly from the leaf segments. Rooting was obtained in the presence of 0.5 mg/l NAA. After 7 days, rooting could be achieved in 90% shoots. The rooted shoots were transplanted to pots and grown initially under glasshouse conditions and later in the field where they flowered true-to-type.

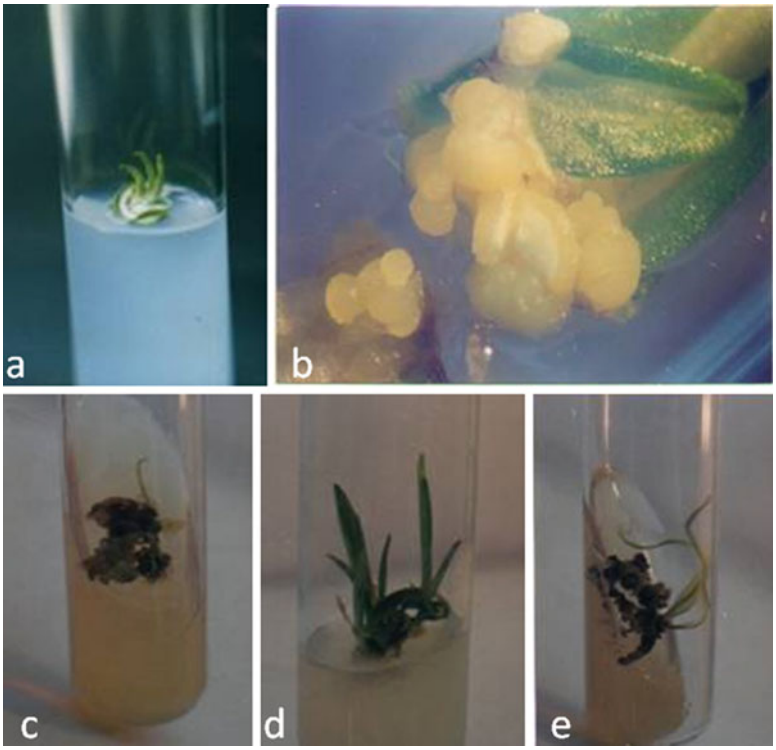

Fig. 14.2 Clonal propagation of *Polianthes tuberose*

14.2.3 Asiatic Hybrid Lilies

From aseptically proliferating shoots, basal leaf portions were used as the explants for in vitro mutagenesis. Three treatments of 0.25, 0.5, and 1.0 Gy γ-radiations were given to the explants under aseptic condition. It was found that 1.0 Gy treatment was lethal to the explants and they could not regenerate shoots, while the other two treatments regenerated shoots and developed two types of mutants, besides several abnormal regenerants. Among the desired ones, Mut 1 was having spotted petals and the other Mut 2 showed white variegation along the margins of leaf lamina. Mut 1 regenerated similar flowers in the next generation; however Mut 2 could not develop flowering for the next two generations, but it was having ornamental value even without flowering.

14.3 Tissue Culture of Some Economically Important Plant Species

14.3.1 *Jatropha curcas*

Jatropha curcas belonging to family Euphorbiaceae is a biodiesel plant, perennial, drought resistant, fast growing, and easy to propagate which can withstand with minimum inputs (Bush and Leach 2007). Its seeds produce oil, which can be combusted as fuel without being refined (Keith 2000; Li et al. 2007). Oil yield of *Jatropha* varieties ranges between 30 and 37 %. Therefore, the use of elite variety is very much necessary for planting material which can assure 20 % more biodiesel yield (Misra et al. 2010a). In view of *Jatropha* plant's life approximately 50 years, the quality of planting material is bound to have long-term effects. To meet the demand of elite *J. curcas* plant for its quality planting material, the development of a standardized protocol for its propagation through tissue culture was urgently required. Screening of four high-yielding elite accessions of *J. curcas* was done for shoot organogenesis from the leaf explants. Leaves were the preferred explants than shoot tips for direct shoot organogenesis as it is the prerequisite for genetic transformation with less chances of chimera development (Misra et al. 2010a). However, indirect shoot organogenesis is likely to produce somaclonal variants.

14.3.1.1 Shoot Organogenesis from Leaf Explants

Evaluation of leaf's position on the plant was performed in terms of number of shoots regenerated, percentage of responding cultures, and condition of the explants in culture. It was observed that the second and third leaves were the best responsive by regenerating maximum number of shoots within 4–6 weeks of incubation, therefore selected as the best. From the cut surfaces of leaf lamina, direct differentiation of shoot buds occurred (Fig. 14.3a–c) in a combination of IBA and BA, where a maximum of seven shoot buds regenerated from one leaf segment within 6 weeks of culture incubation (Table 14.1). TDZ is crucial for indirect differentiation of shoots. Formation of shoot buds was nonsynchronous, where new buds continued to form, while the older ones grew into shoots (Fig. 14.3f) (Misra et al. 2010a).

14.3.1.2 Use of Antibiotics to Control Endophytic Bacteria

The severe problem under in vitro culture of *J. curcas* was the presence of endophytic bacteria that expressed itself in the medium after 2–3 subcultures. Firstly, these bacteria did not interfere in the formation and development of new shoots, but later they influenced the progress of newly developed shoots. These shoots turned yellowish brown and emerged as necrosed making it inconceivable to grow cultures beyond three subcultures. The intractable quandary of endophytic bacterial contamination stands resolved by way of suitable antibiotics, and such cultures are being grown for a longtime period of over 2 years without any contamination (Misra et al. 2010a).

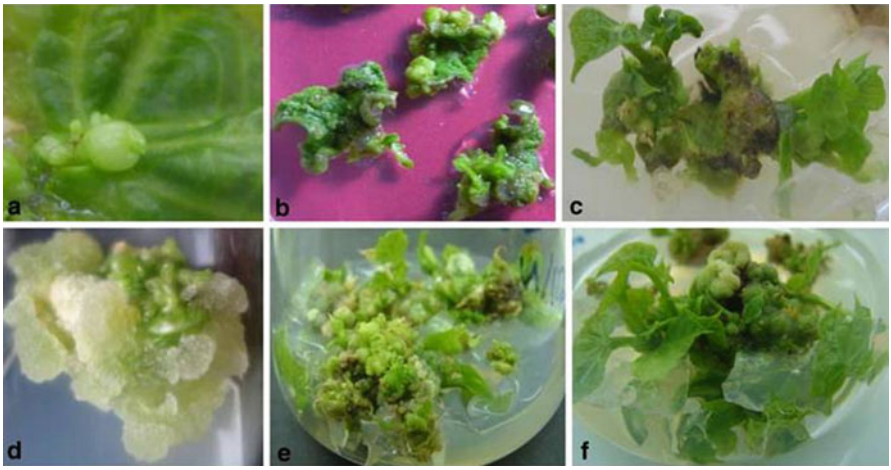

Fig. 14.3 Cultures of *Jatropha curcas*; (**a–c)** Direct shoot organogenesis from leaf lamina; (**a**) from basal portion of leaf having veins; (**b**) from margins/cut surfaces of segments of leaf lamina; (**c**) from adaxial and abaxial surfaces of segments of leaf lamina; (**d–f)** Indirect shoot organogenesis in leaf callus; (**d**) shoot organogenesis from leaf callus; (**e**) different stages of developing shoot buds; (**f**) growing shoots

The bacterial colonies were identified as *Enterobacter ludwigii*. The ring diameter across the antibiotic stab was measured and antibiotics were selected as highly effective (+++), moderately effective (+), and resistant without ring (R, Figs. 14.4a, b and 15.5). Data was recorded on various parameters, such as control of bacterial contamination, number of cycles to control bacterial contamination, and the growth and regeneration of proliferating shoots (Table 14.2). It was observed that the entire tremendously potent antibiotics used were equipped to control the bacterial contamination completely in the first cycle itself; however, those didn't help plant growth and regeneration of shoots. Only augmentin was effective at all the four concentrations used and did not adversely impact regeneration of shoots even at a higher concentration of 400 mg l^{-1} for one cycle. At the beginning, it was used at a higher concentration of 400 mg l^{-1}, where cultures remained fresh, green, and healthy. Later, its concentration was decreased from 300, 200, to 100 mg l^{-1} in subsequent cycles/subcultures depending on the intensity of bacterial contamination. Further, it was observed that 50 mg l^{-1} augmentin was required for maintenance of long-term proliferating cultures. The proliferating shoots of *J. curcas* remained fresh, green, and regenerative in this optimized medium up to 45 days despite of subculture (Misra et al. 2010a).

14.3.1.3 Effect of Antioxidants on Growth of Cultures

Despite the fact that shoot multiplication and their growth was not a problem in *J. curcas*, the regenerating shoots became brown/necrosed within 15–20 days of culture incubation. Owing to browning of tissue in *Jatropha*, it became intricate to

Table 14.1 Influence of growth regulators on direct shoot organogenesis from leaf lamina of *J. curcas* after 6 weeks of culture

Treatment (conc. mg l^{-1})		% of explants responding ± S.E.	Mean of regenerated shoot buds ± S.E.[a]	Associated callus	Health status of regenerated shoots
BA 0.25	IBA 0.1	68.4 ± 1.08	4.6 ± 0.46	–	Shoots were fresh, green, and healthy
BA 0.5		83.6 ± 0.46	6.8 ± 0.60	–	
BA 1.0		70.0 ± 1.52	5.8 ± 0.60	+	
BA 0.25	IBA 0.2	56.8 ± 0.96	4.2 ± 0.34	+	
BA 0.5		63.8 ± 1.22	6.2 ± 0.52	+	
BA 1.0		51.8 ± 1.15	3.8 ± 0.34	++	
Kn 0.25	IBA 0.1	–	–	+	Shoots were yellowish green
Kn 0.5		–	–	+	
Kn 1.0		13.4 ± 0.73	2.6 ± 0.22	++	
Kn 0.25	IBA 0.2	–	–	+	
Kn 0.5		–	–	++	
Kn 1.0		14.6 ± 0.88	1.2 ± 0.34	++	
TDZ 0.25	IBA 0.1	29.2 ± 1.31	3.6 ± 0.46	+	Shoots were vitrified
TDZ 0.5		42.0 ± 0.85	4.0 ± 0.63	++	
TDZ 1.0		35.4 ± 1.43	3.8 ± 0.52	+++	
TDZ 0.25	IBA 0.2	39.6 ± 1.29	2.2 ± 0.34	++	
TDZ 0.5		46.2 ± 0.96	3.4 ± 0.46	+++	
TDZ 1.0		38.0 ± 1.00	3.2 ± 0.52	+++	

Culture incubation: 45 days
"+" sign denotes increasing amount of callus
[a]Mean of 5 replicate cultures

continue long-term cultures. The addition of antioxidants in the medium individually and in combinations analyzing the antioxidant enzyme activity finally could control the browning/necrosis of growing shoots of *J. curcas* (Misra et al. 2010b). We studied the activities and changes in antioxidant enzymes, concentrations of pigments (chlorophylls and carotenoids), and total phenolic compounds in relation to the growth and development of shoots (Misra et al. 2010b).

These studies overcome the hitherto difficulty of browning of in vitro cultures and achieved a foolproof robust protocol for large-scale propagation of tissue-raised plants of elite variety of *J. curcas*. A set of different antioxidants, viz., reduced glutathione, tocopherol, ascorbic acid, and cysteine, were used in the medium to solve the problem of tissue browning and necrosis. The addition of antioxidants proved invaluable for the progress of the shoots. Overall growth of shoots was observed better with the addition of antioxidants and the shoots remained fresh, green, and

Table 14.2 Effect of commonly used plant antibiotics on contaminated regenerating cultures of *J. curcas*. Culture incubation: 30 days

Name of antibiotics (conc. mg l^{-1})		Removal of bacteria	Growth of the proliferating shoots		
			Associated callus	Mean number of regenerated shoots/explant[a]	Health status of the shoots
Novamox	250	+++**	++	0	Yellowish green shoots
	500	+++*	++	0	
Augmentin	100	+++***	+++	4.2 ± 0.52	Green, healthy, and growing shoots
	200	+++**	+++	7.2 ± 0.77	
	300	+++*	++	4.6 ± 0.46	
	400	+++*	++	3.8 ± 0.34	
Pelox®	250	+++**	–	0	Shoot necrosis
	500	+++*	–	0	
Gatiquin®	250	+++**	–	2.7 ± 0.32	Shoot necrosis
	500	+++*	–	0	
Lomadey®	250	+++**	–	0	Shoot necrosis
	500	+++*	–	0	
Genticin®	250	+++**	+++	0	Shoots remained green and healthy
	500	+++*	+++	0	
Ciplox®	250	+++**	+	1.4 ± 0.46	Shoots became yellowish green
	500	+++*	–	0	

"+" sign denotes positive response to some extent; "++" sign denotes positive response to moderate extent; "+++" sign denotes complete positive response
"–" sign denotes no response
"*"sign denotes number of cycles
[a]Mean of 5 replicate cultures

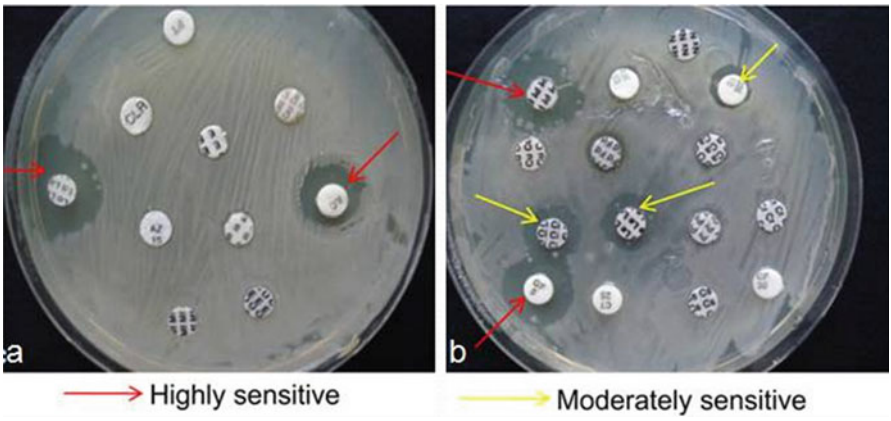

Fig. 14.4 (**a**, **b**) Sensitivity of bacteria toward different antibiotics

healthy for a longer period as against the shoots devoid of antioxidants. Regeneration of shoots was not at all affected by the addition or lack of antioxidants in the medium. The treatment having 25 mg l^{-1} GSH + 10 mg l^{-1} AA was selected as the best for biomass accumulation, where shoots remained fresh, green, healthy, and regenerative up to 40 days (Misra et al. 2010b).

14.3.1.4 In Vitro Rooting and Acclimatization of Plantlets

By the addition of CW, augmentin, and AdS in the multiplication medium, percentage of rooting and survival of acclimatized plants in the field has been improved. Healthy shoots with broad leaves were produced by the addition of 15 mg l^{-1} CW from green coconut in the multiplication medium, which resulted in improved rooting of 85 % as against 52 % reported earlier (Fig. 14.5a). The use of augmentin in the medium not only overcame bacterial contamination but additionally resulted in higher survival and growth of plants during the course of hardening (Fig. 14.5b). The rooted shoots/plantlets were acclimatized in the soilrite with a 100 % transplant success. The plants grew vigorously under net-house conditions (Fig. 14.5c, d).

Even though shoot multiplication used to be just right in *J. Curcas*, the progress of regenerating shoots, low rooting percentage, and problematic acclimatization of tissue-raised plantlets, restrict clonal propagation for quality planting material. Our research work improved the rooting percentage and maximized the survival of plants in the field with the addition of coconut water (CW), augmentin, and adenine sulfate (AdS) in the multiplication medium (Toppo et al. 2012).

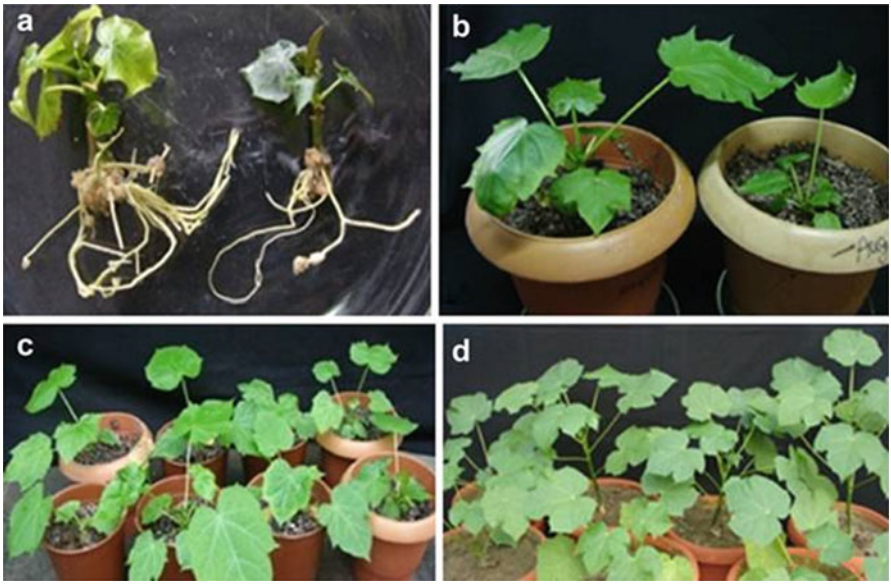

Fig. 14.5 (**a**) Rooting in excised shoots, with augmentin (Lt) and without augmentin (Rt); (**b**) 4-week-old potted plants with augmentin (Lt) and without augmentin (Rt); (**c**, **d**) 8-week-old potted plants in the field without augmentin (**c**) and with augmentin (**d**)

14.3.2 *Withania somnifera*

14.3.2.1 Selection of Maximum Regenerative Explants and Optimization of In Vitro Propagation

Withania somnifera (Fam. Solanaceae) commonly known as ashwagandha, is a high-value medicinal plant since ancient times. Leaves and roots of this plant are widely utilized in preparations of various herbal drugs or home-made remedies for its biological activities as anticancerous, anti-proliferative, anti-oxidative, anti-inflammatory, antiarthritic, antibacterial, and antidiabetic properties. It has different chemotypes varying in their regenerative potentiality. The regenerative potential of different chemotypes of *W. somnifera* was optimized and compared on the basis of shoot organogenesis in leaf segments and their further growth and multiplication.

Highly regenerative protocol for in vitro direct shoot regeneration from different explants of *Withania somnifera* was developed. Leaf, cotyledonary leaf, hypocotyl, and root explants were inoculated on MS medium fortified with different concentrations of BAP, GA_3, and IAA. Regeneration of shoot buds was obtained on MS medium supplemented with BAP and IAA and elongation of shoots was obtained on MS medium supplemented with BAP, IAA, and GA_3. Cotyledonary explants were found best responsive among all the explants. Rooting of excised shoots was obtained on MS medium supplemented with IBA. Thereafter, the in vitro-regenerated plantlets could be acclimatized successfully in soil with 98 % survival percentage.

Nodal explants of *W. somnifera* produced multiple shoots from both the ends of the explants in MS medium containing BAP, Kn, and TDZ within 3 weeks. Maximum number of shoots differentiated in MS medium having BAP. In vitro rooting was induced successfully on half MS medium supplemented with IBA. The plantlets with a well-developed root system were acclimatized and established in pots containing soil/leaf manure (3:1) and grown under greenhouse conditions with survival rate of 94 % (Saema et al. 2015a).

14.3.2.2 A Comparative Analysis of Antioxidant Protection System on Regeneration Potential of Different Chemotypes of *W. somnifera*

The regenerative potentiality of four chemotypes of *W. somnifera* was compared using leaf explants. The study correlated that the chemotype (NIMTLI-130) with high total phenolic content (TPC), high H_2O_2 concentration, and low proline content was less regenerative, whereas the chemotype (NIMTLI-101) with lesser TPC, lower H_2O_2 concentration, and high proline content was more regenerative. In NIMTLI-130, although the activity of SOD, GPX, and APX was higher, CAT being lower in activity along with simultaneous lower proline content had a weak detoxification effect and might be one of the factors for lower regeneration as compared to NIMTLI-101 which has higher CAT activity. The study revealed that the difference in in vitro shoot organogenesis of different chemotypes largely depends on the antioxidant protection system of the plant involving nonenzymatic compounds and activities of antioxidant enzymes (Singh et al. 2015).

14.3.2.3 Monthly Evaluation of Different Withanolide Contents

W. somnifera NMITLI-118 has been propagated on a large scale through tissue culture. About 500 tissue-raised plants were grown in the field. Monthly evaluation of withanolide A, withaferin, and withanone was done for 12 complete months from these field-grown plants. Different parts of plant, e.g., upper and lower leaves, stems, roots, flower buds, and fruit cover, were collected at fifth day of every month from the field for 1 year. For that month, average temperature and humidity was recorded. The plant materials were air dried at room temperature and subsequently chemically analyzed using high performance liquid chromatography. Concentration of different withanolides varied considerably in different plant parts with the variation in the season. Among different tissues, leaves were found to be superior to other plant parts with regard to withanolide production, which can be collected without any damage to the plants. Over the course of the growing season, we found a significant increase in the withanolide contents. The effect of plant maturity on withanolide production was studied in 1-3 month old plant and the data was compared with 1 year old plant. All the three withanolides were found in in vitro shoots, although their amount was far less than field-grown plant material. The site of biosynthesis of withanolides seem to be the leaves, as the concentration of withanolides under in vitro condition remained very high in the leaves both before and after root induction. However, withanolides were in very low quantity in roots under in vitro condition. Total plant withanolide A content was highest (0.081 %) in the month of January, whereas withaferin and withanone (0.158 % each) were highest in the month of November (paper communicated). The present findings might be useful to harvest the material with increased concentration of withanolides throughout the year.

14.3.3 *Hemidesmus indicus*

Hemidesmus indicus R. Br. is a highly reputed plant for its medicinal value in the Indian system of medicine. Recently, this plant has been reported helpful in several free radical-mediated disease conditions. In addition, few antioxidants, lupeol, a triterpene; vanillin, a phenolic; and rutin, a flavonoid, also occur in this plant. This further boosts the medicinal value of the plant. We have developed a regenerative protocol for propagation of this plant from shoot tips and nodal explants where we have observed that the regenerated plants have similar secondary metabolites to their parent plants (Mishra et al. 2003). Due to overexploitation, this plant is becoming increasingly rare; therefore, tissue-raised plants and cultured tissues can be helpful for the extraction of important secondary metabolites.

H. indicus could be established under aseptic culture through shoot tips and nodal stem segments. It has been investigated that defoliation of leaves could be checked with the addition of AdS in the proliferation medium. The plants of *H. indicus* have successfully been transplanted in the combination of sand + soil + leaf manure (1:1:1) (Mishra et al. 2003). The secondary metabolite contents of lupeol,

vanillin, and rutin in the in vitro-grown shoots and the mother plant of *H. indicus* were estimated and found similar to their parents (Mishra et al. 2005). Besides optimization of micropropagation protocol, the purpose of study was to explore whether in vitro production of antioxidants is possible from *H. indicus* cultures, and if so, at what levels of production. This is the first report on in vitro production of antioxidants from this plant to the best of our knowledge.

14.3.4 Gerbera Tissue Culture

Gerbera is a temperate climate plant that grows in the gardens throughout the world. It produces one of the most well-liked ornamental flowers and is used as cut flower and as a potted plant. Therefore it is of high economic importance. Only few cultivars of gerbera could grow in subtropical climate. For availability of this crop in north Indian plains, like Lucknow, tissue culture is the only alternative method that can be applied for commercial purposes. Tissue culture of gerbera has been accomplished, but the determination of compatible cultivars developing in hotter climates, their regeneration advantage, and the selection of proper explants to obtain highest number of shoots in minimal period are the principal concerns of gerbera growers. Different cultivars with their different explants were exploited for in vitro culture. In gerbera, the whole plant is rosette like with short stem which touches the soil. Therefore, it is difficult to establish contamination-free explants in vitro such as nodal segments or shoot tips because they carry soil particles. Inflorescence (young capitulum) and inflorescence stalk is the best material for establishment and regeneration of shoots in gerbera. We have established, regenerated, and grown seven different cultivars of gerbera under in vitro condition. We have done a comparative study of establishment of explants, the regeneration potential, and growth behavior of different cultivars so as to help the growers to select the best cultivar to grow under Lucknow climate (Misra et al. 2010c).

Four cultivars of gerbera, namely, "Dana Ellen," "Sunway," "Salvador," and "Rosaline," were established easily in culture. "Silvester," "Goliath," and "Zingaro" took a long time (4–6 weeks) for establishment. This was caused by bacterial contamination as well as browning of explants. Bacterial contamination was controlled if an unopened inflorescence was used as explant. Data of total phenolic content (Fig. 14.6a) and H_2O_2 concentration (Fig. 14.6b) of seven cultivars revealed that these three cultivars had the higher amount of total phenolics as well as H_2O_2 concentration. 15 mg l^{-1} ascorbic acid and cysteine proved beneficial in controlling browning of explants in "Silvester," "Goliath," and "Zingaro." Capitulum and peduncle both regenerated shoots. Shoot buds regenerated early in peduncle than in capitulum (Table14.3). Upper end of peduncle, which is close to the receptacle, was more regenerative (Fig. 14.7a, b). The purple hair called pappus bristles which cover the florets shed off before starting the developmental process of shoot primordial (Fig. 14.7c). Shoot development in the presence of TDZ was associated with callus formation (Fig. 14.7d), while direct shoot regeneration occurred in presence

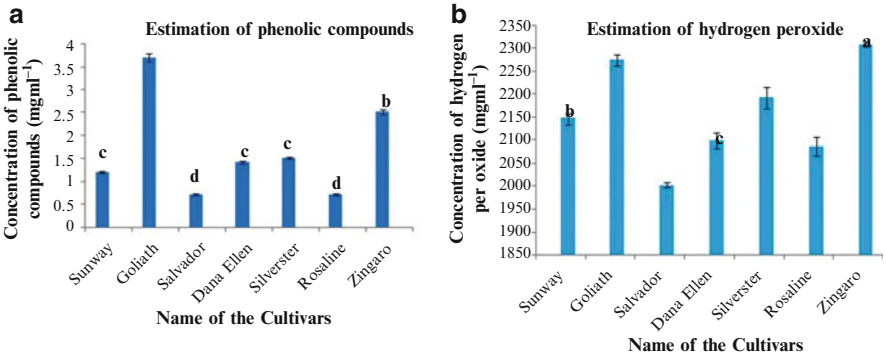

Fig. 14.6 (**a**) Estimation of phenolic compounds in different cultivars of gerbera; (**b**) estimation of hydrogen peroxide in different cultivars of gerbera

Table 14.3 Shoot bud differentiation in different cultivars of gerbera

	Response[1]					
	Type of explant					
	Capitulum[2]		Peduncle[3]		Sepal	
Name of cultivars	Avg. no of regenerated shoots ± SE	Time (d)	Avg. no of regenerated shoots ± SE	Time (d)	Avg. no of regenerated shoots ± SE	Time (d)
Dana Ellen	8.8 ± 0.2[b*]	30	11.2 ± 0.3[a]	20	4.4 ± 0.1[a]	40
Salvador	10.5 ± 0.3[a]	20	9.8 ± 0.2[b]	28	4.0 ± 0.2[a]	42
Rosaline	6.0 ± 0.2[d]	40	8.6 ± 0.4[c]	30	3.2 ± 0.1[b]	45
Silvester	5.2 ± 0.3[e]	42	8.0 ± 0.2[c]	32	–	
Sunway	5.0 ± 0.2[e]	45	7.0 ± 0.2[d]	35	–	
Goliath	8.0 ± 0.4[b]	35	5.0 ± 0.3[e]	42	–	
Zingaro	7.2 ± 0.4[c]	38	5.4 ± 0.2[e]	44	–	
Mean ± S.E.	7.243 ± 0.76		7.857 ± 0.85		1.657 ± 0.79	
F value among the cultivars	36.717***		76.017***		516.463***	

Culture incubation: 45 days
[1]Average of three repeated experiments
[2]Capitulum cut into two to four segments
[3]Longitudinal section of peduncle (ventral view)
*Values followed by the same letters are not significantly different according to Turkey range test
***Significant at $p \leq 0.001$

of BA (Fig. 14.7e). Capitulum when inoculated as such, did not respond, while a good number of shoots regenerated when it was cut into 2–4 segments (Fig. 14.7f, g). It is possible that cut surfaces absorb nutrients and growth regulators, which induced formation of shoots in the growing tissue of florets causing reversal of inflorescence

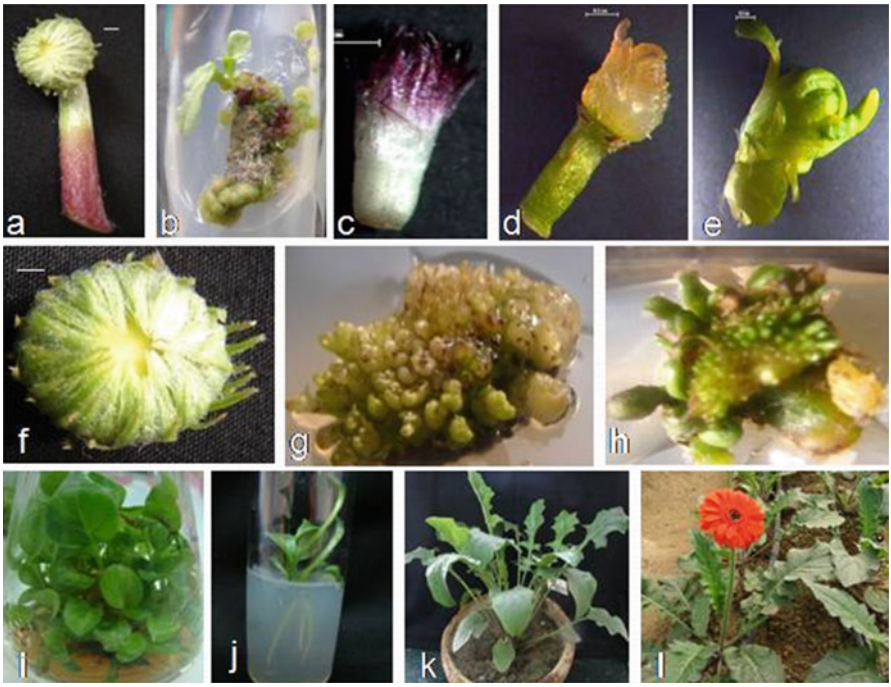

Fig. 14.7 In vitro culture of gerbera; (**a**) an inflorescence; (**b**) shoots differentiating from upper portion of peduncle; (**c**–**e**) conversion of disk floret to shoots; (**f**) A capitulum; (**g**, **h**) shoots differentiating from capitulum; **i** multiplication of gerbera shoots; (**j**) rooting in isolated shoots; (**k**) growing plant in pot; (**l**) a gerbera plant in flowering

axis into vegetative axis (Fig. 14.7h). Differentiated shoots were multiplied, rooted, hardened, and flowered (Fig. 14.7i–l) (Misra et al. 2010c).

14.3.5 *Rosa clinophylla*

Lots of *Rosa* species in India are discovered in temperate Himalayan regions. *R. clinophylla* Thory. is the one species which grows gregariously but not often obvious along the base of the Himalaya eastward to west Bengal near marshy locations and streams. This species grows wildly, hence considered as the wild rose of tropical tracts of India. The species is being used in breeding especially for evolving tropical *Rosa* hybrids to cultivate in the north Indian plains, and that is why it is highly endangered. It provides heat tolerance and evergreen foliage to the breeding strains. A protocol for successful clonal propagation of this important and endangered rose species has been developed.

The plants of *R. clinophylla* were propagated through their nodal segments (Fig. 14.8a). Approximately four to six shoots differentiated from each node but the growth and general health of the regenerated shoots was not very good. However,

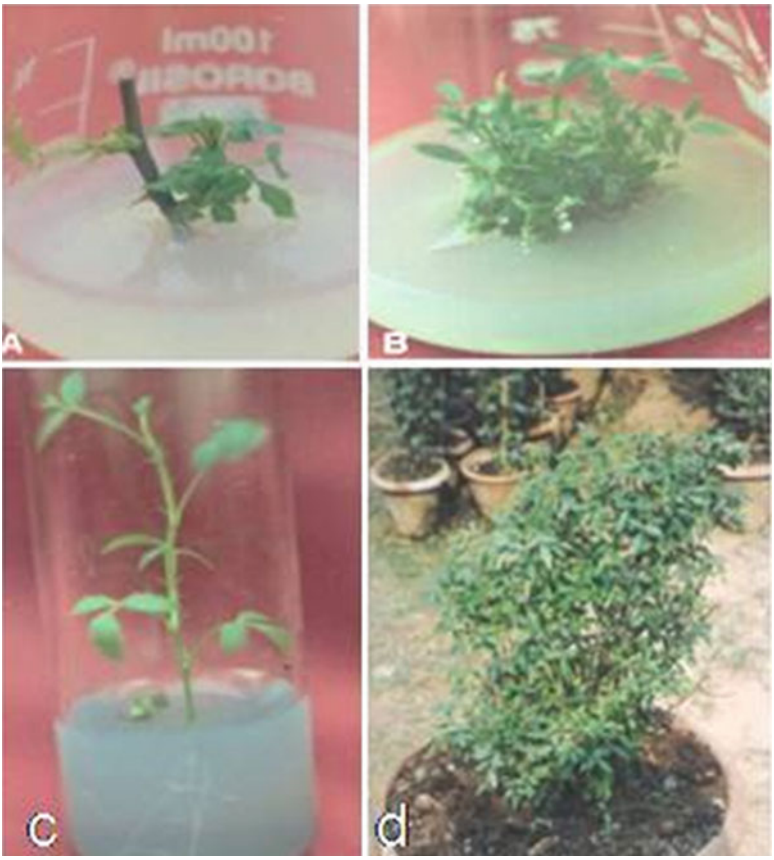

Fig. 14.8 Propagation of *Rosa clinophylla*; (**a**) establishment from nodal segment; (**b**) multiplication of shoots; (**c**) in vitro rooting in isolated shoot; (**d**) in vitro-raised plant in pot

with the addition of $AgNO_3$ in the proliferation medium, their growth improved to some extent (Fig. 14.8b). The well-grown shoots were rooted with IBA in the presence of charcoal (Fig. 14.8c). Activated charcoal played a major role in achieving hundred percent rooting. The rooted plantlets were growing well under field conditions and later flowered also (Fig. 14.8d; Misra and Chakrabarty et al. 2009).

Because the plant species is important for breeding purposes and the planting material is also scarce and not available due to shortage of the plants, there is a necessity of clonal propagation of this plant on a large scale by way of tissue culture. So far, there was no publication reported on micropropagation and field transfer of this plant species; however, a lot more work has already been reported on different aspects of tissue culture of rose.

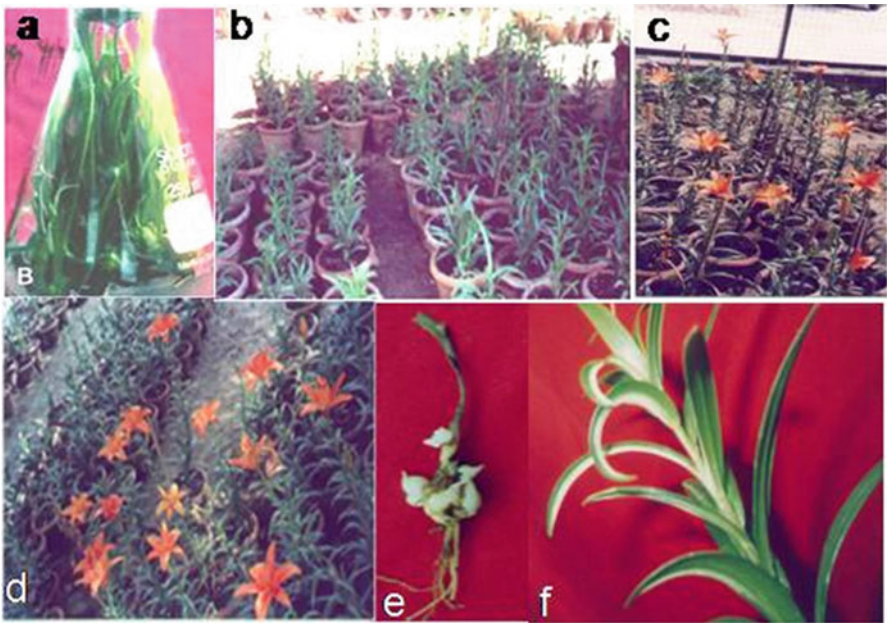

Fig. 14.9 Large-scale propagation of Asiatic hybrid lily; (**a**) multiplication of shoots; (**b–d**) in vitro-raised plants flowering under open field condition

14.3.6 Asiatic Hybrid Lilies (*Lilium* sp.)

The temperate climate floricultural crops are generally loved by all and always remain in great demand all over the world. The cultivators of subtropical climate, such as the fertile tracts of Gangetic basin, are unable to cash this opportunity. For this reason, a protocol for mass multiplication and acclimatization of this temperate climate Asiatic hybrid lily to the subtropical climate of Lucknow was developed using tissue culture (Misra and Datta 2001).

Asiatic hybrid lily was propagated through bulb scale segments. A good rate of proliferation has also been achieved by standardizing the nutrient formulation (Fig. 14.9a). The shoots having well-developed roots were acclimatized initially under controlled conditions and later transferred in the field where they grew well (Fig. 14.9b). The plants flowered with bright orange color in the month of late April to the middle of May 2001 (Fig. 14.9c, d). After flowering, there was a formation of a new bulb at the base of the older ones (Misra and Datta 2001), which could not be formed by conventional methods under Lucknow climate (Fig. 14.9e). Flowering in tissue-raised Asiatic hybrid lily under normal field conditions was achieved continuously under subtropical climate conditions, and after every flowering a new bulb regenerated at the base of the older ones.

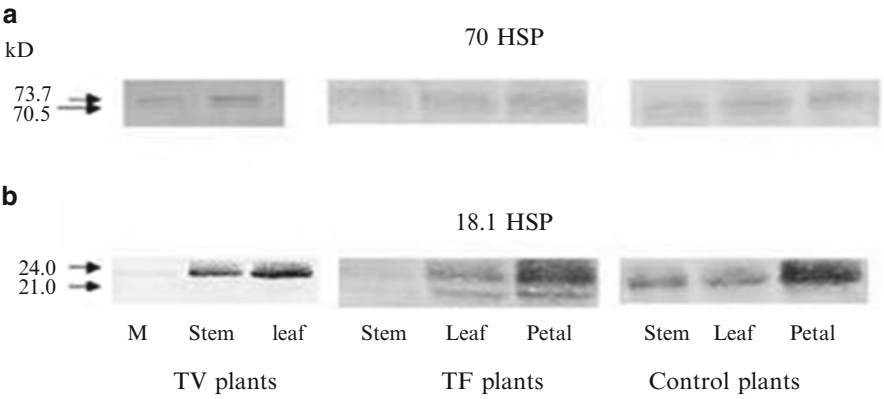

Fig. 14.10 Characterization of (**a**) high and (**b**) low molecular mass heat-shock proteins in tissue-raised flowering plants and control plants of Asiatic hybrid lily under high temperature conditions

With gamma ray treatment, variegation could be developed in the leaves enhancing the ornamental value of the crop even if not flowering (Fig. 14.9f). The control, in vitro-raised plants and their different parts showed differential up- and downregulation of the activity of different antioxidant enzymes such as SOD, POX, CAT, APX, and their isoenzyme patterns. High and low molecular mass heat-shock proteins were characterized using HSP70 and HSP 18.1 antibodies against pea (*Pisum sativum* L.), respectively. The level of high molecular mass proteins did not vary much and was analyzed to be of constitutive nature, whereas in TF plants, a new small molecular mass of 21 kDa was induced indicating a possible role of this stress protein during acclimatization and flowering of Asiatic hybrid lily plants at higher temperature of 43 °C (Fig. 14.10). This protein was much higher in amount in petals as compared to stem and leaf (Misra and Kochhar 2008). The involvement of antioxidant enzymes during the process of shoot and root organogenesis was also studied in callus of Asiatic hybrid lily (Misra et al. 2010d).

14.3.7 *Tagetes erecta* L. (Marigold)

14.3.7.1 F_1 Hybrid of Marigold

In marigold F_1 hybrid varieties are commercially very much exploited. Cytoplasmic/genetic male sterile lines have been developed and used for synthesizing F_1 hybrids of ornamental and horticultural crops. Breeders have to maintain the parents and every time crossing is being done to produce F_1 hybrid. In the same way, users also have to purchase seeds every year from the breeders as there is no seed setting in F_1 hybrids (Misra and Datta 2000). Regeneration protocol has been developed in two cvs. of F_1 hybrids of yellow marigold (*Tagetes erecta* L.), "Pusa Basanti" and "Pusa Narangi."

14.3.7.2 Direct Differentiation from Leaf Segments

Experiments were conducted to directly differentiate shoots from leaf segments of both the cultivars of marigold. Leaves were excised from in vitro-proliferated shoots and cultured on MS medium supplemented with BA alone or with TIBA. TIBA has been used as an antiauxin and played a very crucial role in shoot organogenesis by suppressing root/callus formation. After 3 weeks of incubation, direct shoot bud formation was observed only from basal leaf segments of "Pusa Basanti." Medium containing only BA failed to induce shoots, while all the combinations with TIBA were effective in inducing direct differentiation of shoots (Datta et al. 2002). The maximum number of shoots (8) differentiated in 0.2 mg/l TIBA + 0.25 mg/l BA within 4 weeks. All the responding explants were subcultured onto the same medium for further multiplication. Shoots of 2–3 cm length were excised and transferred to MS basal medium without any growth hormone for rooting. One hundred percent rooting was observed within 1 week. After 2 weeks, rooted shoots were transferred to soil, kept in a hardening chamber for 2 weeks, and then finally moved to the field conditions, where they flowered true-to-type (Misra and Datta 2000).

14.3.7.3 Direct Differentiation from Ray Florets

Surface-sterilized ray florets of both the cultivars of marigold have been grown on MS medium containing either BA + NAA or TDZ + NAA. In cultivar "Pusa Narangi," direct shoot regeneration from the basal portion of the ray floret was observed after 10 days of inoculation in the presence of TDZ and NAA only, while other growth regulator combinations were ineffective (Datta et al. 2002). An average of six shoots per responding explants were differentiated.

14.3.7.4 White Marigold

White marigold (Fam. Asteraceae (Compositae) is among the primary decorative crops, but as a result of nonavailability of planting material, it has not been commercialized properly. Due to low seed viability and poor germination rate (30%), availability of white marigold is not as much as that of the yellow one. Because of its tenderness, white marigold requires totally favorable climatic conditions for vegetative growth as well as for flowering. It is tricky to keep pure line seeds due to its cross-pollinated nature. Tissue culture is the only method to hold genetically identical clones with snow white flower color and fast propagation rate as well as conservation of germplasm for longer periods. The work has already been published for the propagation through shoot tips (Fig. 14.11a) and nodal stem segments (Fig. 14.11b) by Misra and Datta (1999) and through direct differentiation in leaf segments (Fig. 14.11c) by Misra and Datta (2001).

Leaf segments had been used from in vitro-established multiple shoots of white marigold. GA_3 plays an important role for the induction of shoot organogenesis as well as to suppress callus formation. Shoot buds (two to five) could be differentiated from lower petiolar portion of leaf lamina within 4 weeks of incubation in the culture. Differentiated shoots could be grown well with the addition of $AgNO_3$ (Fig. 14.11d). Well-grown shoots were rooted in the medium supplemented with NAA (Fig. 14.11e) and transplanted in the soil. It was observed that all the desired

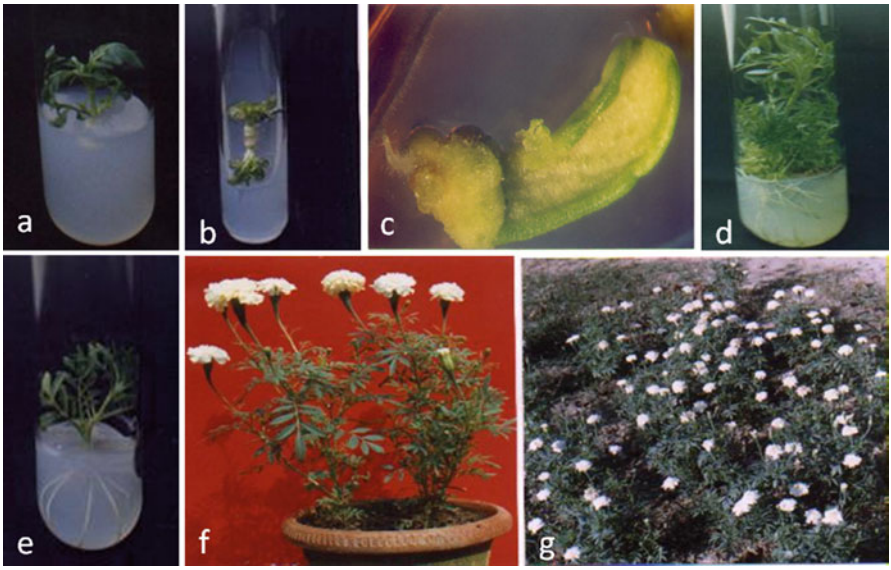

Fig. 14.11 Clonal propagation of white marigold through shoot tips (**a**) nodal segments (**b**) and leaf segments (**c**)

characters, such as plant height, number, and size of flowers per plant, number of viable seeds per flower, and time taken to bloom and the F_1-generation plants were significantly better in tissue-raised plants as compared to control, hence increasing the commercial value of the tissue culture-raised plants in successive generations (Fig. 14.11f, g) (Misra and Datta 2001).

14.3.8 *Papaver somniferum*

Embryos were germinated into plantlets which were later transferred in the field after proper hardening. The plants flowered and developed capsules in due course of time. Acclimatization and field transfer of tissue-raised plants of opium is comparatively difficult and has not been reported earlier. *Papaver somniferum* is an important pharmaceutical plant containing several benzylisoquinoline alkaloids including the narcotic morphine which is analgesic and the codeine which is an antitussive drug. Breeding, selection, and other molecular practices have produced a number of different germplasm with modulated biosynthesis and accumulation of altered particular alkaloids. At present, there is need to modulate biosynthetic pathways of these alkaloids using transgenic technique, for which an efficient regeneration protocol is very much required in opium poppy. We have seen responses of vegetative explants of the seeds for somatic embryogenesis and developed an efficient protocol for *P. somniferum* for regeneration as well as transplantation in the field (Pandey et al. 2010). Root explants induced embryogenic callus in the

presence of 2,4-D and kinetin, maturation in the presence of GA and ABA, and plantlet development into hormone-free 1/2 MS media. Efficient somatic embryogenesis has been achieved but only from the root callus. Alkaloid content was also analyzed in callus and field-grown plants concluding into developmental control of alkaloid biosynthesis (Pathak et al. 2012).

14.4 Optimization of *Agrobacterium*-Mediated Genetic Transformation

14.4.1 *Jatropha curcas*

Genetic transformation of *J. curcas* was already reported in the literature through cotyledon explants (Mazumdar et al. 2010; Pan et al. 2010), but due to origin from seedlings, cotyledons are supposed to be heterozygous in nature. Leaf explants are preferred for direct shoot organogenesis as it is prerequisite for genetic transformation. Moreover, the chances of development of chimeric plants after genetic transformation are lesser when leaf is used as explant. Genetic transformation from in vitro-leaf explants and the hypocotyl segments of in vitro-grown seedlings of *J. curcas* have not been reported earlier. An efficient transformation protocol was developed using these explants through *Agrobacterium tumefaciens* (Misra et al. 2012). Our protocol can be used for transfer of any oil biosynthetic pathway gene in an elite plant.

An efficient transformation protocol has been optimized for *J. curcas* using LS and HS through *A. tumefaciens*. It was demonstrated that the incubation time for the activation of *A. tumefaciens* was optimized to 40 min, while the infection time to 30 min. The explants (both HS and LS) showed similar transient expression of *gus* gene after 3 and 4 days of cocultivation (Fig. 14.12a–d). The putative transformants were confirmed with primary PCR using CaMV (F)-Tnos (R) primers, showed positive plants both from HS and LS which were further confirmed for the presence of transgene by secondary PCR using gus (F) and (R) primers (Fig. 14.13a). The *gus* histochemical assay was also performed to confirm transformants (Fig. 14.14i–l). Expression of gene was checked by RT-PCR (Fig. 14.13a, b). HS produces four transgenics, two highly expressed, one low expressed, and one with very low expression, while LS produced five transgenic plants, two highly expressed, two low expressed, and one with very low expression. Stable TE was more in LS (5 %) as compared to 4 % in HS (Misra et al. 2012).

14.4.2 *Withania somnifera*

An efficient genetic transformation protocol of medicinal plants with unique metabolic pathways is important to understand the molecular basis and secondary metabolism regulation in plants so as to engineer them as per requirement. However, plants with high secondary metabolite content and even with a well-defined

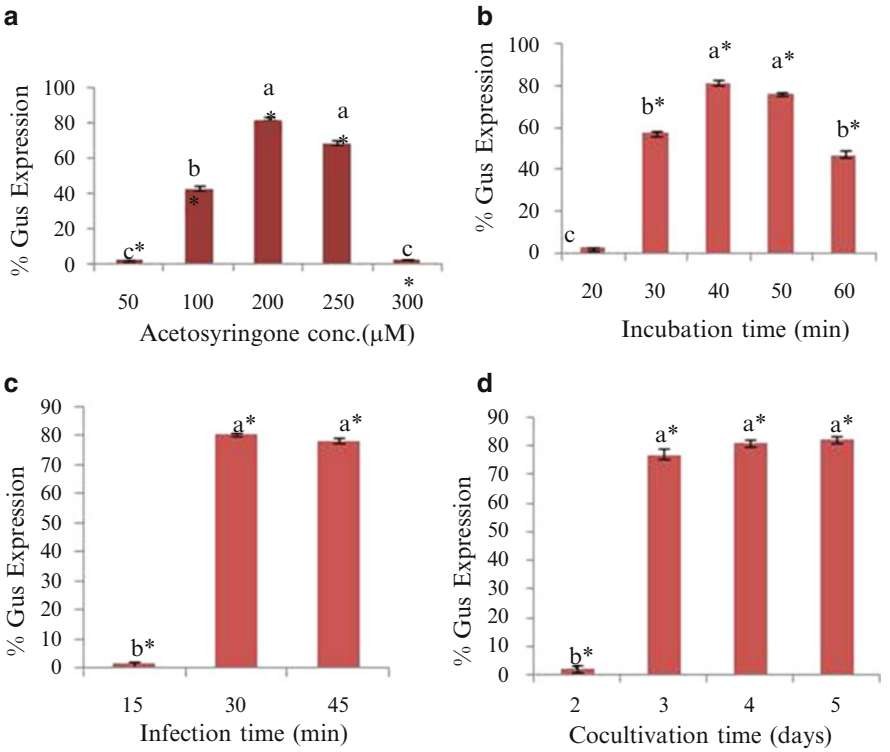

Fig. 14.12 (**a**–**d**) Histochemical GUS expression in respect to (**a**) acetosyringone concentration; (**b**) incubation/activation time (min); (**c**) infection time (min); and (**d**) cocultivation time (days). *Values followed by the same letters are not significantly different according to Duncan's multiple range test at 5 % level

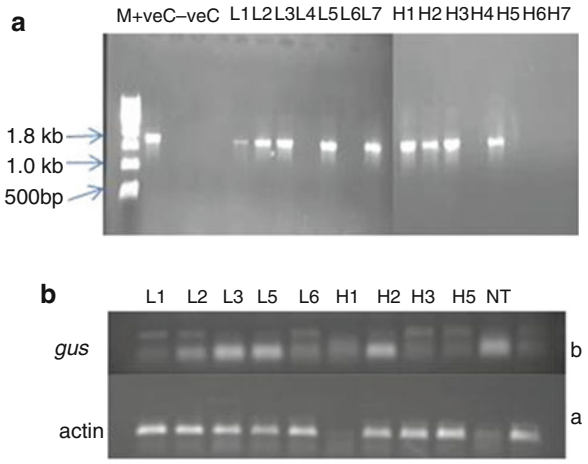

Fig. 14.13 (**a**) PCR analysis of transformants of *Jatropha curcas* with GUS (F) and (R) primers; (**b**) RT – PCR for the detection of GUS expression

Fig. 14.14 GUS histochemical assay of putative transformants

regeneration system have been observed as recalcitrant to regeneration after genetic transformation. In *W. somnifera*, hairy roots were developed after transformation using *A. rhizogenes*, but that results in the production of only those chemical compounds which are being synthesized in the roots itself (Ray et al. 1996; Murthy et al. 2008). Also, development of transgenic plants in *W. somnifera* was not reported

earlier. We have reported for the first time a protocol for genetic transformation of *W. somnifera* through *A. tumefaciens* using pIG121Hm as vector harboring *gusA* as a reporter gene (Pandey et al. 2010). In addition, the plants developed normally and were fertile. The developed protocol of *W. somnifera* could be useful for the transfer of target genes for the production of phytosteroids. It provides a robust tool to study the function of genes of steroidal transformations precisely associated to this medicinal plant and their role in plant growth and development and for biochemical pathway engineering in *W. somnifera*. Moreover, the protocol developed can also be used for the development of stress-tolerant *W. somnifera*.

We have observed the feasibility and effectiveness of the commonly used LBA 4404 strain of *A. tumefaciens* strain, containing the binary vector with pIG121hm plasmid, for transformation of *W. somnifera* through LS. The super virulent strain EHA101 was not found effective for this transformation. Through this study, stable transgenic plants of *W. somnifera* were obtained. The three important aspects were observed crucial for getting stable transgenics. First was the selection of suitable explants, their position on the plant, age, and having high regeneration ability. The second aspect was the standardization of different parameters for transformation, such as the preparation of explants, type of *A. tumefaciens* strain, optical density, sonication, infection time and cocultivation time, etc. The third aspect was the selection of antibiotics and their concentration, selection of transformed tissue, optimization of minimum inhibitory concentration (MIC), regeneration after selection, and their growth, hardening, and acclimatization of the transformed plants to the soil.

An optimization of transformation parameters produced fertile plants and also the increased transient transformation efficiency (TTE). Glasshouse-grown leaves proved as much better explants as compared to the leaves from in vitro-grown plants in terms of shoot regeneration. The second leaf from the tip *W. somnifera* NMITLI 130 was the most regenerative and efficient in comparison to the youngest leaf. Acetosyringone at 100 μM enhanced the efficiency of transformation. The presence and expression of the transgenes in T_1 progeny was confirmed by RT-PCR (Fig. 14.15a, b). Southern blot analysis was performed to confirm the integration of *gusA* gene (Fig. 14.15c). The TE was 1.67 % (Pandey et al. 2010).

14.5 Functional Analysis of Sterol Glycosyltransferase Genes of *Withania somnifera*

Sterol glycosyltransferases (SGTs) are the enzymes that catalyze glycosylation of sterols and play an important role in adaptation of the plant during stress and hold medicinal value in plants like *W. somnifera*. Glucosylation of sterols are catalyzed in the presence of SGTs by transferring sugar moieties to diverse sterol molecules, leading to cellular homeostasis. SGTs are the members of family 1 glycosyltransferases (Chaturvedi et al. 2011). *W. somnifera* is a medicinal plant known for its secondary metabolites and is a rich source of sterols, steroidal lactones, and sterol glycosides. Sterols and their modified glycosides have different medicinal

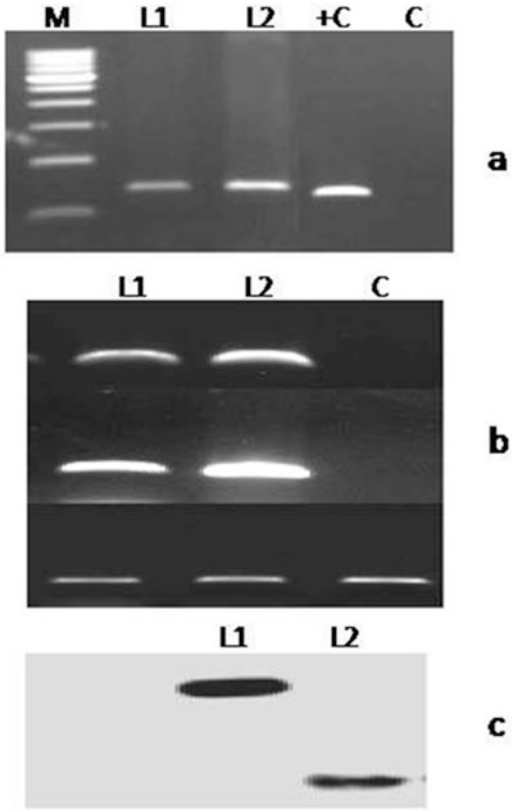

Fig. 14.15 Detection of transformants in W. somnifera RSS-30 plants. (**a**) PCR for the detection of gusA gene in T0 transformants. M 500-bp ladder, L1–L2 putative transformants, +C positive control, C nontransgenic control; (**b**) RT-PCR for the detection of gusA expression in T1 transgenic lines. L1–L2 putative transformants, C nontransgenic control; (**c**) Southern hybridization for the establishment of insertion events in stable T1 transgenic lines. L1–L2 transgenic lines

importance and play a role in adaptation of the plant during stress. In response to external stimuli, the expression of these *sgt*s was differentially modulated in different parts of *W. somnifera*. A family of *sgt* genes is expressed in *W. somnifera*, and these are functionally recruited under stress conditions (Sharma et al. 2007; Chaturvedi et al. 2012). The genes involved in sterol modification are important in view of medicinal value and understanding the stress. We worked on functional characterization of these *sgt* genes of *W. somnifera* in homologous and heterologous expression systems (*N. tabacum* and *A. thaliana*) developing overexpression as well as suppression lines using artificial miRNA and siRNA technology to do their functional analysis.

14.5.1 Overexpression of *WsSGTL1* Gene of *W. somnifera* in *Arabidopsis* Plants

WsSGTL1 is one of the members of *SGT* gene family and has 66 % homology with *AtSGT* of *A. thaliana*. Therefore, we have initially transferred *WssgtL1* gene in the heterologous expression system of *Arabidopsis* to understand its role in plant's

responses to heat, cold, and salt stress and to analyze the phenotypic and physiological changes. The activity of the transgene and the stress-responsive genes was analyzed using quantitative real-time PCR and semiquantitative RT-PCR. The promoter analysis of this gene showed potential cis-acting elements against salt, heat, and cold stress which suggested the regulation of *WssgtL1* gene during stress in *W. somnifera* (Mishra et al. 2013).

The *Agrobacterium*-mediated transformation of *WsSGTL1* gene in *A. thaliana* was done, using the binary vector pBI121 via floral dip method. Different parameters, such as germination, shoot weight, root length, relative electrolyte conductivity, MDA content, relative electrolyte leakage, SOD levels, and chlorophyll measurements, were compared between transgenic and wild-type (WT) *Arabidopsis* plants under different stresses – salt, heat, and cold. Biochemical profiling was done by HPLC-TLC and radiolabeled enzyme assay. The *WsSGTL1* promoter was cloned and the 3-D structures have been predicted.

The transgenic plants transformed with *WsSGTL1* were confirmed to be single copy with the aid of Southern and homozygous by segregation evaluation. The transgenic plants showed higher germination and better tolerance to salt, heat, and cold as compared to WT. The expression of *WsSGTL1* transgene was elevated during heat, cold, and salt stress vis-a-vis other marker genes, such as HSP70, HSP90, SOS3, RD29, and LEA4-5. Biochemical analysis revealed the formation of sterol glycosides and increased enzymatic activity. It was observed that the promoter of *WsSGTL1* gene contained stress-responsive elements. Bioinformatic analysis of the 3D structure of *WsSGTL1* protein demonstrated its functional similarity with sterol glycosyltransferase (AtSGT) of *A. thaliana*.

Transformation of *A. thaliana* with *WsSGTL1* gene conferred tolerance to abiotic stress. The promoter of *WsSGTL1* gene was having stress-responsive elements. The 3D structure illustrated the functional similarity with sterol glycosyltransferases.

14.5.2 Transformation of *WsSGTL1* Gene of *W. somnifera* in *N. tabacum* Plants

WsSGTL1 gene was overexpressed and functionally characterized in transgenic tobacco plants. Transgenic tobacco lines showed an adaptive mechanism by showing late germination, yellowish green leaves, stunted growth, and enhanced antioxidant system. *WsSGTL*1-*Nt* plants were observed with reduced chlorophyll and chlorophyll fluorescence with decreased photosynthetic parameters. These modifications were supposed to be due to the enhanced glycosylation by *WsSGTL1*, as chlorophyll biogenesis-related genes were not observed modulated in transgenic lines as compared to WT plants. More accumulation of major sterols in glycosylated forms, like campesterol, stigmasterol, and sitosterol, was observed in transgenic plants. In addition, other secondary metabolites in relation to plant's antioxidant system were enhanced in *WsSGTL*1-*Nt* along with activities of antioxidant enzymes (SOD, CAT; two to fourfold) when compared to WT plants. It was observed that *WsSGTL*1-*Nt* plants reduced larval weight of *Spodoptera litura* (biotic

stress) up to 27 % and improved survival capacity of leaf disks against salt stress showing a significant resistance toward both biotic and abiotic stresses. This study signifies that increased glycosylation of sterols and enhanced antioxidant system due to the overexpression of *WsSGTL*1 gene confer specific functions in plants to adapt under different environmental stresses (Pandey et al. 2014).

14.5.3 Overexpression of *SGTL1* Gene of *W. somnifera* in Homologous Expression System

WsSGTL1 gene was also expressed in *W. somnifera* to further authenticate its functional significance. *A. tumefaciens*-mediated transformation was performed for the development of transgenics of *W. somnifera* using cotyledonary leaf segments. Overexpressed *WsSGTL1-W. somnifera* lines were confirmed by Southern blot analysis followed by quantitative RT-PCR. The *WsGTL1* transgenic plants showed a number of variations at phenotypic and metabolic level when compared to WT plants. An early and enhanced growth was observed with expanded leaves and increase in number of stomata. An increase in production of glycowithanolide (majorly withanoside V) and glycosylated forms of campesterol, stigmasterol, and sitosterol with reduced accumulation of withanolides (withaferin A, withanolide A, and withanone) were observed. Tolerance toward biotic stress (100 % mortality of *Spodoptera litura*) and improved survival capacity under cold stress was also demonstrated. In addition, transgenic plants showed enhanced recovery capacity after cold stress, as indicated by better performance of photosynthesis, chlorophyll, anthocyanin content, and better quenching regulation of PSI and PSII. Our studies revealed that overexpression of *WsSGTL1* gene was responsible for increased glycosylated withanolide and sterols and conferred better growth and tolerance to plants against both biotic and abiotic stresses (Saema et al. 2015b).

14.5.4 *WsSGTL1* Gene Silencing in *W. somnifera*

14.5.4.1 Effect of RNAi-Mediated Gene Silencing in *W. somnifera*

Silencing of *WsSGTL1* gene via RNAi demonstrated that *WsSGTL1* is responsible for glycosylation of withanolides and sterols in addition to growth and development of *W. somnifera*. RNAi construct (pFGC1008-*WsSGTL1*) was prepared and *A. tumefaciens*-mediated genetic transformation was done in *W. somnifera*. Biochemical analysis by HPLC observed the reduction of withanoside V (the glycowithanolide of *W. somnifera*), increased amount of withanolides (majorly withaferin A) contents, and also major decrease in the level of glycosylated sterols (Saema et al. 2015c).

14.5.4.2 Functional Analysis of Members of *WsSGTLs* Using aMIR-VIGS

We have characterized the function of *SGTs* by silencing *SGTL1*, *SGTL2*, and *SGTL4* in *W. somnifera*. Downregulation of *SGTs* leads to accumulation of withanolide A, withaferin A, sitosterol, stigmasterol, and decreased content of withanoside V in transgenic lines. This was further confirmed by the increased expression of *WsHMGR*, *WsDXR*, *WsFPPS*, *WsCYP710A1*, *WsSTE1*, and *WsDWF5* genes involved in withanolide biosynthesis. These variations of withanolide concentrations in transgenic lines resulted in the pathogen susceptibility as compared to control plants. The infection of *Alternaria alternata* causes increased salicylic acid, callose deposition, superoxide dismutase, and H_2O_2 in transgenic lines. The expression of biotic stress-related genes, namely, *WsPR1*, *WsDFS*, *WsSPI*, and *WsPR10*, were also enhanced in transgenic lines in a time-dependent manner showing that the defense system of the plant turns on the SAR-mediated pathway against the fungal stress. Taken together, our observations revealed that a positive feedback regulation of withanolide biosynthesis occurred by silencing of *SGTLs* which resulted in reduced biotic tolerance (Singh et al. 2015). After downregulation of *SGTL* gene family of *W. somnifera*, the glycosylation of withanolide, intermediate phenolics of PP pathway, and the level of SA decreased which affect the defense system of the plants. After pathogen attack, these intermediate phenolics, salicylic acid, level of reactive oxygen species, and H_2O_2 have significantly increased in downregulated lines as compared to control plants. The expression of defense-related gene *WsPR1*, *WsDFS*, *WsSPI* and *WsPR10*, increased with the infection time showing that defense system of the plant turn on the SAR-mediated pathway against the fungal stress.

14.6 Conclusion

In *J. curcas*, the establishment of field-grown plant material was very problematic because of dormant bacterial contamination, although most of the plant parts regenerated shoots very early. The endophytic bacterial contamination has been removed by adding antibiotics in the medium along with growth hormones after which shoots remained healthy and regenerative without any subculture for 45 days. Multiple shoots could be conserved for long term for over 2 years with no bacterial contamination. Necrosis was also the main constraint for obtaining quality planting material for large-scale propagation of *J. curcas* during long-term culture. We have overcome this problem of necrosis by using different antioxidants and their combinations in the medium. Healthy shoots were observed in the presence of CW and augmentin with 100 % acclimatization success, whereas shoots grown in the absence of CW and augmentin have small leaves and were prone to different soil microbes leading to higher mortality of tissue-raised plants.

Tissue culture is an appropriate technique used to popularize/acclimatize gerberas and Asiatic hybrid lilies in adverse/subtropical climatic conditions of north Indian plains and also to obtain quality planting material on a large scale. An

evaluation has been done on the problems related with establishment, shoot multiplication, and propagation of some distinguished cultivars so as to obtain plants early and in large numbers to benefit gerbera growers. It could be concluded that "Salvador," "Dana Ellen," and "Rosaline" are the best gerbera cultivars to grow in this climate, whereas "Silvester," "Goliath," and also "Sunway" are not performing well under field conditions.

For commercial floriculture, we need novelty in flower color and shape. In vitro mutagenesis is an applied tool to generate different flower colors and shapes of the florets particularly in the case of chrysanthemums. We have developed several varieties using this technique. Leaf color variegation could also be developed in chrysanthemum, tuberose, and lilies. These plants after leaf variegation remained ornamental even without flowering.

We have developed highly efficient transformation protocols of *J. curcas* and *W. somnifera* through *A. tumefaciens* using leaf explants. Various parameters were optimized but the stable transformation efficiency was found to be 1.67 % in *W. somnifera*; however, in *J. curcas* TE was 5 % in the case of LS and 4 % in HS.

The sterol glycosyltransferase (*sgt*) gene family was identified in *W. somnifera*, and *sgtL1* and *sgtL3.1* of this family were characterized in *E. coli*. The expression of these genes was ubiquitous in all the plant parts. The deduced amino acid sequence showed the presence of putative transmembrane domains; however the selection for glucosylation of membrane sterols by *WssgtL1* suggests its membrane functionality. The recombinant Sgts from *W. somnifera* which were partially purified used to be specific to the sterols having hydroxyl group at C3 position. Members of the gene family were expressed to varying levels in different parts of the plant. Rapid increase in the transcript level of some members of *sgtL* gene family, following MeJA or SA treatments, suggests their role in biotic and abiotic stresses. Functional characterization of this gene by its overexpression and suppression in homologous and heterologous expression system suggests that *WsSGTL* gene family plays a very significant role in the growth and development, metabolic balance, and defense mechanism of the plant system.

14.7 Future Prospects

- Control of endophytic bacterial contamination, removal of browning, and control of necrosis in tissue culture is very important. The methods developed by us was followed and cited by many more researchers working on *J. curcas* as well as on other crops. Improvement of 80 % rooting and hardening of in vitro shoots can lead to successful large-scale propagation of any elite plant of *J. curcas* on commercial scale.
- Commercial growers of gerbera and Asiatic hybrid lilies could be benefited from our study for popularization of these crops. They can initiate gerbera and Asiatic hybrid lilies cultivation under subtropical climate of Lucknow by selecting recommended cultivars from our study.

- Using in vitro mutagenesis, different novelties in flower color and shape of the florets could be developed in floricultural crops.
- Difficult-to-propagate crops like *R. clinophylla*, very important for breeding purposes, could be propagated on a large scale using tissue culture.
- The work on *W. somnifera* can further be used for isolation of withanolides, as the plants raised through tissue culture will be uniform.
- Genetic improvement of a plant requires a transformation protocol. We have developed a transformation protocol which can further be used for the transfer of gene of interest or any gene related to oil biosynthetic pathway to increase the oil yield of *J. curcas*. In the case of *W. somnifera*, the developed protocol could be used for transfer of any gene related to the production of phytosteroids or withanolide pathway-related gene, e.g., sterol glycosyltransferase genes.
- The present work is the detailed knowledge base for the involvement of *WsSGTL1* gene of *W. somnifera* in response to environmental stresses. But there is limited information on the multiple functions of *WsSGTL1* and the biological roles of plant *SGT*s. These are the challenging aspects which need further investigation.
- Resolving the glycosylation steps and finally the entire withanolide biosynthetic pathway at molecular stage must be a major target for future WsSGT study.

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Genetic Engineering for Insect Resistance in Economically Important Vegetable Crops

15

D.K. Srivastava, P. Kumar, S. Sharma, A. Gaur, and G. Gambhir

15.1 Introduction

Vegetables play a vital role in human nutrition and health by providing nutrients, vitamins, antioxidants, phytosterols, and dietary fiber. In the developing world, vegetable farming is a considerable part of the agricultural economy of different nations. Vegetable crop quality and quantity are seriously affected by various biotic and abiotic stresses, which destabilize rural economies in many countries. In the last many decades, conventional breeding has contributed significantly for the improvement of vegetable quality, yields, biotic and abiotic stress resistance, and postharvest management, but there are many constraints in conventional breeding, which can only be overcome by techniques of modern biology for genetic advancements.

Plant genetic engineering techniques have enabled investigators to genetically transform various crop species by introducing desirable foreign gene(s) into plants. Insect pest management using plant genetic engineering techniques in crop plants has advantages of user-friendly, environment-friendly, and consumer-friendly method for crop protection to fulfill the demands of sustainable agriculture (Kumar and Srivastava 2016). Food and energy insecurities are currently two foremost global problems. Losses due to pests and diseases have been revealed around 37 % of the agricultural production worldwide, with 13 % due to insects. Engineering insect resistance in transgenic plants has been obtained through the use of insect control protein genes of *Bacillus thuringiensis*. Till date, researchers have focused on the introduction of genes for the expression of modified *Bacillus thuringiensis* (*Bt*) toxins. Successful results on the control of *Bt*-susceptible pests have been achieved, and *Bt* transgenic crops are being commercialized and used worldwide.

D.K. Srivastava (✉) • P. Kumar • S. Sharma • A. Gaur • G. Gambhir
Department of Biotechnology, Dr Yashwant Singh Parmar University of Horticulture and Forestry, Solan 173230, Himachal Pradesh, India
e-mail: dksuhf89@gmail.com

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15.2 Sources of Insect Resistance Genes

Majority of economically important vegetable crops are severely infested by a number of insect pests. Insect pest management using chemical insecticides/pesticides is an effective strategy but often leads to deleterious effects on human health resulting in environmental contamination and ecological imbalances. Plant transgenic technology for insect resistance provides an effective environmentally safe and durable alternative. The various sources of insect resistance gene(s) are microbial, plant, and animal origin. These insect resistance genes have been isolated, cloned, and transferred to various economically important crops.

Insect resistance genes of microbial origin include insecticidal crystal protein, *cry* gene(s), and vegetative insecticidal protein, *vip* gene(s) (Kumar and Srivastava 2016). *Bacillus thuringiensis* (*Bt*) a common soil bacterium was first isolated in Thuringia region of Germany. *Bt* produces insecticidal crystal protein *cry gene*(*s*) (Table 15.1) that paralyzes the larvae of some harmful insects, including the cotton bollworm and the Asian and European corn borers, all of which are common plant pests whose infestations produce devastating effects on important crops. Mode of action of *Bt* toxin is that when it is ingested by the larvae of target insect, *Bt* protein is activated in the insect midgut alkaline condition and punctures the midgut leaving the insect which is not able to feed to die. The cloning of genes expressing the insecticidal proteins of *Bacillus thuringiensis* (Höfte and Whiteley 1989) has allowed the

Table 15.1 Insect resistance gene(s) of microbial origin (*Bacillus thuringiensis*) and crystal protein gene classification

S. No.	Gene designation	Molecular weight (KD)	Toxicity
1.	*cry IA* (*a*)(*b*)(*c*)	131–133	Lepidoptera
2.	*IB*	137	Lepidoptera
3.	*IC*	134	Lepidoptera
4.	*ID*	133	Lepidoptera
5.	*IE*	137	Lepidoptera
6.	*IF*	134	Lepidoptera
7.	*IG*	130	Lepidoptera
8.	*cry IIA*	71	Lepidoptera, Diptera
9.	*IIB*	71	Lepidoptera, Diptera
10.	*IIC*	71	Lepidoptera, Diptera
11.	*cry IIIA*	73	Coleoptera
12.	*IIIB*	73	Coleoptera
13.	*IIIC*(*a*)(*b*)	73	Coleoptera
14.	*cry IVA*	134	Diptera
15.	*IVB*	128	Diptera
16.	*IVC*	77	Diptera
17.	*IVD*	72	Diptera
18.	*cry V*	80	Lepidoptera, Coleoptera

Source: Höfte and Whiteley (1989)

development of transgene strategies for incorporation of resistance to a variety of lepidopteran and coleopteran insect pests in many agriculturally important crops (Fischhoff et al. 1987; Bottrell et al. 1992; Fujimoto et al. 1993; Fromm et al. 1994; Sharma and Srivastava 2014). Different researchers have isolated the *Bt* gene responsible for the production of the insecticidal protein from the bacterium and introduced it into the genome of plants. Thus, these transgenic plants provide mechanism of protection against targeted pests. Sources of *vip* gene(s) are *Bacillus thuringiensis* and *Bacillus cereus*. A large number of vip (approx. 50) have been identified. Ingestion of vip proteins causes swelling and disruption of midgut epithelial cells by osmotic lysis in the target insect. The different types of *vip* proteins are *vip1* and *vip2* active against coleopteran insects and *vip3Aa1* (*vip3A*) and *vip3Bb1* active against lepidopteran insects.

Insect resistance genes of plant origin include *protease inhibitor* (PI) gene and *lectin* gene. Protease inhibitors act as antimetabolic proteins, which interfere with the digestive process of insects. They inhibit the activity of the gut protease of the insects and reduce the quantity of the proteins that can be digested and also cause hyperproduction of the digestive enzymes which enhances the loss of sulfur amino acids, as a result of which the insects become weak, with stunted growth, and ultimately die (Table 15.2). The first time use of a plant-derived PI gene was described and transformed tobacco plants with trypsin inhibitor gene (*CpTI*) from *Vigna unguiculata* were obtained (Hilder et al. 1987). Regenerated plants expressing *CpTI* under the control of cauliflower mosaic virus 35S promoter had significantly enhanced resistance to *Heliothis virescens*. *α-Amylase* gene and protein α-amylase inhibitors have been isolated from a variety of plant species and microorganisms. α-Amylase inhibitors function in a similar manner as proteinase inhibitors, interfering with insect nutrient utilization. When tested in artificial diet, purified α-amylase inhibitors from wheat showed insecticidal effect to coleopteran pests *Collosobruchus maculatus* and *Tribolium confusum* (Gatehouse et al. 1986). Lectins are carbohydrate-binding proteins found in many plant tissues, but are often present in relatively large amounts (usually approximately 1 % of total protein, but in some species, e.g., *Phaseolus vulgaris*, up to 30 %) in seeds and other storage tissues. The toxic effects of lectin are mediated through its binding to the midgut epithelial cell with consequent disruption of the cell function. The bound lectins may inhibit nutrient absorption or disrupt midgut cells by stimulating endocytosis of the lectin and possibly other toxic metabolites present in the midgut. Most importantly, lectins can be used to control sapsucking insects belonging to the suborder Homoptera, which includes some of the most devastating pests spread worldwide. Various lectins have been proved toxic toward members of Coleoptera, Lepidoptera (Czalpa and Lang 1990), and Diptera (Eisemann et al. 1994).

Insect resistance genes of animal origin include *proteinase inhibitors* (*anti-chymotrypsin* from *Manduca sexta*, *anti-elastase* from *Manduca sexta*), *α-antitrypsin* (*α1AT*) (*antitrypsin* from *Manduca sexta*, *bovine pancreatic trypsin inhibitor* (*BPTI*)/*pancreatic*, *spleen inhibitor* (*SI*)), and *chitinase* (*chitinase* from *Manduca sexta*) (Kumar and Srivastava 2016). It is reported that overexpression of protease inhibitors (PIs) provides protection against different insect species (Wolfson and

Table 15.2 Insect resistance gene(s) of plant origin

S. No.	Gene source/genes	Effect evaluated on predators
A few protease inhibitor genes		
1.	Cowpea trypsin inhibitor (*CpTI*)	Coleoptera, Lepidoptera
2.	Tomato proteinase inhibitor-I	Lepidoptera
3.	Tomato proteinase inhibitor-I I	Lepidoptera
4.	Potato proteinase inhibitor-I (*Pot-PT-I*)	Lepidoptera, Orthoptera
5.	Potato proteinase inhibitor – II (*Pot-PT-II*)	Lepidoptera, Orthoptera
6.	Rice cysteine proteinase inhibitor (*OC-1*)	Coleoptera, Homoptera
7.	Soybean Kunitz trypsin inhibitor (*SKT-1*)	Lepidoptera
8.	Barley trypsin inhibitor (*CMe*)	Lepidoptera
9.	Mustard serine proteinase inhibitor (*MTI-2*)	Lepidoptera
10.	Soybean serine proteinase inhibitor (*PI-IV*)	Lepidoptera

S. No	Plant gene	Encoded protein	Plant of origin
Examples of few α-amylase genes			
1.	*α-AI-Pv*	α-Amylase	Common bean
2.	*WMAI-1*	α-Amylase	Cereals
3.	*14K-CI*	Serine and α-amylase inhibitor	Cereals

S. No	Plant gene	Encoded protein	Plant of origin
Examples of few lectin genes			
1.	*GNA* (*Galanthus nivalis* agglutinin)	Lectin	Snowdrop
2.	*p-lec*	Lectin	Pea
3.	*WGA Agglutinin* (wheat-germ agglutinin)	Lectin	Wheat germ
4.	Jacalin	Lectin	Jackfruit
5.	Rice lectin	Lectin	Rice

Murdock 1987). *Chitinase* expression normally occurs in insects during molting when insects shed their old exoskeleton and peritrophic membrane (both contain chitin as major component) and resynthesize new ones. Thus, insect feedings on plants that constitutively express an insect chitinase gene might be adversely affected. Avidin is a glycoprotein from chicken (*Gallus gallus*) egg white that binds its ligand, biotin, with very high affinity. Biotin is a coenzyme required for all forms of life, so feeding avidin to many insects causes a biotin deficiency that leads to a stunted growth and mortality (Ding et al. 1998a) (Table 15.3).

The different approaches for transferring desirable genes into plant cells (monocot or dicot) may be divided into direct and indirect techniques. Transgenic plants

Table 15.3 Insect resistance gene(s) of animal origin

S. No	Gene product	Target insect
1.	Anti-chymotrypsin from *Manduca sexta*	Homoptera
2.	Anti-elastase from *Manduca sexta*	Homoptera
3.	*α-1AT*(α-antitrypsin)	Lepidoptera
4.	Antitrypsin from *Manduca sexta*	Homoptera
5.	*BPTI* (bovine pancreatic trypsin inhibitor)	Lepidoptera, Orthoptera
6.	*SI* (spleen inhibitor)	Lepidoptera
7.	Chitinase from *Manduca sexta*	Lepidoptera

have been produced in different agriculturally important crops by employing direct and indirect gene transfer techniques. These techniques include *Agrobacterium*-mediated gene transfer and direct gene transfer. After delivery and integration of a gene in the genome, a plant cell can be regenerated into a fertile plant, whose germ cells are capable of transferring the gene to the progeny. The genes are stably integrated into the plant genome and further inherited like dominant Mendelian genes.

Plant genetic engineering techniques have tremendous potential for the improvement of crop plants. Most of the transgenic plants produced to date were created through the use of *Agrobacterium* system (Gasser and Fraley 1989; Jain 1993; Singh and Sansavini 1998; Dunwell 2000; Ranjekar et al. 2003; Cardoza and Stewart 2004; Srivastava 1997, 1998, 2001, 2003, 2012a, b). *Agrobacterium* is called as natural genetic engineer, a causative agent of crown gall disease first recognized by Chilton et al. (1977), who demonstrated that the crown gall was actually produced as a result of the transfer and integration of genes from the *Agrobacterium* (Ti plasmid) into the genome of plant cells. The first transgenic plants expressing engineered foreign genes were tobacco plants produced by the use of *Agrobacterium tumefaciens* as a vector (Horsch et al. 1984; De Block et al. 1984). An efficient method for introducing cloned gene(s) into plant cells was given by Fraley et al. (1983), Herrera Estrella et al. (1983). The most widely used method of gene transfer is particle gene gun method and *Agrobacterium*-mediated gene transfer method.

Genetic transformation of plants to confer resistance to insect pests offers an eco-friendly method of crop protection (Kumar and Srivastava 2016). Significant contributions have been made in developing transgenic crops with resistance to the target insect pests over the past two decades (Vaeck et al. 1987; Chakrabarty et al. 2002). Remarkable results have been reported with the expression of *Bt* (*cry*) genes, cowpea trypsin inhibitor gene, serine proteinase inhibitor gene, and cysteine proteinase inhibitor genes in various crops (Gatehouse et al. 1980; Wolfson and Murdock 1987; Houseman et al. 1989; Johnson et al. 1989; Liang et al. 1991; Bai et al. 1992; Ding et al. 1998b; Jin et al. 2000; Cho et al. 1994; Awasthi 2003; Chakrabarty et al. 2002; Zhang et al. 2004; Paul et al. 2005; Lingling et al. 2005; Zhao et al. 2006; Hua et al. 2009; Deng-Xia et al. 2011; Sharma and Srivastava 2013; Kumar and Srivastava 2016). Such transgenic plants have shown considerable promise in reducing insect damage. Conventionally to protect the crop plants from insect pest attack, a massive application of pesticides has been used which

causes adverse effects on nontarget organisms and the environment. In this chapter, we summarize the genetic transformation studies for insect resistance gene(s) being carried out in economically important vegetable crops such as tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), cabbage (*Brassica oleracea* L. var. *capitata*), cauliflower (*Brassica oleracea* L. var. *botrytis*), broccoli (*Brassica oleracea* L. var. *italica*), brinjal or eggplant (*Solanum melongena* L.), pea (*Pisum sativum* L. var. Lincoln), lettuce (*Lactuca sativa* L.), and bell pepper or *Capsicum* (*Capsicum annuum* L.).

15.2.1 Tomato

Tomato (new name *Solanum lycopersicum*, earlier known as *Lycopersicon esculentum* Mill.) is one of the most important vegetable crops of Solanaceae family grown all over the world for its special nutritive value. It is a diploid plant with $2n = 24$ chromosomes. In India, it occupies an area of 845.574 mha with a production of 18,304.54 million tonnes (National Horticulture Board 2014–2015). The Food and Agriculture Organization (FAO) of the United Nations reported about 161.8 million tonnes world production of tomato, where China, the largest producer, accounted for about one quarter of the global output, followed by the United States and India. Tomatoes, eaten throughout the world, are a rich source of carotene and lycopene, one of the most powerful natural antioxidants, which prevents oxidative damage and has been shown to improve the skin's ability to protect against harmful UV rays. Along with lycopene, tomato contains vitamin A, vitamin C, minerals, and a number of other antioxidants (Aggarwal and Rao 2000). Tomato plants produce the plant peptide hormone system, systemin, after an insect attack so that tomato cultivars vary widely in their resistance to disease. Insect pests of tomato cultivar are stink bugs, cutworms, tomato hornworms, tobacco hornworms, aphids, cabbage loopers, whiteflies, tomato fruitworms, flea beetles, red spider mite, slugs, and Colorado potato beetles. Systemin activates defensive mechanisms, such as the production of protease inhibitors to slow the growth of insects (Garcia et al. 2015). However, the tomato crop is severely affected by the caterpillar of *Helicoverpa armigera*. Total yield loss caused by the direct insect pests on tomato is 5–55 % (Selvanarayanan and Narayanasamy 2006). To check this loss, a lot of pesticides/insecticides are being used on tomato crop which have drawbacks such as damage to the ecological system and residual poisoning of humans and animals. Therefore, it is desirable to develop insect-resistant plants through the introduction of foreign insecticidal genes. A number of strategies including the use of genes for insect resistance such as protease inhibitors, lectins, amylase inhibitors, and delta-endotoxin gene present in a soil bacterium *Bacillus thuringiensis* (Bt) assume major significance (Johnson et al. 1989; Schroeder et al. 1995; Naimov et al. 2003; Lingling et al. 2005; Wang et al. 2005; Ignacimuthu and Prakash 2006; Tiwari et al. 2008; Yazdanpanah et al. 2009). Protease inhibitors have been used for genetic engineering in tomato (McGarvey et al. 1994; Dowd and Lagrimini 1997; Gatehouse 1995). Snowdrop lectin *Galanthus nivalis* agglutinin (GNA) in artificial diet resulted in

Table 15.4 Genetic engineering for insect resistance gene(s) transfer studies in tomato

Crystal proteins	Origin	Target insects	References
Insect resistance genes of microbial origin			
Bt gene	Bacterial	Lepidoptera and coleopteran	Fischhoff et al. (1987)
cry IAb	Bacterial	Lepidoptera	Delannay et al. (1989)
Bt gene	Bacterial	Lepidoptera and coleopteran	Delannay et al. (1989)
Bt gene	Bacterial	Lepidoptera and coleopteran	Liang et al. (1994)
Bt gene	Bacterial	Lepidoptera and coleopteran	Rhim et al. (1995)
cry1Ac	Bacterial	Lepidoptera and coleopteran	Mandaokar et al. (2000)
cryIAb gene	Bacterial	Lepidoptera and coleopteran	Kumar and Kumar (2004)
cryIAc	Bacterial	Lepidoptera and coleopteran	Zhang et al. (2006)
cry6A	Bacterial	Lepidoptera and coleopteran	Li et al. (2007)
cry IAa	Bacterial	Lepidoptera	Sharma and Srivastava (2013)
Insect resistance genes of plant origin			
Tomato proteinase inhibitor-I	Plant	Lepidoptera	McGarvey et al. (1994)
CMV-CP	Plant	Lepidoptera	Liang et al. (1994)
Tomato proteinase inhibitor-II	Plant	Lepidoptera	McGarvey et al. (1994)
CpTI (cowpea trypsin inhibitor)	Plant	Lepidoptera	Gatehouse (1995)
GNA(snowdrop lectin)	Plant	Lepidoptera	Gatehouse (1995)
Tobacco anionic peroxidase	Plant	Lepidoptera, Coleoptera	Dowd and Lagrimini (1997)

insect resistance by controlling insect biomass (Gatehouse 1995). Genes encoding for the delta-endotoxins have been cloned since 1980s (Schnepf and Whitley 1981), and expression of first introduced genes in tobacco and tomato provided the first example of genetically modified plants with resistance to insects (Barton et al. 1987; Vaeck et al. 1989). Tomato plants expressing *cry1A*(*b*) and *cry1A*(*c*) genes have been developed to control lepidopteran insects (Delannay et al. 1989; Mandaokar et al. 2000; Kumar and Kumar 2004; Zhang et al. 2006) (Table 15.4). A protocol for insect resistance gene transfer in tomato has been standardized in our laboratory using *Agrobacterium tumefaciens* strain containing *npt-II* and *cryIAa* genes in binary vector pBin-1Aa. Putative transgenic plantlets of tomato have shown the amplification of *cryIAa* gene thereby indicating the presence/integration of *cryIAa* gene into the genome of transgenic tomato. The expression of *cryIAa*

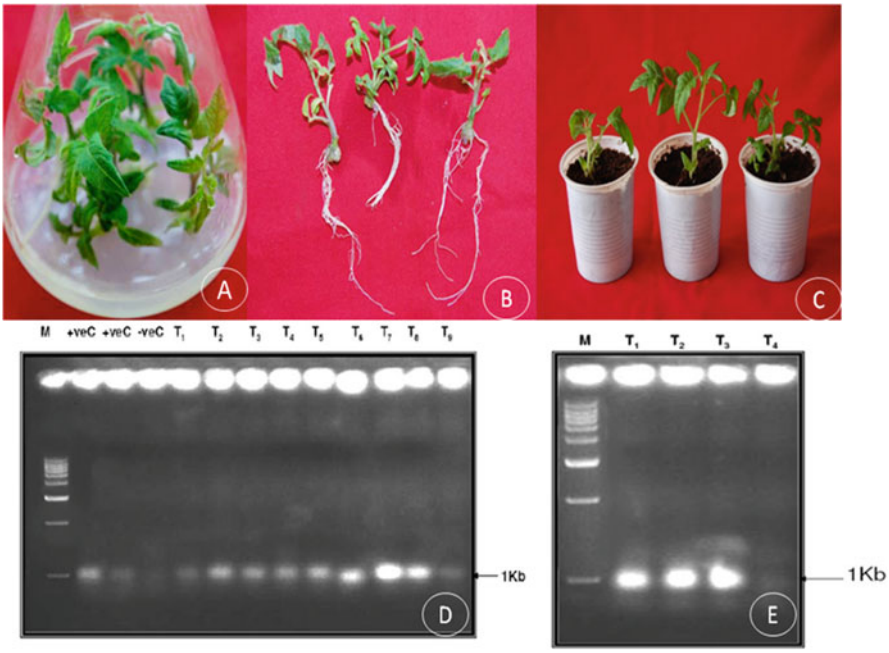

Fig. 15.1 (**a**) In vitro developed transgenic shoots of tomato cv. Solan vajr. (**b**) Root regeneration from in vitro developed shoots of tomato on selective media. (**c**) Young, healthy transgenic plantlets successfully acclimatized on the planting substrate after 4 weeks of hardening and maintained in greenhouse. (**d**) PCR analysis showing amplification of 1 kb of *cryIAa*-specific DNA fragment in regenerated transgenic plantlets of tomato. 1 kb molecular weight marker (M), positive control (+ve C), negative control (-ve C), and (T_1–T_9) independent lineages of putative transgenic plantlets. (**e**) cDNA synthesis by RT-PCR showing the expression of *cryIAa* gene at transcriptional level in the transgenic plantlets of tomato (1 kb molecular weight marker (M), T_1–T_4 independent lineages of transgenic plantlets (plantlets confirmed by PCR using designed primers)) (Source: Sharma and Srivastava 2013)

(insect resistance) gene was studied at the transcriptional level using reverse transcriptase-polymerase chain reaction (RT-PCR) technique. Out of the four samples, three have shown the synthesis and amplification of cDNA thereby indicating that the gene is being expressed at the transcriptional level in these three transgenic tomato lines. A protocol for insect resistance gene transfer in tomato has been standardized (Sharma and Srivastava 2013) (Fig. 15.1).

15.2.2 Potato

Potato (*Solanum tuberosum* L.) is a starchy, tuberous crop from the nightshade family. It is the world's fourth largest food crop, following rice, wheat, and maize. Potato had its origin in southern Peru and extreme northwestern Bolivia, where they were domesticated 7000–10,000 years ago. The potato is best known for its

carbohydrate content (approximately 26 g in a medium potato). Potato is also a rich source of vitamins and minerals, as well as an assortment of phytochemicals, such as carotenoids and natural phenols. Chlorogenic acid constitutes up to 90 % of the potato tuber natural phenols. It provides protection against colon cancer, improves glucose tolerance and insulin sensitivity, lowers plasma cholesterol and triglyceride concentrations, increases satiety, and possibly even reduces fat storage. In India, the total production of potatoes in 2014–2015 was 42,173.977 million tonnes in an area of 1990.053 mha (National Horticulture Board 2014–2015). Due to lack of genetic diversity, the crop is vulnerable to disease. Insects that commonly transmit potato diseases or damage the plants include the Colorado potato beetle, the potato tuber moth, the green peach aphid (*Myzus persicae*), the potato aphid, beet leaf hoppers, thrips, and mites. Various insect resistance strategies have been used by researcher to cope up with the heavy losses caused by insect pest. A gene encoding the mannose-specific lectin from snowdrop expressed in tomato enhanced resistance to potato aphids (Gatehouse et al. 1996, 1997; Shi et al. 1994; Down et al. 1996). Plant-specific protease inhibitors confer insect pest resistance when expressed in potato plants (Burgess and Gatehouse 1997; Lecardonnel et al. 1999). Commercialized GM potato varieties "New Leaf," owned by Monsanto Company, incorporate genes from *Bacillus thuringiensis*, which confers resistance to the Colorado potato beetle; "New Leaf Plus" and "New Leaf Y," approved by US regulatory agencies during the 1990s, also include resistance to viruses (Cornell University); *cryIAb* (Jansens et al. 1995), *cryIAc* (Estrada et al. 2007), and *cry3A* (Douches et al. 1998; Thomas et al. 1997; Perlak et al. 1993) have been expressed in potato plants to provide resistance against insect diseases (Table 15.5).

15.2.3 Cabbage

Cabbage (*Brassica oleracea* L. var. *capitata*) is an important nutritionally rich cole crop of the family Brassicaceae. It is a herbaceous leafy green vegetable crop and a biennial, dicotyledonous flowering plant distinguished by a short stem upon which it is crowded a mass of leaves, usually green but in some varieties red or purplish, which while immature form a characteristic compact, globular cluster. The cultivated cabbage is derived from a leafy plant called the wild mustard plant, native to Eastern Mediterranean region as well as Asia Minor region, where it is common along the seacoast, also called sea cabbage and wild cabbage (Decoteau 2000). Cabbage is a nutritionally rich, good source of beta-carotene, vitamin C, and fibers having anticarcinogenic activity due to the sulforaphane content. Purple cabbage also contains anthocyanins, which in other vegetables have been proven to have anticarcinogenic properties. This vegetable is known for its medicinal properties, declaring that "it is the cabbage that surpasses all other vegetables." This crop is grown in more than 90 countries throughout the world which include countries like China, Japan, Korea, India, and Poland. The Food and Agriculture Organization (FAO) of the United Nations reports that world production of cabbage and other brassicas (these plants being combined by the FAO for reporting purposes) for

Table 15.5 Genetic engineering for insect resistance gene(s) transfer studies in potato

Target gene	Origin	Target insects	References
Insect resistance genes of microbial origin			
cry IIIA	Bacterial	Coleoptera	Perlak et al. (1993)
cry IAb	Bacterial	Lepidoptera	Jansens et al. (1995)
Vip3A	Bacterial	Lepidoptera	Estruch et al. (1996)
Bt cry3A	Bacterial	Lepidoptera	Thomas et al. (1997)
Bt cry3A	Bacterial	Lepidoptera	Douches et al. (1998)
Vip1 and *Vip2*	Bacterial	Coleoptera	Moellenbeck et al. (2001)
cry1Ac	Bacterial	Lepidoptera	Estrada et al. (2007)
Insect resistance genes of plant origin			
p-lec (pea lectin)	Plant	Homoptera and Lepidoptera	Boulter et al. (1990)
GNA (snowdrop lectin)	Plant	Lepidoptera	Gatehouse et al. (1996)
			Shi et al. (1994)
			Down et al. (1996)
BCH (bean chitinase)	Plant	Homoptera and Lepidoptera	Gatehouse et al. (1996, 1997)
CpTI (cowpea trypsin inhibitor)	Plant	Lepidoptera	Burgess and Gatehouse (1997)
C-II (soybean potato serine proteinase inhibitor)	Plant	Lepidoptera	Marchetti et al. (1998)
Rice cysteine proteinase inhibitor	Plant	Lepidoptera	Lecardonnel et al. (1999)
PI-IV (soybean serine-proteinase inhibitor)	Plant	Lepidoptera	Marchetti et al. (2000)
KT13, *SKT1* (soybean Kunitz trypsin inhibitor)	Plant	Lepidoptera	Marchetti et al. (2000)
Insect resistance genes of animal origin			
α-1AT (α-antitrypsin)	Animal	Lepidoptera	Christeller et al. (1992)
SI (spleen inhibitor)	Animal	Lepidoptera	Christeller et al. (1992)
BPTI (bovine pancreatic trypsin inhibitor)	Animal	Orthoptera and Lepidoptera	Christeller and Shaw (1989)

calendar year 2011 was 68,840,531 metric tonnes and cultivated on 2,373,818 ha of land. This was primarily grown in China (43 %) and India (11 % with production of 79,49,000 metric tonnes on 369,000 ha of land). In India, the total production of cabbage in 2014–2015 was 9086.363 million tonnes in an area of 401.481 mha (National Horticulture Board 2014–2015). The cultivation of cabbage is severely infested by a large number of insect pests such as *Plutella xylostella* (diamondback moth, DBM), cabbage looper, imported cabbageworm, cross-striped cabbageworm, and cabbage webworm, which results in great loss of the yield and damage to the quality of cabbage production (Shelton et al. 1982; Gould et al. 1984; Theunissen et al. 1995). The conventional insect control method heavily relies on the intensive and extensive use of chemical pesticides which cause severe environmental

pollution having adverse effects on people and beneficial insects. Moreover, DBM have evolved resistance to chemical insecticides (Hama 1992; Shelton et al. 1993). Genetic engineering has provided a promising method for breeders to obtain insect-resistant plants (Qaim and Zilberman 2003). Various Cry proteins are highly toxic to lepidopterans, coleopterans, or dipterans, but do not harm people or the environment (Crickmore et al. 1998).

Agrobacterium tumefaciens-mediated transformation of cabbage has been reported with *cry1Ab*, *cry1Ac*, and *cry1c* which provided resistance to DBM in tested bioassays of *Bt* cabbage plants (Metz et al. 1995a; Jin et al. 2000; Bhattacharya et al. 2002). Liu et al. (2008) have transformed cabbage with *cry1Ab* gene by direct DNA transfer. Protease inhibitor gene is also effectively used for controlling insect and pest attack. Plant-derived protease inhibitors (PIs) exert lower selection pressure on insect pests and are regarded as a viable alternative to *Bt* endotoxins in insect pest control. PIs have been used to enhance the resistance of transgenic plants to insect pests because of their small size, abundance, stability, and high specificity for a particular class of insect digestive enzymes. Zhang et al. (2004) and Lei et al. (2006) used protease inhibitors in transgenic cabbage. The application of genetic engineering in cabbage cultivation is of great significance to generate new improved and desirable traits like insect- and disease-resistant varieties. Several systems of genetic transformation have been used, but *Agrobacterium tumefaciens* are most common. *Agrobacterium tumefaciens* has been used routinely to obtain transgenic plants, and this technology is now available for many crops (Gasser and Fraley 1989; Day and Lichtenstein 1992; Lindsey 1992; Jain 1993; Dunwell 2000; Srivastava 1997, 1998, 2001 2002, 2003, 2012a, 2012b; Ranjekar et al. 2003; Cardoza and Stewart 2004; Hua et al. 2005; Xing et al. 2008; McPherson and MacRae 2009; Ahmad et al. 2012; Awasthi and Srivastava 2013) (Table 15.6). A protocol for insect resistance gene (*cryIAa*) transfer in cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) has been standardized in our laboratory using *Agrobacterium tumefaciens* strain containing *npt-II* and *cryIAa* genes in binary vector pBin-1Aa. Putative transgenic plantlets of cabbage have shown the amplification of *cryIAa* gene thereby indicating the presence/integration of *cryIAa* gene into the genome of transgenic cabbage. The Southern blot analysis has also been used to confirm copy number of transgene into the genome of cabbage. The confirmation of expression of the transgene *cryIAa* into the genome of cabbage at transcriptional level was confirmed by reverse transcriptase-PCR and real-time PCR and at translational level by bioassay. A protocol for high-frequency plant regeneration and insect resistance gene transfer in cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) has been standardized (Gambhir 2014) (Fig. 15.2).

15.2.4 Cauliflower

Cauliflower (*Brassica oleracea* L. var. *botrytis*) is an important vegetable crop of family Brassicaceae grown for its edible inflorescence (curd). *Brassica* family demonstrates extreme morphological diversity (cauliflower, cabbage, broccoli, Brussels

Table 15.6 Genetic engineering for insect resistance gene(s) transfer studies in cabbage

Plant species/cultivar	Technique of gene transfer	Gene transferred	References
Insect resistance genes of microbial origin			
B. oleracea L. var. *capitata*	*Agrobacterium tumefaciens*-mediated gene transfer	*cryIA*(*c*)	Bai et al. (1992)
B. oleracea L. var. *capitata*	*Agrobacterium tumefaciens*-mediated gene transfer	*cryIA*(*c*)	Metz et al. (1995b)
B. oleracea L. var. *capitata*	*Agrobacterium tumefaciens*-mediated gene transfer	*Bt* gene	Cai et al. 1999
B. oleracea L. var. *capitata*	*Agrobacterium tumefaciens*-mediated gene transfer	*cryIAb3*/*cryI1a3*	Jin et al. (2000)
B. oleracea L. var. *capitata*	*Agrobacterium tumefaciens*-mediated gene transfer	*cryIA*(*b*)	Bhattacharya et al. (2002)
B. oleracea L. var. *capitata*	*Agrobacterium tumefaciens*-mediated gene transfer	*gus* and *cryIAc*	Wang et al. (2003)
B. oleracea L. var. *capitata*	*Agrobacterium tumefaciens*-mediated gene transfer	*cryIB* and *cryIAb*	Paul et al. (2005)
B. oleracea L. var. *capitata*	*Agrobacterium tumefaciens*-mediated gene transfer	*cryIAb*	Venkatesh (2006)
B. oleracea L. var. *capitata*	*Agrobacterium tumefaciens*-mediated gene transfer	*cryIAb*	Liu et al. (2008)
B. oleracea L. var. *capitata*	*Agrobacterium tumefaciens*-mediated gene transfer	*cryIa8*	Lei et al. (2009)
B. oleracea L. var. *capitata*	*Agrobacterium tumefaciens*-mediated gene transfer	*cryIBa3*	Deng-Xia et al. (2011)
B. oleracea L. var. *capitata*	*Agrobacterium tumefaciens*-mediated gene transfer	*cryIAa*	Gambhir (2014)

sprouts, kohlrabi, and kales) in crop forms and therefore holds a great importance in the field of molecular genetics. Most recently the genome sequence of *Brassica oleracea* has been completed by Liu et al. (2014) and reported a 630 Mb genome size with 45,758 protein-coding sequences. Cauliflower is derived from two Latin words *caulos* which means cabbage and *floris* which means flower. Cauliflower is a native of Southern Europe around the Mediterranean coast (Huxley et al. 1992). It was introduced in India by Britishers in the year 1822 (Chatterjee 1986). Cole crops are generally rich in vitamins especially vitamin A and vitamin C. Cauliflower is nutritionally rich in proteins and minerals such as potassium, sodium, iron, phosphorous, calcium, and magnesium (Chaudhary 1996) and low in fat and high in dietary fiber and water content. It also has anticancer value (Zhang et al. 1992). Cauliflower is also reported to have about 70 mg of vitamin A and 75 mg of vitamin C per 100 g of sample and is peculiar in stability of vitamin C after cooking (Singh 1997). India is the second largest producer of vegetables (90 million tonnes) contributing to 9.7 % of the world's vegetable production. Annual production of

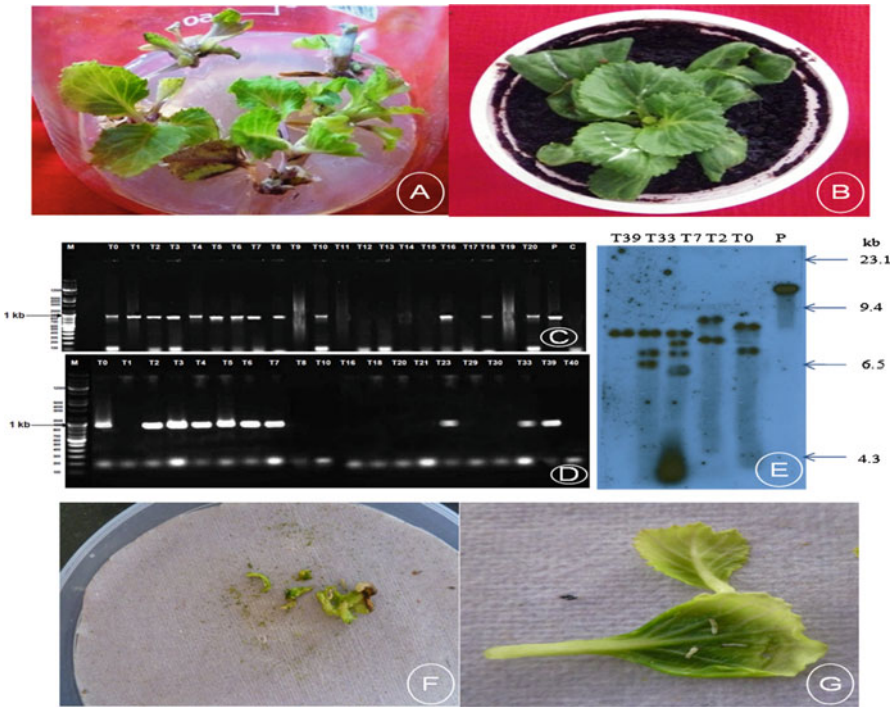

Fig. 15.2 (**a**) Transgenic plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India). (**b**) Acclimatization of in vitro regenerated putative transgenic plantlets of cabbage after 3 weeks. (**c**) PCR analysis showing amplification of 1 kb DNA fragment of *cryIAa* gene in regenerated transgenic plantlets of cabbage cv. Pride of India. (**d**) RT-PCR analysis showing amplification of 1 kb DNA fragment of *cryIAa* gene in regenerated transgenic plantlets of cabbage. (**e**) Southern hybridization of *cryIAa* gene in regenerated transgenic plantlets of cabbage cv. Pride of India. (**f**) In vitro bioassay of non-transgenic (control), live larvae of diamondback moth (DBM) on damaged non-transformed (control) leaves after 72 h. (**g**) In vitro bioassay of transgenic cabbage and dead larvae of diamondback moth (DBM) on damaged transformed leaves after 72 h (Source: Gambhir 2014)

cauliflower in India in 2014–2015 was 8685.302 million tones under the area of 436.052 mha (National Horticulture Board India 2014–2015). However cauliflower is severely infested by a large number of insect pests like diamondback moth (*Plutella xylostella*), cabbage butterfly (*Pieris brassicae*), cabbage semilooper (*Helicoverpa armigera*), cabbage aphid (*Brevicoryne brassicae*), cabbage borer (*Hellula undalis*), cabbage looper (*Trichoplusia ni*), and tobacco caterpillar (*Spodoptera litura*). It leads to economic as well as large yield losses to the nation. Among these insect pests, diamondback moth is responsible for the maximum yield losses in cauliflower and other *Brassica* spp. *Plutella xylostella* (diamondback moth) larvae extensively feed on the leaves and curd of cauliflower, leading to reduced yield and poor quality of produce. Economic yield losses by *Plutella xylostella* may range from 52 to 80% when the attack is severe. To check the losses

caused by these insects, a number of pesticides are being used, but unfortunately major insect pests are developing resistance to most of the applied chemical insecticides; therefore it is important to introduce insect resistance (*cryIAa*) gene in cauliflower which is a natural insecticide.

The application of transgenic technology in cauliflower cultivation is of great significance to generate new improved and desirable traits like insect resistance and disease-resistant varieties. Among *Brassica oleracea*, cauliflower is reported to be the least amenable to genetic transformation (Puddephat et al. 1996; Passelegue and Kerlan 1996). Attempts have been made by various scientists to increase the yield and quality of cauliflower via genetic transformation techniques (Ding et al. 1998a; Ashokan 1999; Jin et al. 2000; Chakrabarty et al. 2002). The first step toward the development of genetic transformation protocol includes the generation of a sound reproducible plant regeneration system. Different direct, indirect, and somatic embryogenesis regeneration systems have been developed by various researchers (Bhalla and Weered 1999; Chikkala et al. 2009; Siong et al. 2011) in cauliflower as a first step toward the genetic improvement of the crop. Several systems of genetic transformation have been used; both direct DNA transfer and *Agrobacterium*- mediated transformation of cauliflower have been attempted. Electroporation and PEG-mediated DNA uptake have been demonstrated with protoplasts (Eimert and Siegemund 1992), but *Agrobacterium*-mediated gene transfer is the most preferred (Dunwell 2000; Husaini 2010) as it results in higher transformation efficiency and a more predictable pattern of foreign DNA integration rather than any other transformation technique (Hiei et al. 1994; Chakrabarty et al. 2002). This technology is now available for many crops. *A. tumefaciens* has been used routinely to obtain transgenic plants (Srivastava 2003) (Table 15.7). A protocol for insect resistance gene (*cryIAa*) and (*cryIAb*) transfer in cauliflower (*Brassica oleracea* L. var. *botrytis* cv. Pusa Snowball K1) has been standardized in our laboratory using *Agrobacterium tumefaciens* strain containing *npt-II* and *cryIAa* and (*cryIAb*) genes in binary vector pBin-1Aa. Putative transgenic plantlets of cauliflower have shown the amplification of *cryIAa* and *cryIAb* gene thereby indicating the presence/integration of *cryIAa* and *cryIAb* gene into the genome of transgenic cauliflower. The confirmation of expression of the transgenes *cryIAa* and *cryIAb* into the genome of cauliflower at transcriptional level was confirmed by reverse transcriptase-PCR and real-time PCR and at translational level by bioassay. A protocol for high-frequency plant regeneration and insect resistance gene transfer in cauliflower (*Brassica oleracea* L. var. *botrytis* cv. Pusa Snowball K1) has been standardized (Awasthi 2003; Gaur 2015) (Fig. 15.3).

15.2.5 Broccoli

Broccoli (*Brassica oleracea* L. var. *italica*) is an economically important nutritionally rich vegetable crop, especially in calcium, antioxidants, vitamin A, vitamin K, β-carotene, riboflavin, and iron content (Vallejo et al. 2003; Abdel-Wahhab and Aly 2003), having anticancer properties which are contributed by sulforaphane

Table 15.7 Genetic engineering for insect resistance gene(s) transfer studies in cauliflower

Plant species/cultivar	Technique of gene transfer	Gene transferred	References
Insect resistance genes of microbial origin			
B. oleracea L. var. *botrytis*	*Agrobacterium*- mediated gene transfer	*cryIA*(*b*)	Chakrabarty et al. (2002)
B. oleracea L. var. *botrytis*	*Agrobacterium tumefaciens*-mediated gene transfer	*gus* and *cryIAc*	Wang et al. (2003)
B. oleracea L. var. *botrytis*	*Agrobacterium tumefaciens*-mediated gene transfer	*cryIc*	Cao et al. (2003)
B. oleracea L. var. *botrytis*	*Agrobacterium tumefaciens*-mediated gene transfer	*cry1a*	Zhang et al. (2004)
B. oleracea L. var. *botrytis*	*Agrobacterium tumefaciens*-mediated gene transfer	*cryIAb*	Awasthi and Srivastava (2013)
B. oleracea L. var. *botrytis*	*Agrobacterium tumefaciens*-mediated gene transfer	*cryIAa*	Gaur (2015)
Insect resistance genes of plant origin			
B. oleracea L. var. *botrytis*	*Agrobacterium tumefaciens*-mediated gene transfer	*Ti* (trypsin inhibitor gene)	Ding et al. (1998b)
B. oleracea L. var. *botrytis*	*Agrobacterium tumefaciens*-mediated gene transfer	*CpTI*	Lingling et al. (2005)

glucosinolate (Keck et al. 2003), quinone reductase, glutathione S-transferase (Zhang et al. 1992; Fahey et al. 1997), and high selenium content (Finley et al. 2001; Finley 2003). It belongs to the family Brassicaceae ($2n = 18$). In India, it is still cultivated on a limited scale, but its cultivation holds promise throughout the temperate and tropical regions. Various environmental stresses, pests, and diseases cause heavy yield losses, so genetic manipulation is necessary for broccoli improvement. Classical breeding techniques alone are not sufficient for the genetic improvement. Sexual incompatibility barriers severely limit the possibilities for gene transfer between species, although some of the *Brassica* can be easily crossed using plant breeding techniques and through somatic hybridization (Puddephat et al. 1996). Thus, genetic engineering can be used to introduce desirable agronomically important characteristics to existing cultivars. However, a prerequisite for transferring genes into plants is the availability of highly efficient, reproducible plant regeneration and genetic transformation standardized protocol. In vitro plant regeneration studies in broccoli were reported by various researchers using different types of explants such as the peduncle (Christey and Earle 1991), anther (Chang et al. 1996), protoplasts (Kaur et al. 2006), hypocotyl (Zhong and Li 1993; Puddephat et al. 2001; Kim and Botella 2002; Ravanfar et al. 2009; Huang et al. 2011, Kumar and Srivastava 2015a; Kumar et al. 2015a), leaf tissue (Robertson and Earle 1986; Cao

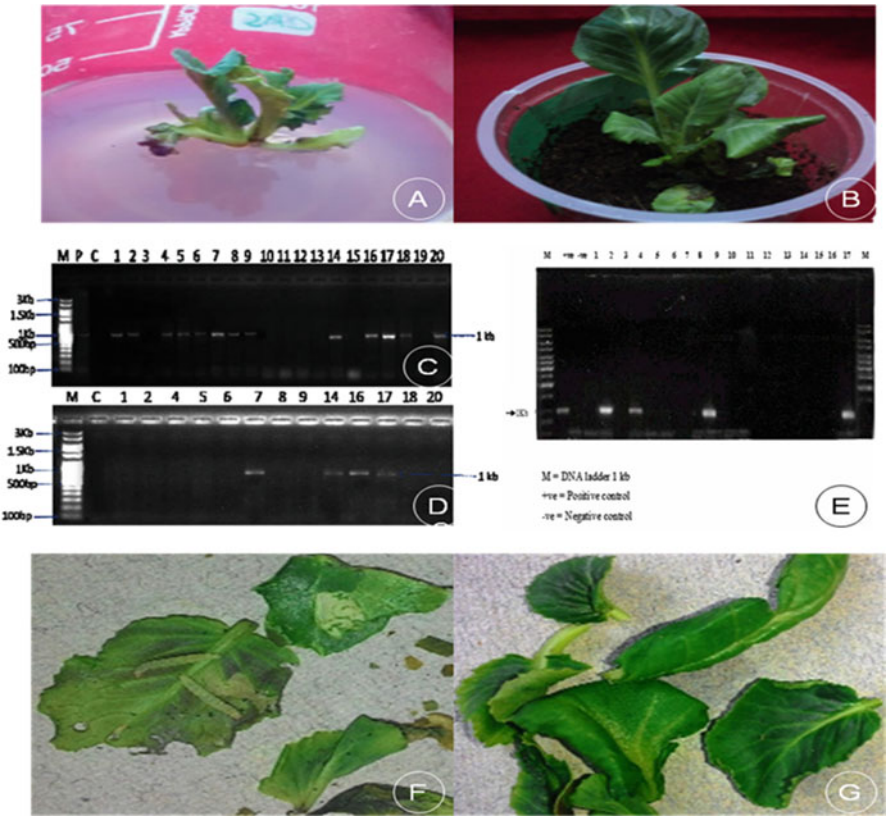

Fig. 15.3 (**a**) Regenerated transgenic shoots of cauliflower (*Brassica oleracea* L. var. *botrytis* cv. Pusa Snowball K1). (**b**) Acclimatization of in vitro regenerated transgenic plantlets of cauliflower cv. Pusa Snowball K1 after 3 weeks. (**c**) PCR analysis showing amplification of 1 kb DNA fragment of *cryIAa* gene in regenerated transgenic plantlets of cauliflower. (**d**) RT-PCR analysis showing amplification of 1 kb DNA fragment of *cryIAa* gene in regenerated transgenic plantlets of cauliflower (*Brassica oleracea* L. var. *botrytis* cv. Pusa Snowball K1). (**e**) PCR analysis showing amplification of 1 kb DNA fragment of *cryIAb* gene in regenerated transgenic plantlets of cauliflower. (**f**) In vitro bioassay of non-transgenic (control), live larvae of diamondback moth (DBM) on damaged non-transformed (control) leaves after 72 h. (**g**) In vitro bioassay of transgenic cabbage and dead larvae of diamondback moth (DBM) on damaged transformed leaves after 72 h (Source: Awasthi 2003; Gaur 2015)

and Earle 2003; Farzinebrahimi et al. 2012; Kumar and Srivastava 2015a), cotyledon (Qin et al. 2006; Ravanfar et al. 2011, 2014, Kumar and Srivastava 2015a, b), and petiole (Kumar et al. 2015a, b).

Broccoli and other cole crops are highly susceptible to large numbers of insect pests and diseases (Kumar and Srivastava 2016). Insect pests of agricultural importance include the cabbage looper (*Trichoplusia ni*), cutworm (*Spodoptera littoralis*), cabbage fly (*Delia brassicae*), cabbage aphid (*Brevicoryne brassicae*), imported cabbage worm (*Pieris rapae*), cabbage butterfly (*Pieris canidia*), cabbage moth (*Crocidolomia binotalis*), and the diamondback moth (*Plutella xylostella*). The

diamondback moth is considered to be the major insect pest of the crucifers worldwide (Kumar and Srivastava 2016) and has become resistant to all major categories of insecticides (Tabashnik et al. 1991). Metz et al. (1995b) were the first to report *Agrobacterium*-mediated gene transfer studies in broccoli, followed by other workers (Puddephat et al. 1996, 2001; Chen et al. 2004, 2007; Huang et al. 2005; Higgins et al. 2006; Bhalla and Singh 2008; Kumar and Srivastava 2016). High-frequency plant regeneration and genetic transformation of the broccoli are highly dependent on genotype and need to be standardized for each cultivar. Metz et al. (1995a) have reported a large number of transgenic broccoli lines carrying the *cry1A*(*c*) gene which provides 100 % mortality to first instar larvae of the diamond moth, a major insect pest of crucifers in tested bioassay studies. Southern blots of some resistant transformants confirmed the presence of *cry1A*(*c*) gene. Cao et al. (1999) developed transgenic broccoli with high levels of *Bacillus thuringiensis cry1C* protein to control diamondback moth larvae resistant to *cry1A* or *cry1C*. They reported that high production of *cry1C* protein can protect transgenic broccoli not only from susceptible DBM larvae but also from DBM selected for moderate levels of resistance of *cry1C*. Cao and Earle (2003) studied transgene expression in broccoli clones propagated in vitro via leaf explants by using *cry1Ac* and *cry1C* genes from *Bacillus thuringiensis* associated with kanamycin and hygromycin selectable markers, respectively. Molecular analysis using polymerase chain reaction indicated that the *cry1Ac* and *cry1C* genes were both maintained. ELISA showed that all of the clones produced a high level of *cry1Ac* protein similar to the original transgenic plant; however, most clones had significantly lower levels of *cry1C* protein than the original plant. Viswakarma et al. (2004) studied insect resistance in transgenic broccoli cultivar Pusa Broccoli KTS-1 expressing a synthetic *cryIA*(*b*) gene (Table 15.8). A protocol for insect resistance gene (*cryIAa*) transfer in broccoli (*Brassica oleracea* L. var. *italica* cv. Solan green head) has been standardized in our laboratory using *Agrobacterium tumefaciens* strain containing *npt-II* and *cryIAa* genes in binary vector pBin-1Aa. Putative transgenic plantlets of broccoli have shown the amplification of *cryIAa* gene thereby indicating the presence/integration of *cryIAa* gene into the genome of transgenic broccoli. A protocol for high-frequency plant regeneration and insect resistance gene transfer in broccoli (*Brassica oleracea* L. var. *italica* cv. Solan green head) has been standardized (Kumar 2016) (Fig. 15.4).

15.2.6 Brinjal

Brinjal or eggplant (*Solanum melongena* L.) is an agronomically important solanaceous non-tuberous crop grown primarily for its large oval fruit. It is also known as eggplant, aubergine, baingan, mad apple, and brinjal. In India the total production of brinjal is 12986.583 million tonnes with the cultivation on an area of 680.398 mha (National Horticulture Board 2014–2015). Brinjal has a high nutritive value and medicinal value used for the treatment of several diseases, including diabetes, arthritis, asthma, and bronchitis. It is reported that brinjal extracts have a significant effect in reducing blood and liver cholesterol rates in humans (Khan 1979) and adult rats (Silva et al. 1999). Nasunin is a major component of anthocyanin pigment of

Table 15.8 Genetic engineering for insect resistance gene(s) transfer studies in broccoli

Method	Crystal protein	Target insect	References
Insect resistance genes of microbial origin			
Agrobacterium-mediated gene transfer	*cryIA*(*c*) (*c*)	*Plutella xylostella*	Metz et al. (1995a, b)
Agrobacterium-mediated gene transfer	*cry1C*	*Plutella xylostella*	Cao et al. (1999)
Agrobacterium-mediated gene transfer	*cry1A*, *cry1C*	*Plutella xylostella*	Cao et al. (2003)
Agrobacterium-mediated gene transfer	*cryIA*(b)	*Plutella xylostella*	Viswakarma et al. (2004)
Agrobacterium-mediated gene transfer	*cryIA*(a)	*Plutella xylostella*	Kumar et al. (2015a, b)

brinjal, which has free radical scavenging and iron-chelating activities. Eggplant is also having antimutagenic activity (Yoshikawa et al. 1996). Major insect pests and diseases which cause crop yield losses in brinjal crops are particularly shoot and fruit borer (*Leucinodes orbonalis* Guenée), Colorado potato beetle (*Leptinotarsa decemlineata* Say), aphid (*Aphis gossypii* Glover), *Fusarium* wilt (*F. oxysporum* f.sp. *melongenae*), nematodes (*Meloidogyne spp.*), etc. *Cry1ab* gene has been transferred in brinjal against fruit borer (*Leucinodes orbonalis*) and root-knot nematode (*Meloidogyne incognita*) by Kumar et al. (1998) and Phap et al. (2010). *Cry3a* and *cry3b* genes have been transferred against Colorado potato beetle (*Leptinotarsa decemlineata*) by Iannacone et al. (1997), Arpaia et al. (1997), Acciarri et al. (2000), Hamilton et al. (1997, Mennella et al. (2005), and Jelenkovic et al. (2000), whereas other genes which possess insect resistance against fruit and shoot borers have been transferred by Narendran et al. (2007) (*cry2Ab*), Pal et al. (2009) (*cry1Ac* gene), and Rai et al. (2013) (*Cry 1Ac*) (Table 15.9). A protocol has been standardized for plant regeneration and genetic transformation of brinjal with *npt-II* and *gus* genes in our laboratory using genetically engineered *Agrobacterium tumefaciens* LBA4404 strain containing reporter β-glucuronidase (*gus*) gene in binary system (pBI121) along with reporter kanamycin resistance gene, i.e., neomycin phosphotransferase (*npt-II*), which can be further exploited to develop transgenic plantlets of brinjal with insect resistance gene(s) (Bardhan et al. 2013) (Fig. 15.5).

15.2.7 Pea

Pea (*Pisum sativum* L. var. Lincoln) is a cool season grain legume and economically important pulse crops in the world in an area of 2,241,318 ha with a production of 1,697,4983 M tonnes (FAO). However, India, being the fourth largest producer of peas in the world, has a production of 3961.07 million tonnes with the cultivation

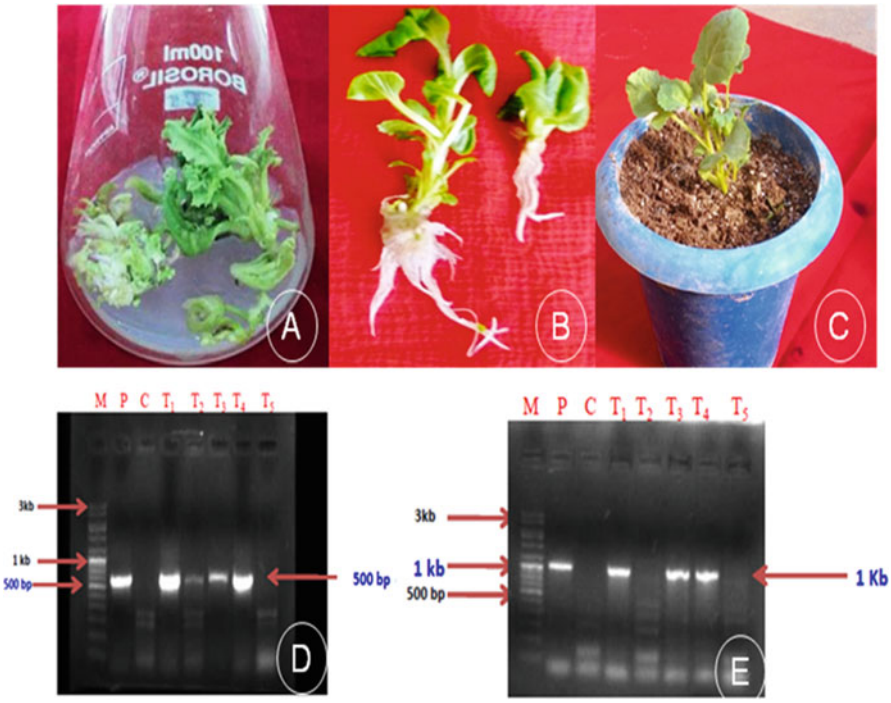

Fig. 15.4 (**a**) In vitro regenerated plantlets of broccoli cv. Solan green head. (**b**) Root regeneration from in vitro developed shoots of broccoli cv. Solan green head. (**c**) Successful acclimatization of the plantlets on planting substrate after 4 weeks of hardening and maintained in greenhouse. (**d, e**) PCR analysis showing amplification of 500 bp of *nptII* and 1 kb of *cry1Aa*-specific DNA fragment in regenerated transgenic plantlets of broccoli (*M* marker (step-up ladder 100 bp–3 kb), *P* plasmid DNA, *C* non-transformed DNA (control DNA), *T1- T5* DNA of putative transformed shoots) (Source: Kumar 2016)

area of 432.84 mha (National Horticulture Board 2014–2015). Pea belongs to family Leguminosae with chromosome number ($2n$) = 14 for cultivated pea. Being a legume it fixes atmospheric nitrogen in the roots and increases the soil fertility. Pea is a rich source of proteins; carbohydrates; fats; vitamins A, B, and C; and minerals like calcium and phosphorus (Cherian et al. 1995). It also contains high levels of essential amino acids lysine and tryptophan and hence plays an important role in the economy of growers (Pownall et al. 2010). Pea is susceptible to several biotic and abiotic stresses. Major biotic stresses are wilt/root rot, powdery mildew, ascochyta blight, and rust. Additionally, insects such as the pea leaf weevil (*Sitona lineatus*) and bruchid larvae cause damage to peas and other pod fruits. The weevil larvae feed on the root nodules of pea plants, which are essential to the plants' supply of nitrogen and thus diminish leaf and stem growth. Bruchid larvae are reported to cause major losses of grain legume crops throughout the world. The most commonly available means to deal with the severity of pest damage is the use of insecticides, which are causing threat to environment. To deal with all these problems, many researchers have carried

Table 15.9 Genetic engineering for insect resistance gene(s) transfer studies in brinjal

Method	Gene transferred	Target insect	References
Insect resistance genes of microbial origin			
Agrobacterium -mediated gene transfer	*cry3b* gene	Colorado potato beetle	Iannacone et al. (1997)
Agrobacterium-mediated gene transfer	*cryIIIB*	*Leptinotarsa decemlineata*	Arpaia et al. (1997)
Agrobacterium-mediated gene transfer	*cryIIIA*	*Leptinotarsa decemlineata*	Hamilton et al. (1997)
Agrobacterium-mediated gene transfer	*cry1Ab*	Fruit borer (*Leucinodes orbonalis*)	Kumar et al. (1998)
Agrobacterium-mediated gene transfer	*cry3B*	Colorado potato beetle	Acciarri et al. (2000)
Agrobacterium-mediated gene transfer	*cry3B*	Colorado potato beetle	Mennella et al. (2005)
Agrobacterium-mediated gene transfer	*cry2Ab*	Fruit and shoot borer (*Leucinodes orbonalis*)	Narendran et al. (2007)
Agrobacterium-mediated gene transfer	*cry1Ac* gene	Fruit borer larva	Pal et al. (2009)
Agrobacterium-mediated gene transfer	*cryIAb* gene	Root-knot nematode, *Meloidogyne incognita*	Phap et al. (2010)
Agrobacterium-mediated gene transfer	*cry1Ac*	Fruit and shoot borer	Rai et al. (2013)

out gene transfer studies in *Pisum sativum* against lepidopteran and other classes of insects. α-Amylase inhibitor and proteinase inhibitor have been transferred to *Pisum sativum* against *Helicoverpa armigera*, bruchid beetles, and pea weevil by Charity et al. (1999), Morton et al. (2000), Shade et al. (1994), Majer et al. (2007), and Schroeder et al. (1995). Attempts have been done for the transfer of *cry1Ac* gene by Negawo and coworkers in 2012 against various lepidopteran insects. Amylase inhibitors are reported to inhibit the α-amylase enzyme thus causing the death of the insects by carbohydrate starvation, while *cry* genes cause the death of the insect by the formation of pores in gut membrane leading to ion imbalance in the gut. Alemayehu et al. (2012) and Griga et al. (2009) developed insect-resistant transgenic pea plants using *Agrobacterium*-mediated transformation. Griga et al. (2009) produced transgenic pea having improved tolerance to insect pests and fungal pathogens. Putative transformants containing protease inhibitor transgene *gmSPI2* were histochemically tested for *GUS* expression and were then further characterized using molecular/PCR analysis, while Alemayehu et al. (2012) produced insect-resistant transgenic pea using *Agrobacterium*-mediated transformation containing *cry1Ac* gene for insect resistance and *bar* gene for herbicide resistance. And the putative transgenic shoots were characterized using molecular and functional analysis (Table 15.10). A protocol has been standardized for plant regeneration and genetic transformation of pea (*Pisum sativum* L. var. Lincoln) with *npt-II* and *gus* genes in our

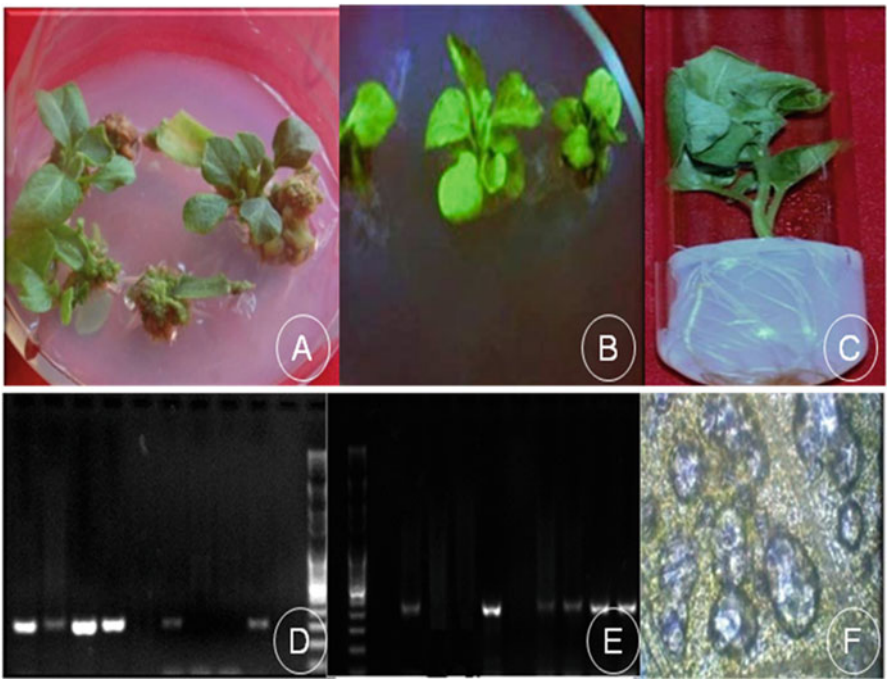

Fig. 15.5 (**a**, **b**) In vitro regenerated transgenic plantlets of brinjal (*Solanum melongena* L.) (**c**) Root regeneration from in vitro developed shoots of brinjal on selective media. (**d**, **e**) PCR analysis showing amplification of 500 bp of *nptII* and 439 bp of *gus* gene in regenerated transgenic plantlets of brinjal. (**f**) Histochemical determination of GUS assay in the PCR-positive transgenic shoots of brinjal (Source: Bardhan et al. 2013)

laboratory using genetically engineered *Agrobacterium tumefaciens* LBA4404 strain containing reporter β-glucuronidase (*gus*) gene in binary system (pBI121) along with reporter kanamycin resistance gene, i.e., neomycin phosphotransferase (*npt-II*), which can be further exploited to develop transgenic plantlets of pea with insect resistance gene(s) (Sharma 2013) (Fig. 15.6).

15.2.8 Lettuce

Lettuce (*Lactuca sativa* L.) belongs to family Asteraceae (Compositae). There are approximately 100 species of *Lactuca*, although only *L. serriola*, *L. saligna*, and *L. virosa* share any sexual compatibility with *L. sativa*. The chromosome number of lettuce is most commonly $n=9$, although $n=8$ and $n=17$ have been found (Ryder 1999). It is an annual, self-fertile species and major fresh leafy vegetable which is consumed raw. It is known as a water plant for its refreshing properties. Originating from the Mediterranean area, lettuce was first introduced into America by Christopher Columbus when he sailed "the ocean blue" in 1492. The center of origin of lettuce is probably the Middle East and Southwest Asia, while today, the main

Table 15.10 Genetic engineering for insect resistance gene(s) transfer studies in peas

Method	Gene transferred	Target insect	References
Insect resistance genes of microbial origin			
Agrobacterium-mediated gene transfer	*cry1Ac*	*Helicoverpa armigera*	Griga et al. (2009)
Agrobacterium-mediated gene transfer	*cry1Ac*	*Helicoverpa armigera*	Alemayehu et al. (2012)
Insect resistance genes of plant origin			
Agrobacterium-mediated gene transfer	α-Amylase inhibitor	Bruchid beetles	Shade et al. (1994)
Agrobacterium-mediated gene transfer	α-Amylase inhibitor	*Bruchus pisorum*	Schroeder et al. (1995)
Agrobacterium-mediated gene transfer	Proteinase inhibitor	*Helicoverpa armigera*	Charity et al. (1999)
Agrobacterium-mediated gene transfer	α-Amylase inhibitor	*Bruchus pisorum*	Morton et al. (2000)
Agrobacterium-mediated gene transfer	α-Amylase inhibitor	Pea weevil	Majer et al. (2007)

areas of production and consumption of lettuce are the USA and Europe (Ryder 1986). Lettuce is a good source of vitamin A, vitamin K, potassium, carbohydrates, protein, and some dietary fibers.

Lettuce is a rich source of antioxidants such as quercetin and caffeic acid. Its stem and leaves contain many active ingredients such as mannitol (which takes effect on diuretic and blood circulation promotion) and lactucerin (which plays a role on hypnosis, analgesia, and adjuvant treatment of neurasthenia). It is used in the production of nicotine-free cigarettes and the isolation of sesquiterpene lactones from the milky sap, for use in medicine (Ryder 1999). Nowadays, extracts from *Lactuca sativa* L. have been used for curing sunburn and rough skin in cream and latexes (Odu and Okomuda 2013). Lettuce generally suffers from viral and fungal diseases, but crowding in lettuce tends to attract pests and diseases. Some of the works in this field include the use of plant protease inhibitors for protection against Lepidoptera and orthopteran insects (Gatehouse 1995; Burgess and Gatehouse 1997; Burgess et al. 1996) (Table 15.11). A protocol has been standardized for plant regeneration and genetic transformation of lettuce (*Lactuca sativa* L. cv. Sol- Let-1) with *npt-II* and *gus* genes in our laboratory using genetically engineered *Agrobacterium tumefaciens* LBA4404 strain containing reporter β-glucuronidase (*gus*) gene in binary system (pBI121) along with reporter kanamycin resistance gene, i.e., neomycin phosphotransferase (*npt-II*), which can be further exploited to develop transgenic plantlets of lettuce with insect resistance gene(s) (Sharma 2014) (Fig. 15.7).

15.2.9 Bell Pepper

Bell pepper or *Capsicum* (*Capsicum annuum* L.) is the best-known domesticated species in the world belonging to family Solanaceae of the order Solanales. Also it

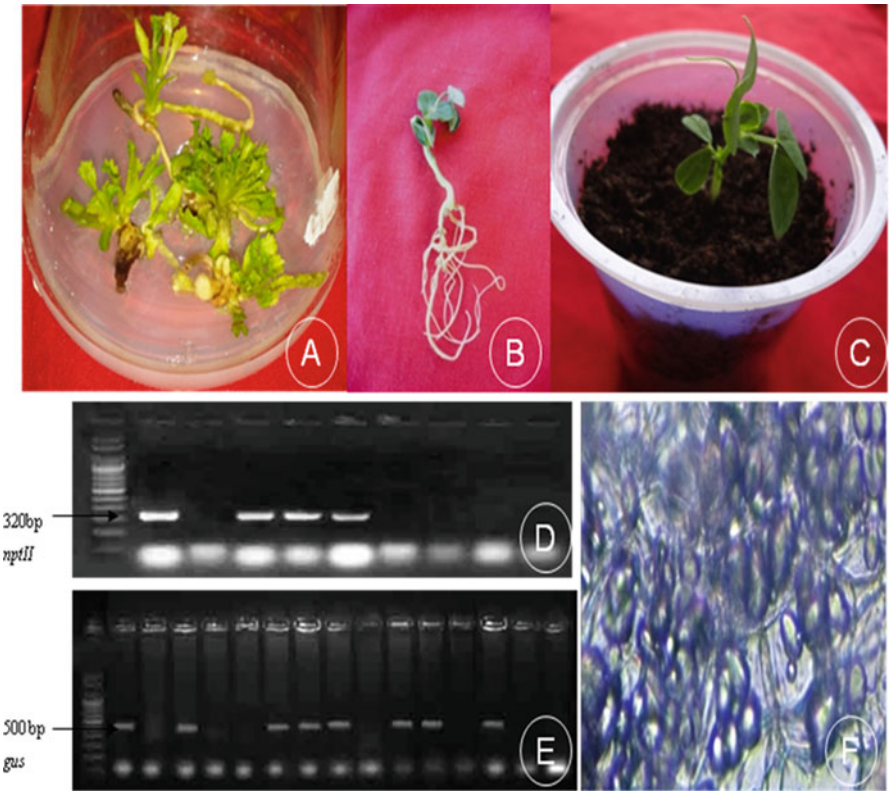

Fig. 15.6 (**a**) In vitro regenerated transgenic plantlets of pea (*Pisum sativum* L. var. Lincoln). (**b**) Root regeneration from in vitro developed shoots of pea on selective media. (**c**) Acclimatization of transgenic plantlets successfully on the planting substrate after 3 weeks of hardening and maintained in greenhouse. (**d**, **e**) PCR analysis showing amplification of 500 bp of *gus* and 320 bp of *nptII* gene in regenerated transgenic plantlets of pea. (**f**) Histochemical determination of GUS assay in the PCR-positive transgenic shoots of pea (Source: Sharma 2013)

is the most economically important species (Greenleaf 1986; Bosland et al. 1988). This crop is widely consumed as fresh vegetable or condiment and is used for pharmaceutical and cosmetic purposes. It became the dominant pepper globally in part because it was the first pepper discovered by Columbus. Among the various cultivars, California Wonder is one of the oldest cultivars being in cultivation and typical of pod type. Peppers are grown in most of the countries of the world. California produces the most bell peppers in the world and bell group is the most economically important pod type. Bell pepper (*Capsicum annuum* L.) is a rich source of alkaloids (capsaicin), fatty acids, flavonoids, volatile oils, and carotene pigment. It is rich in vitamin C (ascorbic acid) and zinc which are vital for strong and healthy immune system. It is high in vitamin A, reactin (a bioflavonoid), and β-carotene and also contains magnesium, phosphorus, vitamin B complex, sodium, and selenium.

In India, it occupies an area of 30.052 mha with a production of 171.079 million tonnes (National Horticulture Board 2014–2015). Productivity of bell pepper in

Table 15.11 Genetic engineering for insect resistance gene(s) transfer studies in lettuce

Gene transferred	Origin	Target insects	References
Insect resistance genes of plant origin			
CpTI (cowpea trypsin inhibitor)	Plant	Lepidoptera	Gatehouse (1995)
Pot-PT-II (potato proteinase inhibitor-I)	Plant	Lepidoptera, Orthoptera	Burgess and Gatehouse (1997)
BPTI (bovine pancreatic trypsin inhibitor)	Plant	Lepidoptera, Orthoptera	Burgess et al. (1996)

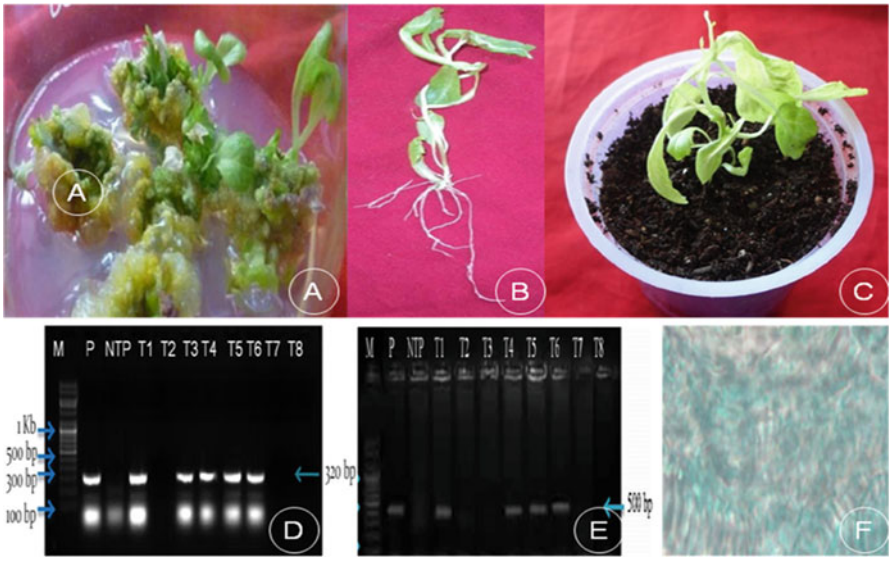

Fig. 15.7 (**a**) In vitro regenerated transgenic shoots of lettuce (*Lactuca sativa* L. cv. Sol- Let-1). (**b**) Root regeneration from in vitro developed shoots of lettuce on selective media. (**c**) Acclimatization of transgenic plantlets successfully on the planting substrate after 3 weeks of hardening and maintained in greenhouse. (**d**, **e**) PCR analysis showing amplification of 500 bp of *gus* and 320 bp of *nptII* gene in regenerated transgenic plantlets of lettuce. (**f**) Histochemical determination of GUS assay in the PCR-positive transgenic shoots of lettuce (Source: Sharma 2014)

India is low as compared to western countries because it suffers from many diseases, pests, and disorders that reduce fruit quality and yield. Damage can be caused by a wide range of biological agents including fungi, bacteria, viruses, insects, and nematodes. Bell pepper suffers great losses due to infection by various viruses and fungal pathogens. In India, attack by mites and thrips is the major handicap in cultivation of bell pepper which is often followed by viral infection. Production in India is also affected by pepper mottle virus (PeMV-1) to a larger extent. Preliminary research in the area of genetic transformation of *Capsicum* has resulted in a few transformed sweet and hot pepper (Kim et al. 1997). Genetic engineering offers a direct method of plant breeding that selectively targets one or a few traits for

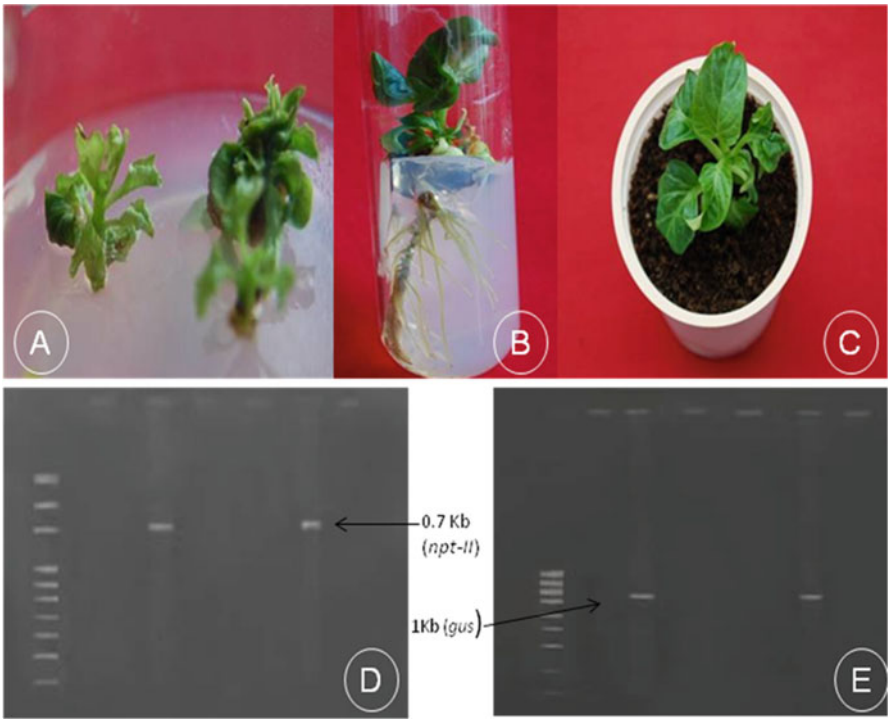

Fig. 15.8 (**a**) In vitro developed transgenic plantlets of bell pepper (*Capsicum annuum* L.) (**b**) Root regeneration from in vitro regenerated shoots of bell pepper on selective media. (**c**) Acclimatization of transgenic plantlets successfully on the planting substrate after 4 weeks of hardening and maintained in greenhouse. (**d**, **e**) PCR analysis showing amplification of 1 kb of *gus* and 700 bp of *nptII* gene in regenerated transgenic plantlets of bell pepper (Source: Verma et al. 2013)

introduction into the crop plant such as disease and insect resistance and is dependent on an efficient and reliable transformation and regeneration protocol. A protocol has been standardized for plant regeneration and genetic transformation of bell pepper (*Capsicum annuum* L.) with *npt-II* and *gus* genes in our laboratory using genetically engineered *Agrobacterium tumefaciens* LBA4404 strain containing reporter β-glucuronidase (*gus*) gene in binary system (pBI121) along with reporter kanamycin resistance gene, i.e., neomycin phosphotransferase (*npt-II*), which can be further exploited to develop transgenic plantlets of bell pepper with insect resistance gene(s) (Verma et al. 2013) (Fig. 15.8).

15.3 Conclusion

With changing global climate conditions and depleting natural resources, traditional farming practices alone are not sufficient to sustain the quality and quantity of the produce. With the introduction of modern plant molecular biotechnological tools, which permit gene transfer across the species, transgenics opened an avenue for solving the serious threat regarding food and energy insecurity. Some of the limitations in plant transgenic technology applications need to be resolved for its wider application and global acceptance. Various biosafety measures and ethical issues regarding environmental risks such as cross-pollination with closely related wild relatives of the crop plants and effect of transgene products on human health and environment need to be assessed carefully on a case-by-case basis. Globally, major concerns regarding the use of selectable markers such as antibiotic and herbicide resistance genes from the ecological and human safety point of view. Today major breakthrough in transgenic, to obtain marker-free transgenic plants which may enhance the public acceptance of transgenic crops. Development of binary vectors or mini-chromosomes for multiple gene transfer such as gene stacking and improvement in transformation technology for vegetable crops may further increase our capability to introduce different agronomically important traits with long-lasting value. This chapter provides an insight into genetic engineering for insect resistance in economically important vegetable crops.

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RNA Interference (RNAi) and Its Role in Crop Improvement: A Review

16

Amanpreet Kaur, Anil Kumar, and M. Sudhakara Reddy

Abstract

Genetic modifications using contemporary biotechnological tools hold immense potential for improvement of the quality of crops. The copious paraphernalia of genetic engineering has been adopted to identify functions of various genes with an eventual aim of crop improvement. Among these, RNA interference (RNAi), a double-stranded RNA (dsRNA)-based gene silencing technology, serves as an extending platform for genetic interventions intended at the improvement of crops, by modifying these for important agronomical traits. RNAi is a gene silencing process initiated by the presence of dsRNA molecules which are ultimately responsible for complementary mRNA-specific degradation through a series of steps. Two important enzymatic complexes crucial in RNAi technology are Dicer and RNA-induced silencing complex (RISC). Dicer is a multidomain enzyme complex belonging to ribonuclease (RNase) III class. It plays an important role in the cleaving of dsRNA to produce small interfering RNA molecules. Another complex, RISC, is composed of Argonaute proteins, which uses interfering RNA as guide molecules for complementary mRNA degradation. This review focuses on the RNA interference (RNAi) technology and its impending role in the crop improvement.

Keywords

Argonaute proteins • Small interfering RNAs • RISC • Dicer • Functional genomics • Reverse genetics

A. Kaur • A. Kumar (✉) • M.S. Reddy
Department of Biotechnology, Thapar University,
TIFAC-CORE Building, Patiala 147004, India
e-mail: adatta@thapar.edu

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16.1 Introduction

Till the dawn of the twentieth century, the scientific history was witnessing RNA as sheer "transcriptional product." Later on, the empirical and scientific observations led to the enlightening innovation that RNA could be used for gene-specific silencing. These annotations led to the revolutionary technology of antisense RNA, which further paved the path for miraculous RNA interference (RNAi). In plants, concepts of RNAi came into existence in 1990 in petunias, though at that time it was not recognized as RNAi but co-suppression. The following years were to witness the commotion in certain deliberate and chance discoveries, for instance, the year 1995 witnessed RNAi in animals as a chance discovery during gene knockout experiment carried out on *Caenorhabditis elegans* (Guo and Kemphues 1995). Later on in 2000, efficient RNA silencing with the help of RNAi construct was established in *Arabidopsis thaliana* (Chuang and Meyerowitz 2000). RNAi is reported to affect the central dogma by disrupting genomic stability, which further distorts the cellular metabolism (Kulinska et al. 2003; Hannon 2003). These reports unveil the facts that duplex RNA molecules are able to block translation (Nordstrom and Wagner 1994) and endorse sequence-specific mRNA degradation (Duan et al. 2012). Therefore, RNAi affects the overall echelon of gene expression (Hannon 2003). Among the plethora of necessary and sufficient conditions for RNAi, triggers serve an indispensable function. Triggers of the RNAi are small interfering RNA (siRNA) molecules which are formed by cleavage of double-stranded RNA molecules by ribonuclease III-like enzyme, commonly known as Dicer (Blevins et al. 2006). The machinery of RNAi operates through targeting mRNA molecule by a protein complex called RNA-induced silencing complex (RISC) carrying guide strand of double-stranded RNA (dsRNA) with sequence complementary to target mRNA. RISC is known to contain Argonaute family proteins, which are endonucleases and also possess slicing capabilities (Pratt and MacRae 2009). Once the target sequence is identified then it is sliced into small fragments with the help of RNase activity of RISC (Blevins et al. 2006). This interfering response undergoes rapid amplification through the involvement of RNA-dependent RNA polymerase (RdRp) (Forrest et al. 2004). RNAi occurs as a natural defense in plants against viruses by protecting the cell from inappropriate expression of viral genomes (Ding 2010), repetitive sequences, and transposons (Haasnoot et al. 2007). An adaptive immunity can also be achieved through siRNA introduction which helps plant in recognizing nucleic acids such as those derived or delivered from virus and inactivates them through RNA cleavage (Waterhouse et al. 2001). A large number of in-depth review articles are focused on functions and role of RNAi in plant growth, development, morphogenesis, stress tolerance, etc. leading to enhancement of our knowledge of gene function and regulation (Rao and Sockanathan 2005; Tomari and Zamore 2005; Artymovich 2009; Jagtap et al. 2011; Duan et al. 2012; Deng et al. 2012; Wang et al. 2014; Sanchez et al. 2014; Zhang et al. 2015). This fascinating technique has immense potential for various applications in crop sciences through development of improved varieties for pest and pathogen management (Ellendorff et al. 2009; Tabassum et al. 2011; Ogwok et al. 2012), improved nutritional value (Kumar et al.

2006; Eck et al. 2007), establishment of host-parasite interactions (Younis et al. 2014), modification of crop product for human benefit (Ogita et al. 2003; Regina et al. 2006), and also to assign specific function to genes (Deng et al. 2012). Moreover, the role of RNAi in human therapeutics such as in the treatment of cancer, hepatitis B, HIV and genetic disorders makes the technique much more enlivening (Kusov et al. 2006; Jia et al. 2006).

16.1.1 Definition

"RNAi" is a natural mechanism and can also be introduced through various molecular tools. Due to the ability of RNAi to determine functions of genes before its sequence is known, it is often referred as reverse genetics (Waterhouse et al. 2001). Moreover, this is a conserved regulatory mechanism for gene silencing, resulting either from inhibition of gene transcription or through degradation of transcriptional product (Mette et al. 2000). RNAi plays a crucial role in many biological activities such as gene expression regulation, maintaining genome stability, heterochromatin formation, and as a defense against the virus (Brodersen and Voinnet 2006; Vaucheret 2006).

16.1.2 Discovery

Genetic manipulation for desirable characters in crops has always been important in plant sciences. Scientific crop improvement approaches are spread over a vast arena, from conventional breeding methods (Johnston and Rowberry 1981) to recently developed genetic engineering. A striking progress in genetic engineering, which revolutionized the scientific world, is a precisional biotechnological approach that offered crop improvement through manipulation of genes for desirable characters. In the era of functional genomics, gene silencing methods have received increased consideration (Zamecnik and Stephenson 1978). Prior to the discovery of RNAi, homologous recombination was used to generate knockout organisms (Smithies 1985, 1987; Capecchi 1987) with the purpose to understand the intricate mechanism of gene functioning in vivo, but this method was found inefficient due to prolonged time requirement, high financial requirement, intensive labor requirement, weak suppression of gene expression (Guru 2000), and difficult oligonucleotide delivery (Caplen et al. 2001). However, its major drawback was that it sometimes remained non – informative due to death of embryo (Capecchi 1990; Li et al. 1992). Therefore, the necessity for an efficient technology led to the development of ribozyme technology (Cech et al. 1981; Kruger et al. 1982), but this technology was also limited due to the low frequency of homologous recombination and inaccurate integration (Morton and Hooykaas 1995). Furthermore, genetic methods raised the apprehensions about possible dangers in terms of human health and the environment through ecological disturbances, especially when gene transferred to an edible part of the plant holds its origin from non-plant species. These

unequivocal factors lead to convoluted research to ensure the safety of transgenic plant and hence shooting the expense.

Aforementioned drawbacks paved the way for the discovery of RNAi as a powerful and efficient approach in the field of molecular biology, which stepped forward as a phenomenon termed as "co-suppression" (Napoli et al. 1990). This technique evolved as serendipity in an experiment to enhance purple coloration of petunias by overproduction of chalcone synthase enzyme, which is a precursor in anthocyanin biosynthesis (Jorgensen et al. 1994). However, unexpected results were observed with the blooming of white or variegated flowers. This observation led to a series of experiments, conducted by Fire and Mello, to conclude that double-stranded RNA molecules, which are homologous with the target sequence, play a vital role in gene silencing (Fire et al. 1998).

16.1.3 Advantages of RNAi

RNA interference is a targeted approach of functional genomics to determine gene function. This approach has got following advantages:

- It is a targeted approach to determine gene function.
- Silencing of a single gene or multiple genes could be achieved.
- Silencing effect generates a mobile signal (Mlotshwa et al. 2008) known as a systemic response that spreads from the site of initiation to neighboring cells through plasmodesmata (Molnar et al. 2007).
- RNAi avoids problems related with knockout techniques such as abnormalities or early embryonic death, which affects observation (Preuss and Pikaard 2003).
- RNAi eliminates the need for laborious transgenic knockout identification and isolation.
- Silencing is systemic. Silencing travels through vascular system from few leaf cells to whole tissue and further to whole plant (Kalantidis et al. 2008).
- It is a highly efficient technique, which can be amplified and obtained with the minimum trigger.

16.2 Mechanism of RNA Silencing

Current technology of inducing RNAi in plants derives its root from the observations of Waterhouse and coworkers to demonstrate silencing of the target gene as a result of expression of mRNA containing inverted repeats (Waterhouse et al. 1998). It is also observed that silencing efficiency is increased by the addition of introns interposed between inverted flanking target sequences within RNAi transgene (Smith et al. 2000). As shown in Fig. 16.1, RNA-mediated silencing machinery performs through a set pattern of three descriptive steps which are detailed below along with their elements:

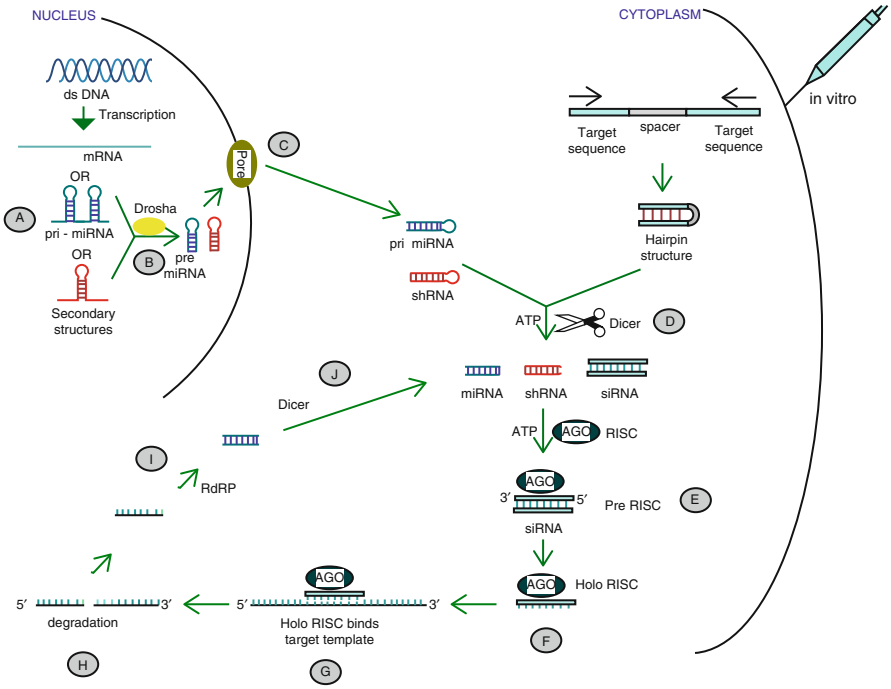

Fig. 16.1 Mechanism of RNA interference (RNAi) (*A*) after transcription apart from mRNA, formation of hairpin structures such as primary miRNA (pri-miRNA), and several secondary structures are formed. (*B*) miRNAs and secondary structures are cleaved by nuclear RNase III endonuclease enzyme, called Drosha, to liberate stem structures to form pre-miRNA. In plants, function of Drosha is also carried out by nuclear protein Dicer. (*C*) Pre-miRNA moves out of nucleus with the help of Exportin 5 protein. (*D*) Within cytoplasm, either pre-miRNA or in vitro introduced hairpin structures undergo cleavage to remove hairpin structures by a multidomain RNase III enzyme called Dicer to produce (*E*) miRNA, shRNA, and siRNA. They combine it with RNA-induced silencing complex, which at this stage is known as pre-RISC. (*F*) Helicase activity of pre-RISC unwinds siRNA retaining antisense strand with pre-RISC, and the complex is known as holo-RISC. (*G*) The antisense strand acts as guide strand for holo-RISC to target complementary RNA strand for cleavage. *(H)* Complementary strand is cleaved between 5' and 3' ends by holo-RISC. (*I*) Cleaved single-stranded RNA acts as template for RNA-dependent RNA polymerase (RdRp) and is again converted into double-stranded fragment for signal amplification. (*J*) This double-stranded fragments are again processed by Dicer and hence enter in RNAi cycle causing amplification of response

1. *Initiation*: Double-stranded RNA (dsRNA) once present inside the cell is automatically recognized by an ATP-dependent enzyme complex called Dicer, which cleaves dsRNA into small fragments of 21–23 bp small interfering RNA molecules, commonly known as siRNA. Under natural conditions, processing of short hairpin RNA or primary miRNA molecules is carried out in the nucleus by endonuclease called "Drosha," which liberates stem-loop containing miRNA between 3′ and 5′ overhangs. Processed primary miRNA molecules are known as pre-miRNA which moves out to cytoplasm with the help of a protein called

Table 16.1 Various Dicer or Dicer-like proteins reported

Initiator	Molecule formed	Length of molecule	Function	References
DCL 1	miRNA	21 nucleotides	Release miRNA through processing of foldback precursor (tasiRNA)	Dong et al. (2008) and Grigg et al. (2005)
DCL 2	siRNA	24 nucleotides	Synthesize natural stress-related transcripts (nat-siRNA)	Bouche et al. (2006) and Mlotshwa et al. (2008)
DCL 3	siRNA	24 nucleotides	Heterochromatin formation	Pontes et al. (2006) and Wierbicki et al. (2009)
DCL 4	siRNA	21 nucleotides	Mediate posttranscriptional gene silencing for some genes. It also terminates transcription if normal mechanism fails	Gasciolli et al. (2005), Yoshikawa et al. (2005), and Liu et al. (2012)

Exportin 5 (Meister et al. 2004; Blevins et al. 2006). Further, it has been cited that 3′ adenine 5–8 bp/thymine 5–8 bp overhangs enhance siRNA-mediated silencing up to tenfold by increasing complex stability (Bellemin et al. 2007). Elements of initiation include initiators and triggers as discussed below:

1.1. *Initiator*: Dicer or DCL (Dicer-like) protein is a large ATP-dependent multiprotein RNase III enzyme. Many forms of Dicer protein have been identified and are classified based on their activities (Table 16.1). The basic role of Dicer lies in processing and preparing small RNA molecules for activation of RNA-induced silencing complex. Dicer is expressed in different tissues at different stages of development or against various environmental stresses (Deleris et al. 2006). It is a multidomain ribonuclease generally containing six domains: DEAD box, helicase C, DUF283, PAZ, ribonuclease III, and double-stranded RNA-binding domain (dsRBD) (Liu et al. 2009). Forms of Dicer protein vary from organism to organism, Dcr1 and Dcr2 exist in animals, and Dcl1, Dcl2, Dcl3, Dcl4, and Dcl5 exist in plants, out of which Dcl5 has evolved in monocots only (Marqis et al. 2006).

1.2. *Triggers*: Triggers include various RNA molecules like RNA virus, transposon, exogenously introduced siRNA, and endogenous small noncoding miRNA and siRNA. Various types of triggers are discussed below:

1.2.1. MicroRNAs (miRNAs): These are naturally occurring small noncoding RNA molecules which play a vital role in the regulation of transcriptional and posttranscriptional gene expression (Kevin and Nikolaus 2007). Biogenesis of miRNAs includes processing of primary miRNAs (pre-miRNAs) by various endonucleases such as Drosha, Serrate, Dicer, etc. with a purpose to generate duplex named as pre-miRNA. This duplex is transported to the cytoplasm where the RNAi response is elicited. Endonucleic protein “Drosha” is conserved among eukaryotes (Tanzer and Stadler 2004; Molnar et al.

2007) with few variations in mode of target recognition (Brodersen et al. 2008; He and Hannon 2004; Lewis et al. 2003, 2005). miRNAs are known for its role in normal cell functioning (Mraz and Pospisilova 2012). Any mutation in miRNAs leads to diseases. For instance, a mutation in miR-96 of seed region causes hearing loss (Mencia et al. 2009). Many literary reviews also link miRNA mutations with cancer (He et al. 2005; Mraz et al. 2009).

1.2.2. Short hairpin RNAs (shRNAs): These are artificial ribonucleic acid polymers, analogous to miRNAs. They are delivered to the nucleus with viral vectors and form a hairpin-like structure called pre-shRNA which is cleaved by an endonuclease, "Drosha," along with double-stranded RNA-binding protein to relieve stem structures between 3′ and 5′ overhangs. This cleaved fragment is transported out to the cytoplasm where it enters RNAi pathway in a manner similar to miRNAs (Rao et al. 2009).

1.2.3. Natural siRNAs (nat-siRNAs): Biogenesis of nat-siRNAs includes overlapping of transcriptional product from two genes (Borsani et al. 2005). They also move into similar RNAi pathway as of other siRNAs. One example of nat-siRNA biosynthesis cited in literature includes overlapping of salt stress-induced gene transcript (SRO5) with Δ1-pyroline-5-carboxylate dehydrogenase (P5CDH) for generation of 24-nucleotide-long nat-siRNA.

Four types of endogenous siRNA have been discovered: trans-acting siRNA (tasiRNA) in *Arabidopsis thaliana*, repeat-associated siRNA (rasiRNA) in *Schizosaccharomyces pombe* and *Arabidopsis thaliana*, small-scan (scn)RNA in *Tetrahymena thermophila*, and PIWI-interacting (pi)RNA in mammals (Rana 2007). tasiRNAs are among few recently found siRNAs. tasiRNAs are dsRNAs derived by synthesis of the second strand of the antisense strand of miRNA with the help of an enzyme, RNA-directed RNA polymerase 6, also known as RdRP6 (Allen et al. 2005). Double-stranded tasiRNAs are protected from degradation by a suppressor of gene silencing (Yoshikawa et al. 2005) and are processed by analogs of RNase III enzyme called Dicer-like proteins 4 (DCL4). DCL4 works in association with dedicated double-stranded RNA-binding protein 4 (DRB4) to form 21-nucleotide-long tasiRNA molecules. During activation of RNA-induced silencing complex, tasiRNAs act as substrates for RISC (Adenot et al. 2006).

2. *Activation of RISC*: RNA-induced silencing complex (RISC) or RNA-induced transcriptional silencing complex (RITS) plays a crucial role in the regulation of gene expression. RISC is an ATP-dependent multiprotein complex. The core of RISC is formed by Argonaute proteins, which bind to guide strand through N-terminal and C-terminal (Fig. 16.2). Argonaute proteins are present diffusely at the cellular level and are predominantly expressed in germ lines for transposon silencing (Aravin et al. 2006). An Argonaute protein family consists of three

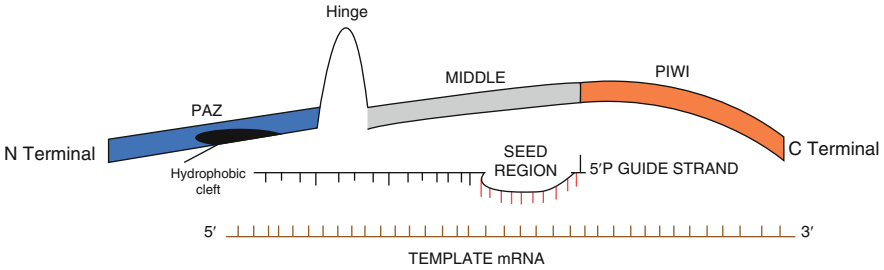

Fig. 16.2 Diagrammatic representation of Argonaute protein in RISC. Composition and working of RISC, the Argonaute protein forms the core of RISC. Interaction between silencing complex and target mRNA takes place through guide strand in RISC complex. Argonaute is composed of two terminals, held together by a hinge which adds flexibility to protein. N-terminal includes a single domain called PAZ which harbors a cleft and is able to bind 3′ end of guide strand. C-terminal includes two domains, namely, middle domain and PIWI domain. 5′ phosphate of guide strand is able to bind in pocket of both domains. During binding of guide strand, its structure gets distorted, and 4–6 bases extrude out and can easily interact with target mRNA

clades, and a largest clade of the family is called Argonaute and is present in the nucleus and cytoplasm of most of plants, animals, fungi, and protists. Argonaute proteins are also known as AGO against the name of plant "*Arabidopsis*," in which it was reported initially. The second clade is called PIWI, named after PIWI protein found in *Drosophila*. The third clade belongs to worms and hence named as worm Argonaute (Liu et al. 2004). Homology of prokaryotic Argonaute proteins to that of eukaryotes provides a platform to discover the functions of Argonaute proteins in RNA interference mechanism. Structurally, Argonaute proteins are bilobed structures, in which both the lobes are interconnected by a hinge composed of secondary proteins. Each lobe is capable of binding to ends of antisense strand which acts as a guide strand in RNAi mechanism (Pratt and MacRae 2009). N-terminal of protein contains single PAZ domain which is capable of binding at 3′ terminal of guide strand whereas C-terminal is able to bind to 5′ phosphate of the guide strand. Two domains of C-terminal lobe also bear some hydrolase activity and are capable of cleaving target RNA molecules. Through Argonaute proteins, RISC binds to siRNA to form a complex called pre-RISC (Nykanen et al. 2001). For activation of RNA-induced silencing complex, pre-RISC is converted into holo-RISC through a series of reactions which include unwinding of siRNA and then holding of the antisense strand. Anchoring of guide strand through 5′ phosphate group with a pocket between middle and PIWI domain of C-terminal lobe of Argonaute protein acts as a checkpoint of RNAi pathway (Zhang et al. 2006; Carthew and Sontheimer 2009) and guides holo-RISC to the complementary target template. The first nucleotide of guide strand also tucks in the pocket and hence does not contribute in target recognition (Batista et al. 2008). Rest nucleotide bases of strand interacts with the protein through their phosphodiester backbone and hence makes AGO non-sequence specific (Wang et al. 2008). Two bases at 3′ terminal get clamped into a hydrophobic cleft in PAZ domain, but 11–18 bases lie in disordered form to provide

mobility to guide and to make AGO non-length specific (Pratt and MacRae 2009). Moreover, the hinge between C- and N-terminal of Argonaute proteins helps RNA-induced silencing complex to attach to target mRNA strand. Diagrammatic representation of the interaction of Argonaute protein present in RISC with target mRNA molecule is discussed in Fig. 16.2.

3. *Degradation of target mRNA*: Well-established contact between Argonaute and guide strand enables recognition of target sequence from a pool of RNAs. Two to six bases of guide expose themselves fully to ease the formation of a double helix with a complementary region of target RNA molecule (Haley and Zamore 2004; Ameres et al. 2007) and are known as "seed region" (Lewis et al. 2003; Pratt and MacRae 2009). Protruding seed region is used as an initial probe by RISC to locate the target and hydrolyze it to break reading frame (Tolia and Joshua 2007). The cleaving ability of RISC is catalyzed by divalent ion-dependent RNase activity of PIWI domain of Argonaute protein. Target is sliced from the point where 10–11 bases of guide interact with it and thus two fragments are produced (Forstemann et al. 2007; Eulalio et al. 2008). Besides degrading posttranscriptional product (mRNA), RISC is also able to target genome directly and is known as RNA-induced transcription silencing (RITS) complex which modifies histones through methylation and also interacts with RNA-dependent RNA polymerase (RdRp) to convert RNA transcript to dsRNA (Buhler et al. 2006; Volpe et al. 2002; Moazed 2009).
4. *Amplification of interference response*: The RdRp is capable of synthesizing double-stranded RNA molecules by using single-stranded antisense strand, also known as passenger strand. This strand is removed from sense strand through helicase activity of RNA-induced silencing complex (RISC) while activating pre-RISC to holo-RISC. This dsRNA once again becomes substrate for Dicer, and RNAi pathway is repeated leading to amplification of response, also known as transitivity (Forrest et al. 2004; Smardon et al. 2000).

16.3 Applications

A huge number of RNAi applications in agriculture biotechnology include crop improvement by enhancing the nutritional value of crop, reduction or modification of undesirable component(s) present in the edible part of plant, enhancements of coloration in ornamentals, development of biotic and abiotic stress-tolerant plants, etc. Though, crop improvement holds major attention in the application due to potential benefit in the attainment of food security. One good example cited in literature includes a reduction in the level of a terpenoid, that is, gossypol in cotton (Kumar et al. 2006). Gossypol is present throughout the cotton plant and provides protection from insects and pathogen (Kumar et al. 2006). However, cardio- and hepatotoxic nature of the terpenoid makes cotton seeds unsuitable for human and animal consumption. Biosynthesis of gossypol includes a key enzyme, delta-cadinene synthase. With a purpose to reduce gossypol levels in seeds, RNAi was implemented to silence delta-cadinene synthase gene during seed development.

Early ripening of tomatoes is another challenge in the way of agroeconomy. Ripening of fruits is triggered by ethylene. With a final objective to delay ripening of tomatoes, an enzyme involved in ethylene biosynthesis, 1-amino cyclopropane-1-1carboxylate (ACC) synthase, was targeted and downregulated through RNAi. As an additional benefit, the level of polyamines and ascorbic acid was increased, which in turn increases shelf life and improves juice quality (Gupta et al. 2013). Other instances of crop improvement through gene silencing include reduction of beta-carotene content in potato through silencing of beta-carotene hydroxylase gene (Eck et al. 2007) and production of non-stimulatory decaffeinated coffee through RNAi-mediated suppression of CaXMT1 gene encoding theobromine synthase enzyme, which catalyzes addition of methyl group to xanthosine and its conversion to caffeine in biosynthetic pathway (Ogita et al. 2003).

Another application of RNAi in functional genomics is to assign functions to unknown genes by reverse genetics and to throw light on various plant interactions. Last but not least, the antiviral role of RNA silencing cannot be left untouched. RNAi exists as a natural antiviral defense in plants. Most of the plant viruses have sense-stranded RNA (Schuck et al. 2013) which plays a role similar to mRNA and also acts as a template for the enzyme, RdRp in host cell cytoplasm and synthesize double-stranded virus RNA (Ortin and Parra 2006). Replicated RNA acts as key silencing inducers (Ding 2010; Pantaleo 2011) and helps in siRNA production (Wang et al. 2008) which gets accumulated throughout the plant and reduces virus titer (Szittya et al. 2002; Moissiard and Voinnet 2004). Various reports have demonstrated the potential of exogenous small dsRNA molecules against various pathogens, e.g., reduction of Israeli acute paralysis virus (IAPV) disease in honeybees (*Apis Mellifera*) (Hunter et al. 2010), defense against vascular fungi causing *Verticillium* wilt disease in *Arabidopsis* (Ellendorff et al. 2009), development of resistance to banana bract mosaic virus (BBrMV) in banana (Rodoni and Dale 1999), and development of resistance to barley yellow dwarf virus (BYDV) in barley (Wang et al. 2000). Many other examples of the use of RNAi in crop sciences have been enlisted in Table 16. 2.

16.4 Conclusion

With the first demonstration of double-stranded RNA-mediated silencing in *Arabidopsis thaliana*, phenomena of RNAi have established itself as a most potent biotechnological tool for crop improvement through the decrease of disease pressure and enhancement of important agronomic traits. A better understanding of triggers, initiators, and pathways of RNA interference is continuously adding to the simplicity of the technique. Although various plants have been modified for better resistance to diseases, still there is a long way to understand the technique for efficient and effective use. The efficiency of RNA silencing is a result of interactions between protein complexes and double-stranded RNA molecules, sequence similarity between the target sequence and initiator, etc. Thus prior prediction of silencing

Table 16.2 Alteration in various agronomic traits through RNAi technology for crop improvement

Plant	Gene modified/silenced	Modification through RNAi	References
Cotton	ghFAD2-1, ghSAD1, delta-cadinene synthase	Increased oleic acid and stearic acid content, reduced gossypol level	Liu et al. (2002) and Kumar et al. (2006)
Tomato	ACS, LeETR4, SINCED1	Reduced early ripening, increased lycopene, beta-carotene content	Gupta et al. (2013) and Sun et al. (2012)
Potato	SBE II, DET 1, SYR1	Increased amylose and carotenoid, flavonoid content, increased resistance to late blight	Shimada et al. (2006), Davuluri et al. (2005), and Eschen-Lippold et al. (2012)
Maize	SDH, SBE II	Improved lysine content and increased amylopectin levels	Tang et al. (2007), Regina et al. (2006), and Guan et al. (2011)
Tobacco	CYP82E4, STM	Reduced nicotine levels, improved seed germination, understanding role of a gene	Lewis et al. (2008)
Melon	Cm-elF4E	Resistance to four viruses: CVYV, MNSV, MWMV, ZYMV	Rodriguez et al. (2012)
Cassava	Delta CP, CYP79D1, CYP79D2	Resistance to brown streak disease, reduced linamarin content	Ogwok et al. (2012) and Jorgensen et al. (2005)
Coffee	CaMXMT1	Reduced caffeine content	Ogita et al. (2003)
Onion	Lachrymatory factor synthase gene	Tearless onion	Kamata et al. (2011)
Rice	OsDSG1, OsGA20ox2, PNS12	Enhanced drought resistance, improved grain yield, resistance to rice dwarf virus	Wang et al. (2011), Qiao et al. (2007), and Shimizu et al. (2009)

efficiency is difficult. Moreover, the involvement of mixed pathogens in a disease outbreak presents another challenge to modify plants to resist infection outside polyhouse facilities. Therefore, much experimentation is still needed in the field of RNA-mediated silencing for strengthening of a technique for its effective utilization for crop improvement.

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In Vitro Selection of Disease-Resistant Plants

17

Srinath Rao and H. Sandhya

17.1 Introduction

Worldwide crops are attacked by several diseases, and due to these diseases, heavy loss in the yield of crop plants is a common phenomenon. This is more common in tropical regions. Diseases affect plants during all stages of their life cycle and even during storage stage. Controlling the pathogens through chemical pesticides is a very costly affair and labor intensive (Bezier et al. 2002). In addition, they also pose environmental hazards as they are nonbiodegradable; they accumulate in the soil and reduce the production of crops. They also cause loss of local flora and fauna. Hence, it has become pertinent to find alternative biotechnological methods to develop disease-resistant crop plants. An important step in the breeding of crops is to develop an effective strategy for selection of desirable traits (Roane 1973; Van den Bulk 1991; Novak and Brunner 1992; Lebeda and Savabova 2010). Conventionally, selection of traits of interest is carried out in the field; this involves identifying resistant plants, crossing them with the superior yielding variety, and studying their inheritance pattern, which may require 12–15 years to release a new variety. Compared with the techniques of in vitro selection (Novak and Brunner 1992; Jin et al. 1996a; Patade et al. 2008). Genetic engineering is another approach that can be utilized to develop disease-resistant plants, but limitations in the form of transgene silencing (Manners and Casu 2011) reduced gene expression, and frequency of low transformation (Mondal et al. 1997) and tough legislations (Burnquist 2006) hampers the use of this approach (Table 17.1 and Plates 17.1, 17.2, and 17.3).

S. Rao (✉) • H. Sandhya
Plant Tissue Culture and Genetic Engineering Laboratory, Department of Botany, Gulbarga University, Kalburgi 585106, Karnataka, India
e-mail: srinathraomm@gmail.com

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Table 17.1 List of plants where culture filtrate/toxin has been used for in vitro selection of disease-resistant plants

Sl. No.	Name of the plant	Name of the pathogen	Selection agent	References
1.	*Arachis hypogaea* (groundnut)	*Cercosporidium personatum*	CF	Venkatachalam and Jayabalan (1996)
2.	*Ananas comosus*	*Fusarium subglutinans*	CF, FA	Borras et al. (2001) and Hidalgo et al. (1998)
3.	*Amaranthus hybridus*	*Fusarium oxysporum*	CF	Chen and Swart (2002)
4.	*Allium sativum*	*Fusarium oxysporum* f. sp. *cepae*	CF	Zill-e-Huma Aftab and Saira Banarus (2013)
5.	*Allium cepa*	*Alternaria pori*	PCF	Tripathi et al. (2008)
6.	*Ananas comosus*	*Fusarium subglutinans*	CF	Hidalgo and Bermudez (2010)
7.	*Avena sativa*	*Helminthosporium victoriae*	Toxin	Rines and Luke (1985)
8.	*Azadirachta indica*	*Phomopsis azadirachtae*	PCF	Girish et al. (2009)
9.	*Brassica oleracea*	*Phoma lingam*	TMs	Sjodin et al. (1988)
10.	*Brassica napus*	*Phoma lingam*	PT	Sacristan (1985)
11.	*Cajanus cajan*	*Fusarium odum* Butler	GF	Rao et al. (2006)
12.	*Carthamus tinctorius* (safflower)	*Alternaria carthami*	CF	Kumar et al. (2008a, b)
13.	*Citrus limon* (lemon)	*Phoma tracheiphila*	CF	Gentile et al. (1992)
14.	*Citrus jambhiri*	*Phytophthora parasitica*	CF	Savita et al. (2011)
15.	*Cucumis sativus*	*Fusarium oxysporum*.	CF	Malepszy and El Kazzaz (1990)
16.	*Curcuma longa* (turmeric)	*Pythium graminicolum*	CF	Gayatri et al. (2005)
17.	*Dendrobium* sonia-28	*Fusarium proliferatum*	CF	Dehgahi et al. (2014)
	D. grandiflorum	*Dendranthema grandiflorum*	CF	Kumar et al. (2008a, b)
18.	*Dianthus caryophyllus*	*Fusarium oxysporum* f.sp. *dianthi*	CF	Mosquera et al. (1999)
		Alternaria dianthi	CF	Thakur et al. (2002) and Esmaiel et al. (2012)
		Rhizoctonia solani	CF	Metha et al. (2007) and Sharma et al. (2009)
19.	*Gladiolus grandiflorus* (gladiolus)	*Fusarium oxysporum*	FA	Remotti et al. (1997)

(continued)

Table 17.1 (continued)

Sl. No.	Name of the plant	Name of the pathogen	Selection agent	References
20.	*Glycine max*	*Fusarium solani* f. sp. *Glycines*	CF	Li et al. (1999)
		Septoria glycines		Song et al. (1994)
		Fusarium solani		Jin et al. (1996a, b)
				Hashem et al. (2008)
21.	*Gossypium hirsutum* (cotton)	*Fusarium oxysporum*, *Alternaria macrospora*	CF	Ganesan and Jayabalan (2006)
22.	*Helianthus annuus*	*Diaporthe helianthi*	CF	Quaglia and Zazzerini (2007)
		Alternaria helianthi	CF	Rao and Ramgopal (2010)
23.	*Hordeum vulgare* (barley)	*Fusarium* sp.	FA	Chawla and Wenzel (1987)
24.	*Humulus lupulus*	*Verticillium albo-atrum*	CF	Connel et al. (1990)
25.	*Linum usitatissimum* (flax)	Fusarium oxysporum	CF	Krause et al. (2003)
26.	Lycopersicon *esculentum*	*Fusarium oxysporum*	FA	Shahin and Spivey (1986)
		F. oxysporum f. sp.	CF	Toyoda et al. (1984)
		Lycopersici	FA	Fuime and Fuime (2003)
		Pyrenochaeta lycopersici	CF	
		Verticillium albo-atrum	CF and cell wall components	Koike et al. (1993b)
		Alternaria alternata		Kodama et al. (1991)
				Arcioni et al. (1987)
27.	*Medicago sativa*	*Fusarium oxysporum* f. sp. *medicaginis*	Toxins	Binarova et al. (1990)
		Fusarium spp.	CF	Hartman et al. (1984)
		Fusarium oxysporum	CF	
28.	*Musa textilis* nee	*Fusarium oxysporum* f.sp. *cubense*	CF	Purwati et al. (2007)
29.	*Musa* sp.	*Fusarium* sp.	FA	Matsumoto et al. (1995)
		Fusarium oxysporum f. sp. *cubense*	CF	Matsumoto et al. (2010)
30.	*Nicotiana tabacum* (tobacco)	*Pseudomonas syringae*, *Pseudomonas*, and *Alternaria* TMV	Methionine sulfoximine/ CF	Carlson (1973)
				Thanutong et al. (1983)
				Toyoda et al. (1989)

(continued)

Table 17.1 (continued)

Sl. No.	Name of the plant	Name of the pathogen	Selection agent	References
31.	*Oryza sativa* (rice)	*Helminthosporium oryzae*	CF	Vidhyasekaran et al. (1990)
		Pyricularia oryzae	CF	El-Banna and Khatab (2012)
32.	*Pelargonium graveolens*	*Alternaria alternata*	CF	Saxena et al. (2008)
33.	*Prunus persica* (peach)	*Xanthomonas campestris*	CF	Hammerschlag (1988)
34.	*Saccharum* sp. (sugarcane)	*Colletotrichum falcatum*	CF	Sengar et al. (2009)
35.	*Saccharum officinarum*	*Colletotrichum falcatum*	PPT	Ali et al. (2007)
36.	*Solanum melongena*	*Verticillium dahlia*	CF	Koike et al. (1993)
37.	*Solanum tuberosum*	*Phyrophthora infestans*	CF	Behnke (1979, 1980)
38.	*Vitis vinifera*	*Botrytis cinerea*	CF	Reustle and Matt (2000)
		Elsinoe ampelina		Jayashankar et al. (2000)
39.	*Zea mays* (maize)	*Helminthosporium maydis*	PT	Gengenbach et al. (1977)
40.	*Zingiber officinale*	*Fusarium solani*	CF	Gupta et al. (2006)

CF culture filtrate, *FA* fusaric acid, *TMs* toxic metabolites, and *PPT* partially purified toxin

17.2 Use of Pathogens in Selection

In the 1980s new method of dual culture was developed in which in vitro developed callus tissue of the host and the disease-causing pathogen are cultivated together; Hrib and Rypacek (1983) and Johnson and Whitney (1988) were the first to study the pathogenicity in dual cultures at the level of embryonic stage. Later on several reports appeared on this topic (Hendry et al. 1993; Sirrenberg et al. 1995; Hrib et al. 1995; Terho et al. 2000; Kvaalen and Solheim 2000; Kvaalen et al. 2001). The pathogen responsible for causing the disease can be used as the in vitro selection agent for resistance (Daub 1986; Van den Bulk 1991; Lebeda and Savabova 2010. The concentration of the inoculums, temperature, and the composition of the medium are the important factors which influence the expression of resistance to the disease for which screening is done (Xue and Hall 1992; Bertetti et al. 2009), which may lead to inconsistent results being obtained (Daub 1986). Moreover, this option has limitations including (1) uneven exposure of the cells to the pathogen, (2) whether resistance can be expressed in in vitro cultured cells, and (3) the overgrowth of the pathogen on the cells and medium, which makes it difficult to make

Plate 17.1 (**a**) Effect of increasing concentration of *Alternaria helianthi* culture filtrate on seed germination in sunflower. *1* Control seeds *2*, *3*, and *4*. Seeds treated with 10, 20, and 30% culture filtrate (CF). Note decrease in percent germination with increasing concentration of CF. (**b**) Seedlings raised from 0, 10, 20, to 30% CF-treated seedlings. Note decreased seedling growth with an increase in concentration of CF. (**c**) Seedlings treated with increasing concentration of CF. Note increase in disease symptoms with an increase in the concentration of CF (Ramgopal 2003; Rao and Ramgopal 2010)

observations (Daub 1986). Fungal conidia can be inoculated onto shoot cultures, and these are visually monitored for resistance to the fungus, provided there is a correlation with the effect of the fungus in vivo (George 1993). Several successful experiments have been carried out in vitro with live inoculums, i.e., powdery mildews (*Blumeria graminis*, *Erysiphe pisi*, *Sphaerotheca pannosa*, *Uncinula aceris*) (Webb and Gay 1980). *Plasmodiophora brassicae* (Buczacki 1980), *Colletotrichum trifolii* (Cucuzza and Kao 1986), *Peronospora tabacina* (Tuzun and Kuc 1987), *Phytophthora cinnamomi* (McComb et al. 1987; Cahill et al. 1992), *Alternaria alternata* (Takahashi et al. 1992), and *Fusarium solani* (Huang and Hartman 1998). The in vitro conditions (higher humidity, reduced air velocity, media rich in

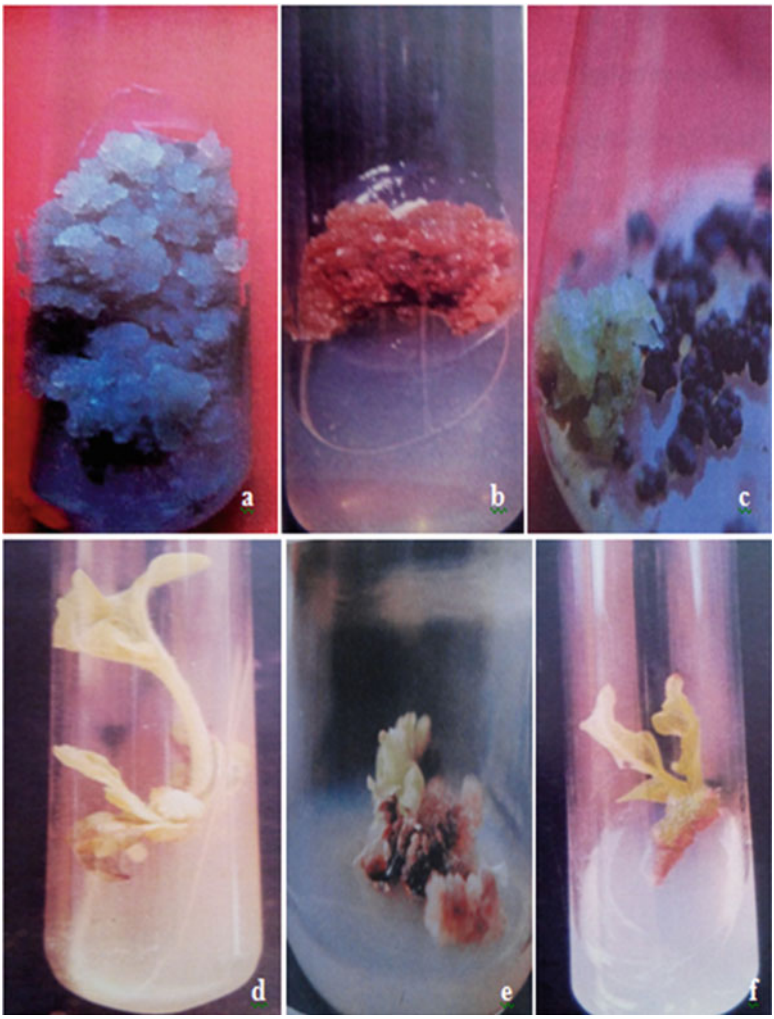

Plate 17.2 (**a**) Callus grown on medium devoid of *A. helianthi* culture filtrate. (**b**) Callus grown on medium containing 40 % CF; note extensive browning and reduction in growth of callus. (**c**) Portion of resistant callus growing on 40 % CF. (**d**) Shoots from control callus (**e** and **f**) shoots initiated from callus resistant to 40 % CF (Ramgopal 2003; Rao and Ramgopal 2010)

nutrients) are very hospitable and favor growth of microorganisms in general. In a number of studies, different live pathogens were tested as agents for in vitro selection but were found to be too devastating for the plant tissues/organs and, therefore, of limited use.

Growing resistant varieties is the most effective control measure. As resistant varieties are not available, it is essential to generate variability and screen the variants. Genetic variation as a result of spontaneous mutations is noticed during culturing of plants in vitro. Larkin and Scowcroft (1981) named them as

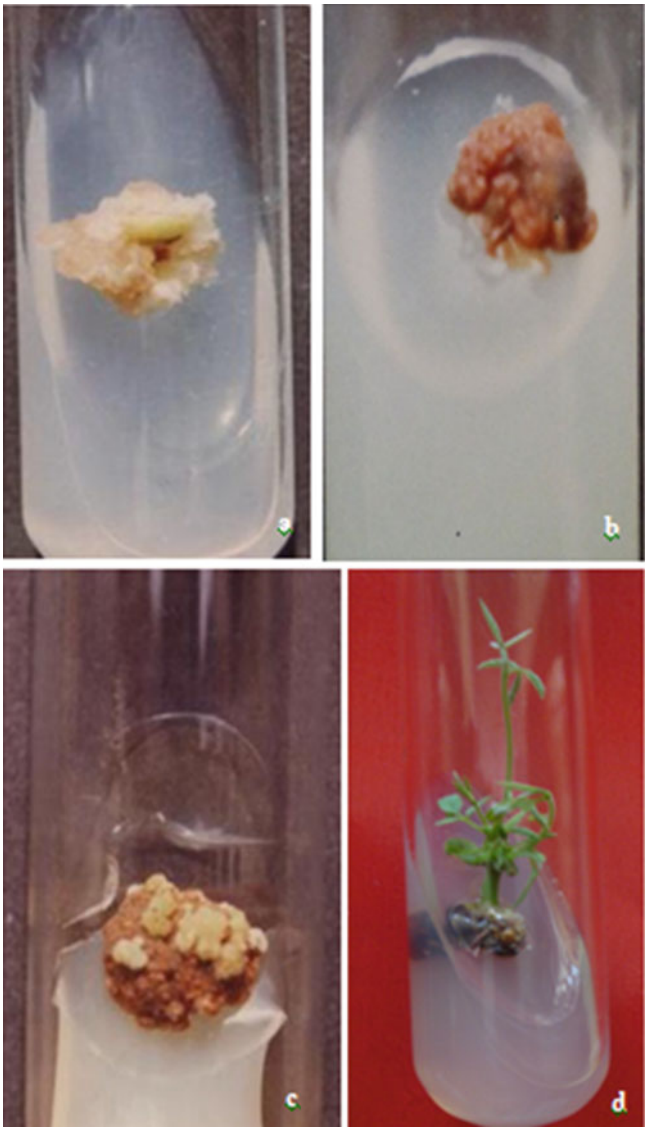

Plate 17.3 (**a**) Callus of *Cajanus cajan* growing on medium devoid of *Fusarium odum* culture filtrate. (**b**) Callus of *Cajanus cajan* growing on medium supplemented with 40 % CF; note extensive browning and reduced growth of callus. (**c**) Surviving sector of callus showing resistance to 40 % CF. (**d**) Regeneration of plantlet from CF-resistant callus (Basavaraj 1992; Rao et al. 2006)

somaclonal variations, and they realized its potential application in crop improvement. Since then, somaclonal variation has been successfully used in crop improvement (Breiman et al. 1987; Van den Bulk 1991; Lakshmanan 2006; Snyman et al. 2011). Further, the exposure of cultured cells to physical or chemical mutagens can be used together with in vitro culture to increase mutation

frequency, thus enhancing somaclonal variation (Maluszynski et al. 1995; Snyman et al. 2011). An in vitro screening protocol utilizing an appropriate selecting agent, such as toxins produced by the pathogen or culture filtrates of the pathogen, can then be used to obtain disease-tolerant plants (Van den Bulk 1991; Chandra et al. 2010). With this approach the screening of large numbers of plants in a limited space over a short period of time is possible (Ahloowalia and Maluszynski 2001; Patade et al. 2008; Suprasanna et al. 2009). The partially purified or purified toxins or culture filtrates of pathogen containing toxins involved in disease development are suitable selection agents for use in vitro (Daub 1986; Svabova and Lebeda 2005). However, it is important to expose the plants selected in vitro to the pathogen in order to confirm tolerance (Thakur et al. 2002; Tripathi et al. 2008; Sengar et al. 2009).

17.3 Rational Behind Using Fungal Culture Filtrate/Toxins for In Vitro Selection

Fungal metabolites which are present in the culture filtrates are able to produce disease-like symptoms and trigger elicitation of defense responses. Wheeler and Luke (1955) first used phytotoxin in resistance breeding. Several studies have confirmed a correlation between tolerance of plants to toxins or culture filtrates and that obtained by inoculating with the pathogen (Gengenbach et al. 1977). It is reported that when calli of soybean susceptible to *Fusarium-caused* disease were exposed to elevated levels of culture filtrate of *Fusarium solani*, it will show reduced growth and regeneration (Jin et al. 1996a, b). Regenerated plants which are not affected by the toxin are also disease resistant. *Helminthosporium maydis*, the casual agent of southern corn leaf blight, produces T-toxin, which acts specifically on mitochondria of susceptible cells (Miller and Koeppe 1971). In this segregation for virulence but not for pathogenicity was observed from a cross between race T (producing toxin) and race 0 (not producing toxin), suggesting that the toxin is a virulence factor (Yoder and Gracen 1975). Toxin produced by *Alternaria alternata and A. mali* is considered to be pathogenicity factors; mutation eliminating toxin production genes resulted in loss of pathogenicity (Nishimura et al. 1974, 1979). Pathogenicity and toxin production could be restored by another mutation. The culture filtrate from nonpathogenic isolates of *P. gregata*, which causes the brown stem rot of soybean, did not affect the growth of either susceptible or resistant calli (Gray et al. 1986). The virulent isolates of *Helminthosporium oryzae* lost its ability to produce toxin because of repeated subculturing; hence it also becomes nonpathogenic (Vidhyasekaran et al. 1986). It is observed that the phytotoxicity of crude filtrates from *Fusarium subglutinans* is the causal agent of fusariose in pineapple (Borras et al. 1998); further he reported that the callus tissue obtained from the disease-susceptible varieties was more affected by the disease than the calli obtained from resistant cultivars. They further reported that the growth of the callus was reduced, and browning of the callus tissue was more when treated with the crude culture filtrate in susceptible cultivars. Macky et al.

(1994) reported reduced callus growth of *Cucumis melo* exposed to toxins produced by *Myrothecium roridum*. Hidalgo et al. (1998) reported that *F. subglutinans* culture filtrate was toxic to pineapple calli and leaves. Similarly Hidalgo et al. (1998, 1999) observed in pineapple that the plantlets obtained from the calli resistant to culture filtrate of *Fusarium subglutinans* showed resistance to the disease. It is reported that if resistance is found in vivo, then toxin tolerance is also detected in the host cultures (Gentile et al. 1992). From the results it is obvious that these characteristics can be used for in vitro screening of germplasm for resistant lines (Behnke 1979, 1980; Thanutong et al. 1983; Sacristan 1985; Ludwig et al. 1992; Song et al. 1994). Girish et al. (2009) reported that partially purified culture filtrate of *Phomopsis azadirachtae* caused growth reduction and necrosis of neem callus. These reports show that phytotoxins produced by plant pathogens/or their culture filtrate can contribute to pathogenicity and are important in symptom development and can be used to screen for disease resistance/selecting tolerant cell lines (than regenerate plantlets from the resistant cell lines) using in vitro system.

17.4 Advantages and Limitations of In Vitro Selection

In vitro selection for disease resistance is advantageous for several reasons: (1) Cultured cells get uniformly exposed to the selective agent; thus, the number of escapes is highly reduced. (2) Small space is sufficient to maintain the culture systems, thus eliminating the need of expensive green houses required for field testing. (3) Spread of the disease-causing agent can be prevented and confined within the laboratory. In addition, the technique allows for the introduction of a pathogen in a controlled environment, negating the need for strict quarantine if carried out ex vitro (Chandra et al. 2010). In vitro selection can be applied regardless of the source of genetic variation (e.g., "somaclonal variation" or induced mutations). Also the distinction between monogenic and polygenic inheritance is rather irrelevant, since at the selection stage only the phenotype difference between susceptible and resistant is important. However, in vitro culture conditions must be suitable to screen for resistance to the various plant pathogens. It is important that such screening should be performed immediately after the generation of genetic variation, e.g., protoplasts/cells subjected to mutagens could subsequently be placed on toxin-containing media. The plants which have survived the toxin pressure should also be tested for their resistance to pathogen under field conditions. During in vitro selection, a selection pressure can be applied to in vitro cultured cells and/or to the regenerated plants in the culture medium and subsequently to the field plants (Maluszynski et al. 1995; Chandra et al. 2010). This approach allows for selection of a large number of tolerant plants/cells in a limited space under specific environment that can be controlled which is free from abiotic and biotic factors that might negatively influence selection (Chaleff 1983; Duncan and Widholm 1990). This strategy also allows uniform exposure of the cells to the selection pressure by culturing them on media containing the toxin (Daub 1986). For example, a potato breeder may screen in the

greenhouse or the field perhaps 50,000–100,000 seedlings per year for disease resistance. On the other hand, 20 million protoplasts obtained from only 1 g of leaf tissue could be easily cultured in a laboratory. If a given beneficial mutation such as disease resistance has a probability of 10^{-5}, then perhaps one such mutant would occur among the 100,000 seedlings. However, 200 mutant plants might be obtained from protoplast culture screening. Thus, a serious consideration of the potential of in vitro selection both with respect to various pathogen types and to various host plant materials is clearly warranted.

However, limitations of in vitro selection are that traits expressed at the cellular level might not be expressed at the plant level (Daub 1986). Furthermore, the technique cannot be used to select certain phenotypic traits (e.g., agronomic traits), which require cell differentiation and organization (Chaleff 1983). When the desired traits are dominant and homozygous recessive, resistant cells and plants can be selected immediately, but crossing is necessary in cases of heterozygous plants in order to obtain plants with recessive traits (Van den Bulk 1991). The traits expressed in cells as a result of epigenetic variation may not be expressed in the progeny of the plants, as the epigenetic effects are reversed by meiosis during sexual reproduction (Chaleff 1983; George 1993; Suprasanna et al. 2009).

17.5 Requirements for the In Vitro Selection of Variant Cells and Plants

The tolerant plant can be generated if (1) there should be high variations in the cells available, (2) selection method can be easily applied, (3) the tolerant cells/tissue should easily regenerate into a complete plant, and (4) tolerance to toxins or culture filtrates expressed by somaclonal variants should correlate to tolerance to the pathogen (Van den Bulk, 1991; Svabova and Lebeda 2005). (5) According to Koch's postulates (Parry 1990), plants susceptible to the pathogen should exhibit symptoms similar to those displayed by diseased plants from which the pathogen was initially isolated. The tolerant plants should display no or minimal symptoms in the presence of the pathogen in the plant tissue (Gengenbach et al. 1977; Arcioni et al. 1987; Botta et al. 1994). Cells should not be exposed to high levels of the toxin early in selection as this might affect the ability of resistant cells to regenerate (Gengenbach et al. 1977). (6) Inheritance of the desired character is an essential component (Widodo and Sudarsono 2005).

17.6 Studies on Selection for Disease Resistance Through In Vitro Technique

Carlson (1973), for the first time, reported that it is possible to select plants for resistance through in vitro techniques. He used methionine sulfoximine (MSO) as an agent for selecting cells and protoplasts of tobacco. Since that time, several

researchers have used this technique which has yielded sufficient data on in vitro selected disease-resistant plants and regeneration of plants from callus selected for resistant toward fungal culture filtrate or toxin produced by the pathogens. *Helminthosporium maydis* induces southern corn leaf blight in maize. Gengenbach et al. (1977) used the purified toxin produced by this fungus to select for cells that were resistant to the corn leaf blight disease in maize. Hartman et al. (1984) reported selection of alfalfa cell lines which showed resistance to the toxin(s) produced by *Fusarium oxysporum* f. sp. *medicaginis* and regenerated plants from the selected cell line which showed resistance to the disease caused by this fungus. Plants resistant to eyespot disease have been selected using a toxin produced by the *H. sacchari* in sugarcane (Chaleff 1983; Prasad and Naik 2000). Amusa (1998) tested cassava plants for resistance to anthracnose disease using phytotoxic metabolites of *Colletotrichum gloeosporioides* f. sp. *manihotis* and reported that the plants are resistant to disease even in field conditions. Ali et al. (2007) partially purified a toxin produced by *Colletotrichum falcatum* and used it to select mutants resistant to red rot in sugarcane. Culture filtrates can be used when there is no reliable description of the toxins produced by the fungus. The fungal CF is prepared by passing the liquid culture through a series of filters in order to remove the mycelia and conidia (Sengar et al. 2009). Potato callus was selected showing resistance to culture filtrates of *Phytophthora infestans*, and plants were regenerated from the resistant callus (Behnke 1979, 1980). Toyoda et al. (1984) selected *F. oxysporum* f. sp. *lycopersici* culture filtrate-resistant tomato cells after exposure to the mutagen N-methyl-N-nitro-N-nitrosoguanidine (MNNG). Hammerschlag (1988) selected peach cells resistant to culture filtrates of *Xanthomonas campestris* pv. pruni and regenerated plants which also showed resistance to disease caused by *Xanthomonas campestris* pv. pruni. Alfalfa plants resistant to *Fusarium oxysporum* f. sp. *medicaginis* were selected through in vitro techniques by Arcioni et al. (1987). Hidalgo et al. (1998, 1999) reported selection and regeneration of plantlets in pineapple from calli resistant to culture filtrate of *Fusarium subglutinans* and observed that the plantlets obtained from the resistant calli showed resistance to the disease. Ahmed et al. (1996) isolated *Fusarium-resistant* somaclones in wheat using culture filtrate of *Fusarium*. Krause et al. (2003) reported flax plants (*Linum usitatissimum*) raised from another culture and callus tissue showing *resistance* to *Fusarium oxysporum. Vitis vinifera* plants showing resistant to *Elsinoe ampelina* culture filtrate were obtained by Jayashankar et al. (2000) and further added that the plants thus obtained were resistant to the fungus. Somatic embryos of cotton (*Gossypium hirsutum* L. cv. SVPR 2) plants were screened with fungal culture filtrate, and disease-tolerant plants were obtained (Ganesan and Jayabalan 2006).

The above reports show that culture filtrates are potential for in vitro screening for disease resistance (Ludwig et al. 1992; Song et al. 1994). This is an easy and effective method as culture filtrates have been shown to be phytotoxic (Suprasanna et al. 2009; Chandra et al. 2010).

17.7 Phytotoxins from Plant Pathogens

A number of plant pathogens produce toxins (Wood et al. 1972; Dublin 1981) as a mode of protection against a host plant's defenses, enabling them to kill host cells and in the process induce disease symptoms (Markham and Hille 2001). Toxic metabolites isolated from fungal species often cause symptoms similar to those caused by the live pathogens. Over 250 fungal and bacterial phytotoxins have been extracted and characterized (Lebeda and Savabova 2010). Such toxic metabolites include colletotin from *C. fuscum* (Goodman 1960; Lewis and Goodman 1962) and from *C. nicotianae* (Masatoshi et al. 1976, 1978), phomopsin A by *Phomopsis leptostromiformis* (Lanigan et al. 1979), cercosporin from *Cercospora* sps. (Daub 1982), enniatin from *Fusarium tricinctum* (Burmeister and Plattner 1987), dihydrofusarubin and isomarticin from *Fusarium solani* (Nemic 1988), malseccin from *Phoma tracheiphila* (Gentile et al. 1992), colletotrichin and colletopyrone from *Colletotrichum gloeosporioides* (Jayasankar et al. 1999), solanopyrone A, B, and C from *Ascochyta rabiei* (Hamid and Strange 2000), phomalide from *Phoma lingam* (Pedras et al. 1999), destruxin B (Pedras and Biesenthal 2000), and AM toxin from *Alternaria alternata* (Saito et al. 2001). The phytotoxic metabolites of most of these pathogens have been reported to play a significant role in pathogenesis (Chandraskharan and Ramakrishnan 1973; Walker and Templeton 1978; Amusa 1991; Amusa et al. 1993). Some of these metabolites, also known as pathotoxins, are chemopathogens of biological origin that can be used to replace the pathogens in studies to investigate the nature and the development of pathogenesis (Wheeler and Luke 1963). This is because the pathotoxins play a causal role in the onset of diseases and produce symptom characteristic of the disease in susceptible plants. These toxins cause wilting, necrosis, and chlorosis of plants (Chandra et al. 2010). They can, therefore, be used as in vitro selection agents (Chandra et al. 2010). Thus, phytotoxic metabolites have been used to screen crops for disease resistance (Wheeler and Luke 1955; Hartman et al. 1986; Amusa et al. 1994; Amusa 1998, 2000).

A prerequisite for the use of a toxin is to determine that it contributes to pathogenesis, i.e., that it is a pathotoxin (Van den Bulk 1991). To determine this, various approaches can be undertaken, viz.: (1) the phytotoxin can be extracted from the infected plant; (2) the phytotoxin's presence at a crucial stage of the disease can be tested; and (3) the phytotoxin's ability to induce disease symptoms on the plant can be assessed (Yoder 1980; Hamid and Strange 2000). Further, the gene(s) responsible for the synthesis of the toxin can be made dysfunctional, and pathogenesis of the mutated fungus can then be assessed (Desjardins and Hohn 1997). In this strategy, it is postulated that cells resistant to the phytotoxins will also be resistant to the pathogen (Daub 1986; Van den Bulk 1991; Desjardins and Hohn 1997; Chandra et al. 2010). Consequently, initial tests should be conducted to establish the effect of the toxin or filtrate on the plant tissue cultures to determine a suitable concentration of the toxin or filtrate that can be used in selection (Lebeda and Savabova 2010). The purified toxins can be used in selection strategies (Remotti et al. 1997; Khan et al. 2004). They can be either host specific or nonhost specific (Markham and Hille 2001) and can be purified from culture filtrates

(Mayama et al. 1990) or acquired from commercial suppliers (Desjardins and Hohn 1997; Remotti et al. 1997). Purification of the culture filtrates can be done using ultrafiltration and fast protein liquid chromatography (Bailey 1995). However, due to the conditions provided in vitro, the concentration of toxins produced is likely to be greater than that produced by the fungus in vivo (Yoder 1980). This might result in a weak correlation between the amount of toxin in vitro and virulence of the fungus in vivo (Yoder 1980; Tripathi et al. 2008). Hence, the toxin-tolerant plants should be inoculated with the pathogen to confirm tolerance (Chen and Swart 2002). Since inoculation is usually carried out in non-sterile environments and there is, therefore, potential for secondary infection by other pathogens, it is important to confirm that the inoculated pathogen is the causal agent of observed symptoms (Harris 1999). This can be achieved by re-isolation of the pathogen onto appropriate culture media and identification of the isolates (Chen and Swart 2002; Abdel-Monaim et al. 2011).

17.8 Possible Mechanism of In Vitro Variability and Stability

Tissue culture- or mutation-induced variations are manifested in three ways: (1) the tissue culture system itself acts as a mutagenic system because cells experience traumatic conditions during explant isolation and culture and may undergo a type of reprogramming during plant regeneration that is different to that under natural conditions (Jain 2001), and thus, we get variation within cell cultures, in which individual cells within a culture may vary in morphology and genetic makeup; (2) primary regenerants are showing a nonheritable phenotypic effect but that can be maintained as asexually propagated plants if the variation is somatically stable; and (3) heritable variation is sexually transmitted to the offspring in a seed-derived population (Kaeppler et al. 1998; Jain 2001).

17.9 Methodology

A major problem associated with the evaluation of somaclonal variation for disease resistance is the availability of efficient, reliable screening methods (Sebastiani et al. 1994). The effect of the selection agent (pathogen culture, culture filtrate, phytotoxins, etc.) must be demonstrated in a preliminary experiment, where a suitable concentration range allows for a comparison of the toxic effects on susceptible and tolerant/resistant germplasm. To apply a selection pressure in vitro, the concentration of the selection agent that kills or inhibits the growth of cells has to be established for incorporation into the selection medium (Suprasanna et al. 2009). Exposure of cells to the selection agent can either be single step with two to three times the lethal dose of the agent or multiple step where the concentration of the selection agent is gradually increased, starting at the lethal concentration (Suprasanna et al. 2009).

The outcome of such preliminary experiments is to ascertain the precise dosage of the selection agent that is optimal for screening resistant material while killing or drastically reducing growth of susceptible.

In vitro cultures can be initiated by using various explants, viz., stems, leaves, apical tissues, floral parts, gametes, meristematic regions, lateral buds, shoot tips, immature cotyledons, zygotic embryos protoplasts, cell suspensions, and organogenic or embryogenic calli (Bhojwani and Razdan 1983).

Two types of selection agents, live pathogen/spores or culture filtrate and phytotoxin/pathotoxin, can be used incorporated in the selection medium (Svabova and Lebeda 2005; Lebeda and Savabova 2010).

When appropriate selection pressure is applied through a selection agent, plant organs/tissues/cells that survive the selection pressure are potential sources of tolerant/resistant subclones. The difference between the selected tolerant/resistant lines and the original plant material may originate from somaclonal variation or induced mutagenesis.

17.9.1 In Vitro Selection

The perfect model of in vitro selection for disease resistance should comprise: (1) an explant culture able to generate genetic variation with high ability to regenerate resistant/tolerant, genetically stable fertile plants; (2) a selection agent which induces similar biochemical reactions as the pathogen in vivo; (3) a verification system of resistance/tolerance of in vitro lines to prove acquired character via cocultivation with the pathogen isolate, assessing parameters such as reduced growth of hyphae and reduced weight of mycelia as compared to control, and via greenhouse and field tests; and (4) molecular tools to characterize the selected resistant lines at the DNA level.

These preliminary experiments must be performed for each combination of plant tissue culture and selection agent combination.

The general scheme for experiments which combines biotechnological and phytopathological approaches also has to include the following steps: (1) collection of pathogen isolates and their biological characterization; (2) establishment of pathogen culture; (3) derivation of an effective selection agent; (4) establishment of the plant tissue culture; (5) testing of the effect (phytotoxicity) of the selection agent on the cultures in vitro and a statement of the selection concentrations for a particular plant-pathogen model; (6) serial selection cycles in vitro; (7) regeneration of explants surviving selection pressure to plants; (8) testing in vitro, in vivo, and heritability analysis; and (9) assessment in field conditions under natural infection.

17.10 General Scheme of In Vitro Screening for Disease Resistance and Selection

- Establishment of explant culture (stem, leaves, cells, protoplasts)
- Establishment of the regeneration protocol (organogenesis, embryogenesis)
- Treatment with various mutagens (for increasing the somatic variability) an optional step
- Establishment of the pathogen culture (purification, propagation)

- Biological characterization of isolates (virulence, pathogenicity)
- Isolation of the selection agents: inoculation, double layer, culture filtrate, crude × purified, autoclaved × filtered through Millipore, phytotoxins/pathotoxins
- Establishment of the evaluation method for the assessment of the effect of selection agents on in vitro cultures: selection of suitable concentrations for a particular explant culture
- Several cycles of in vitro selection
- Regeneration of explants that survived the selection pressure
- Biochemical and molecular analyses of selected plant material
- Screening of selected plant material for resistance to pathogen in greenhouse and/or field conditions
- Multiplication of the lines with improved resistance for further use in breeding programs or direct farming

17.11 Concluding Remarks and Future Issues

In recent years, considerable progress has been made regarding the development and isolation of disease-tolerant cell/callus lines using in vitro technique. In vitro selection will save the time required for developing disease-resistant/tolerant lines of important plant species.

The basic advantages of using in vitro cultures as compared to natural conditions are that (1) unfavorable weather and climate conditions are avoided, which enable the assessment of quantitative differences in polygenic traits more easily and precisely; (2) a large number of individuals can be tested in a small space; (3) it is easier to manipulate mutants, haploids, and somaclones with higher variability in the genome; and (4) mass screening of mutants for resistance is facilitated. In vitro select variants should be finally field-tested to confirm the genetic stability of the selected trait. Major problem with in vitro selection is low frequency of selected explants; this can be overcome by the use of explants with high morphogenic potential which may ensure successful regeneration.

Although there are not many reports of cultivars whose resistance is based on in vitro selections, many resistant breeding lines were obtained; hence, interest in utilizing in vitro methods for improving resistance to plant pathogens remains (Upadhyay and Mukerji 1997). Thus, selection of resistance in vitro must be considered as one of the methods which, in combination with conventional resistance screening and plant breeding methods including biotechnological procedures, may offer plant breeders a new approach to accelerate the development of disease-resistant plants (Crino 1997).

These results suggest that the selection approach could have two major limitations: (1) the lack of knowledge concerning the genotypic dependence of the characterized toxins or extracellular metabolites that confer host-pathogen selectivity and (2) no assurance that the susceptibility and/or resistance of cultured tissues to the culture filtrate or toxins reflect those of the whole plant.

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Role of Rol Genes: Potential Route to Manipulate Plants for Genetic Improvement

18

Sana Khan, Syed Saema, Suchitra Banerjee, and Laiq ur Rahman

Keywords
Metabolic engineering • Genetic engineering • *Rol* genes • *Agrobacterium rhizogenes* • Hairy root

Abbreviations

ARMT	*Agrobacterium rhizogenes*-mediated transformation
GA	Gibberellic acid
HR	Hairy root
IAA	Indole acetic acid
ORF	Open reading frame
RBF	Rol binding factor

S. Khan • S. Banerjee • L. ur Rahman (✉)
Plant Biotechnology Division, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow 226015, UP, India
e-mail: faizslaiq@gmail.com

S. Saema
CSIR, National Botanical Research Institute, Lucknow 226001, India

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18.1 Introduction

The art of genetic engineering not only complements conventional breeding strategies by providing opportunities to incorporate foreign genes/enzymes but also serves as a wide platform to strategically manipulate the endogenous rate-limiting steps for desired or elite traits. The transgenic approaches have made possible to recuperate the genetic architecture in order to improve various biotic and abiotic aspects necessary for plant adaptation and survival. *Agrobacterium*-mediated transformation is the viable approach that has been widely utilised as a convenient method for the improvement of particular trait through incorporation and functional characterisation of genes involved in biochemical, phenotypical and genotypic routes of the metabolic pathways.

In the past few years, *Agrobacterium rhizogenes*-mediated hairy root culture technique has been proven as an ideal platform to imitate the metabolite production profile of plant roots (Karuppusamy 2009; Palazon et al. 1997a, b). HRs are differentiated root cultures that are characteristically highly stable, lacking geotropism, hormone independent, fast growing, lateral branching and productive in terms of metabolites. HRs are means of potentially active compounds which can be derived from crop with high risk of extinction, plants associated with limited geopolitical or climatic condition, etc. (Verpoorte et al. 2002). The growing demand for valuable pharmacologically bioactive compounds has led the researchers to search for an alternative which they have got a new hairy root technology. Using *A. rhizogenes*-mediated transformation (ARMT) method, large numbers of chemical compounds were successfully produced under in vitro condition (Guillon et al. 2006; Giri and Narasu 2000). Therefore, by applying *A. rhizogenes*-mediated transformation, it is possible to introduce only gene of interest in the recipient's body, while genetic architecture of the recipient's plant remains the same.

The hairy root disease is caused by *rol* genes which are considered as the natural precursors for tumour formation (Riker et al. 1930; Cardarelli et al. 1987a; Spena et al. 1987; White et al. 1985). Rol genes are oncogenic in nature, which are inherently present on the TL-DNA region of Ri plasmid. The TL-DNA region consists of 18 open reading frames (ORF1-ORF18) which correspond to different *rol* genes. ORF 10 corresponds to *rol A*, ORF 11 to *rol B* and ORF 12 to *rol C*, and likewise ORF15 represents *rol D* (Slightom et al. 1986). During agrobacterial infection, the T-DNA region of the plasmid gets transferred and integrated in the host plant genome. The expression of *rol* genes interferes with hormonal balance and is able to alter the plant's normal functioning, and in particular this alteration has been proven beneficial to researchers in terms of improved traits, characters, varieties and production of important secondary metabolites. The existence of *rol* genes has additionally broadened the scope to investigate *Agrobacterium rhizogenes*-based transformation events that served as an extremely powerful and attractive tool for crop improvement. Besides facilitating the study of developmental biology and gene regulation in a wide

range of crops, *rol* genes have also proved useful in manipulating the metabolic pathways to enhance the accumulation of desired products of commercial interest. The manipulation of *Rol* genes has fuelled the possibility to modify the plant to obtain desired trait/characters.

Based on the present knowledge, it will be of great interest to explore the potential of *rol* genes in relation to its transfer, integration and inheritance and its significant concomitant result/alteration in the host plant. Over the decades many significant roles of *rol* genes have been described (Bulgakov 2008; Binns and Costantiano 1998; Meyer et al. 2000; Nilsson and Olsson 1997). The application of *rol* genes in floriculture was also summarised by Casanova et al. (2005).

The modulation in the set of these *rol* genes will surely made the researchers capable of understanding the differentiation pattern of abundant roots in the presence or absence of an adequate plant growth regulator. The exploration and identification of similar genes (*rol A*, *B*, and *C*, aux, ORF8, ORF13) are capable of stimulating the state of responsiveness in particular to biotechnological aspect, and undoubtedly analysing/practising the selected group of *rol* genes will be of great help in understanding the physiology of root differentiation pattern and enhancing the quality and yield of the desired products. The chapter summarises and explores biochemical, physiological, morphological and inheritance pattern of both individual and constitutive expression of *rol* genes *in planta*.

18.1.1 The Saga of Genus *Agrobacterium* and Host Range

The genus *Agrobacterium* consists of three species, namely, *A. tumefaciens*, *A. rhizogenes* and *A. radiobacter*, out of which *A. tumefaciens* and *A. rhizogenes* are virulent while *A. radiobacter* is an avirulent strain. The type of disease developed in any host species depends upon the type of plasmid available within a particular strain. Likewise, hairy root disease is caused by *A. rhizogenes*, crown gall disease is caused by *A. tumefaciens* and *A. rubi*, a new species, causes cane gall disease in plant system. *Agrobacterium* has a broad spectrum infectivity ranging from human cells to sea urchin embryos, dicots and monocots plant species (Kunik et al. 2001; Bulgakov et al. 2006; Hohn et al. 1989). A wide range of monocotyledonous plants like sugarcane, wheat, rice and corn has been reported to be transformed by *Agrobacterium* strains. Successful transformed plants like banana, *Alstroemeria* and *Typha latifolia* (Matsumoto et al. 2009; Akutsu et al. 2004; Nandakumar et al. 2005) have been reported so far.

Agrobacterium rhizogenes invokes phenotypic variations in infected host plant and lets the transformed host species tissue to grow in vitro in the absence of exogenous hormonal supply. The emerging hairy root from the site of infection is highly stable and can be regenerated into plants (depending upon the genotype of the host plant species), in many species, shown to have characteristic phenotype like shortened internodes, stunted growth, wrinkled/necrotic

leaves, reduced apical dominance and altered flower phenotype with low fertility (Tepfer 1984).

As the Ti plasmid has a central role in crown gall tumorigenesis, few years later it was revealed that *A. rhizogenes* contains a Ri plasmid called T-DNA region which in turn translocates and integrates into host plant cell (White and Nester 1980a, b; Moore et al. 1979; Chilton et al. 1982). The morphology of the hairy root closely mimics to *A. tumefaciens* tmr mutants, and therefore it was initially hypothesised that a defective Ti plasmid is present in *A. rhizogenes*. Studies like insertional mutagenesis have revealed that pRiA4b (a root-inducing plasmid) contains a T-DNA region homologous to TR-DNA (called as tms) and a second TL-DNA region is also present. Likewise, in *Kalanchoe daigremontiana*, transposon insertion and deletion study in the aux region and in TL-DNA region have shown to affect or even oppose the induction of hairy root (White et al. 1985). Initially, four oncogenes were identified from *A. rhizogenes* TL-DNA and termed as rol A, B, C and D (root locus). In some plants, TR-DNA alone is sufficient to induce hairy root formation, although the expression and phenotype of hairy root were not proper (Vilaine and Casse-Delbart 1987). Studies therefore suggested that TL-DNA may be involved in extending the host plant interaction in *A. rhizogenes* strains having agropine-type binary T-DNAs (Nilsson and Olsson 1997; Porter 1991). The aux factor is thought to be linked with nongeotropic property of root (both transformed and non-transformed), as it was observed that geotropism characteristics of the roots are lost when IAA is supplied exogenously in the culture (Capone et al. 1989a).

The Ri and Ti DNA oncogenes are thought to be homologues in nature and therefore appear to be descendants of a single ancestor. The TL-DNA of *A. rhizogenes* consists of eukaryotic 5ϙ and 3ϙ regulatory elements and left and right border sequences, and the oncogenes (rol A, B, C and D and ORFs 8, 13 and 14) were identified and found to be located in T-DNA region. These include Ri genes rolB, $rolB_{TR}$, rolC, ORF8, ORF13, ORF14 and ORF18 as well as Ti genes tms1/iaaM, ons, tml, 5, 6a and 6b (Otten and Helfer 2001; Levesque et al. 1988). Earlier, the plants were transformed with four rol gens, in combination and individually (Capone et al. 1989a; Spena et al. 1987; Cardarelli et al. 1987a). The variations in phenotypes totally depend on how the endogenous plant growth regulator may interact with rol gene product, also depending upon the genotype of the host plant species. Due to complexity of the interaction between Ri plasmid and the genes of the host plant species, the focus has been diverted to the interaction of individual or specific genes. The subsequent studies have been in queue in order to identify the interaction between endogenous and exogenous plant growth regulators at genotypic and phenotypic levels. Inducible and constitutive promoters (typically the cauliflower mosaic virus 35S promoter) have also been used to determine specific phenotype in relation to particular hormonal levels. Nowadays, T-DNA promoters containing GUS and GFP reporter genes have widely been used to elucidate the gene expression and specificity (localisation) at developmental and tissue stage. Researchers must have to focus on the T-DNA (ROl A, B, C) and their probable functions. In pRiA4 strain of *A. rhizogenes*, T-DNA consists of different TL-DNAs and TR-DNAs which are physically separated by DNA sequence of ca. 18 kb. TL-DNA and

TR-DNA regions get transferred either as complete T-DNA or may be separately during infection process (Christey 2001). The 18 open reading frames present in TL-DNA region found enough for induction and proliferation of HRs (Hong et al. 2006; Spena et al. 1987). TL-DNA region is solely responsible for the induction of hairy root. Other genes like mas1, mas2, aux1, aux2 and ags are located on TR-DNA region and known to encode products which stimulate the function of auxin and various opines (Bouchez and Tourneur 1991; Camilleri and Jouanin 1991). Taneja and coworkers showed that HR cultures, in the absence of TR-DNA region, have shown higher accumulation of transcripts and alkaloid level concentration also (Taneja et al. 2010) in concordance with earlier findings by Moyano and coworkers (Moyano et al. 1999; Robin 1998). They have observed that when synthetic auxin is added exogenously to the cultures, an increase in biomass of callus was seen, although negative effect on alkaloid production was also observed. In *Nicotiana rustica* the capacity of nicotine metabolism was lost, when cultures were treated with exogenous auxin (Robins 1998). This could be the reason why TL-DNA region must be studied to get proper knowledge related to rol A, B, C and D in plant morphogenesis and secondary metabolism. Ri plasmid consists of *rolA*, *rolB*, *rolC*, orf8, orf13 and orf14 responsible to induce HR (Matveeva et al. 2015).

18.2 Rol Genes: Expression and Mechanism of Action

There are four genes responsible for induction and proliferation of hairy roots. These are designated as *rol A*, *rol B*, *rol C* and r*ol D*. The rol genes (A, B, C and D) are restricted to Ri plasmid region, resulting in both positive and negative effects on HR. However, the regenerated plants via hairy roots, owing genes *rol A*, *B*, *C* and *D*, may exhibit abnormalities (phenotypical, morphological and genotypical). Although, these abnormalities led researchers to take advantage and discover new probabilities of evolution, function during the course time.

Rol A: The open reading frame of *rol A* gene is about 300 bp in length, which encodes 100 amino acid protein (Nilsson and Olsson 1997). R*ol A* gene, however, is found to cause functional impairment in phytohormonal level. Transgenic plants expressing *rol A* show reduced length of internode with curled and wrinkled small leaves (Sinkar et al. 1988). The promoter region of *rol A* gene is similar to the sequences of genes involved in auxin regulation (Carneiro and Vilaine 1993). R*ol A* gene is known to be expressed maximally during the stationary phase of growth, and therefore the bacteria is able to resist under nutrient-confined environment for their survival (Pandolfini et al. 2000).

Studies have suggested that the activity/expression of *rol A* could be diminished if any mutation occurs in the splice site 5′ UTR intron region; however, no effect was observed in the transcript level of *rol A* (Magrelli et al. 1994). *Rol A* gene is said to be a non-integral membrane protein, as the *rol A* and GUS fusion protein are restricted to plasma membrane having no transmembrane motifs. *Rol A* gene is involved in the stabilisation of expression of GUS (Vilaine et al. 1998). However, *rol A* gene may also be involved in the protein degradation steps (Barros et al. 2003).

But, the exact function and insights of *rol A* gene/protein are still unknown and need to be surveyed more.

R*ol A* protein has shown its significant activity as transcription factor (TF) and is involved in the polyamine metabolism via interfering in the conjugation mechanism of polyamines. *Rol A* gene was also suggested to be involved in the metabolism of gibberellins resulting in the decrease of its content in *rol A*-transformed tobacco plants (Dehio et al. 1993). This report is in concordance with an earlier study in which a similar kind of phenotype has been obtained by inhibiting gibberellin biosynthesis (Dehio et al. 1993). It has been well documented that the sequence of *ORF-13* is highly conserved in *mannopine*-, *mikimopine*-, *agropine*- and *cucumopine*-type plasmids. However, in *Nicotiana* species, there are two counterparts found, i.e. *Ng ORF-13* in *N. glauca* and *Nt ORF 13* in *N. tabacum* (Aoki et al. 1994; Fruendt et al. 1998). *ORF-13* is involved in the phytohormone signalling pathways in plants that has interaction with cytokinin activities and thus known to encode plant growth regulators (Hansen et al. 1993). Recently, it has been observed that various ORFs have been involved in the regulation of cell cycle and can induce an ectopic expression in KNOX (KNOTTED 1-like homeobox) TF in transgenic tomato. Furthermore, *ORF-13* is found to have retinoblastoma RB binding motif site and binds with retinoblastoma in vitro (Stieger et al. 2004). The expression of *ORF-13* involved in the production of spikes in between the minor veins of petals and leaves in *Nicotiana*. These kinds of similar structures and protuberations have been observed in overexpressed KNOX genes (KNOTTED 1-like homeobox) (Sinha et al. 1993; Chuck et al. 1996; Sentoku et al. 2000). Apart, accelerated cell divisions in vegetative phase of apical meristem and increased production of leaf primordia are the characteristics of plants expressing *rol A*/*ORF13*. In *A. rhizogenes*-infected cells, *ORF-13* binds to RB and hence results in promoting the transitions of cells from G1 to S phase, respectively (Stieger et al. 2004).

Rol B: This is the most essential and important gene for induction of HRs. Any alteration/mutation results in the loss of function rendering to avirulency (inactivation of vir genes) and ultimately results in the inhibition of HR formation. Hereby, *rol B* gene is known to encode a functional protein named indoxyl-β- glucosidase (1259 amino acid), and the open reading frame (ORF) for rol B gene is approx. 777 bp in size (Estruch et al. 1991a). Functionally, *rol A* and *rol B* are antagonistic in nature. Although, all other rol genes have been important for proper functioning and induction of HRs, *rol B* when introduced as a single gene has been found capable of inducing HR in the host plant. The plant owing *rol B* expressed advanced roots which seem to be phenotypically abnormal with heterozygosity in flower (Cardarelli et al. 1987a, b; Spena et al. 1987).

Rol B gene is involved in the regeneration of new apical meristems depending upon the local hormonal concentration, and these newly formed meristems subsequently showed organogenesis (Altamura 2004). This study can be correlated with the earlier experiment of tobacco TLC, in which *rol B* enhances adventitious flowering and stimulates adventitious roots by interfering with developmental stage (Altamura et al. 1994). Based on the evidences, r*ol B* has showed a strong relationship between local hormonal balances with differentiation patterns (Altamura et al. 1998). *Rol B* is universally present in all *Ri* plasmid with 60 % similarity among the

strains (Meyer et al. 2000). Expression of *rol B* gene is restricted to phloem rays, root, pericycle, phloem parenchyma, shoots and flowers (Altamura et al. 1991). The plants transformed with *rol B* gene show formation of adventitious roots along with modified shoot architecture such as altered leaf shape and heterostyly and increase in flower size and necrotic leaves (Schmülling et al. 1989).

R*ol B* and *rol C* genes are present on opposite strands and both can be controlled by a single bidirectional promoter although a close association between *rol B* and auxin gene has been suggested. For example, in carrot, TR-DNA aux genes (to supply auxin in culture cell) are essential for inducing HR rather than *rol B* gene alone (Capone et al. 1989a; Cardarelli et al. 1987b). R*ol B* and *rol C* genes share an intergenic conserved region or sequence that was found to be essential for promoter activity; therefore it may be possible that some DNA-binding proteins take part in the expression of both proteins (Leach and Aoyagi 1991). Apart, various endogenous nuclear proteins that could interact with rol C promoter were also found *in planta* (Matsuki and Uchimiya 1994; Fujii 1997; Suzuki et al. 1992).

Synergistic effects of auxin were determined in cultured tissues, where the expression of *rol B* and *rol C* genes was increased to a much higher fold (*rol B* expression up to 20–100 and *rol C* expression up to fivefold). However, the increment in *rol B* gene was observed after several hours when auxin is added exogenously to the culture medium (Maurel et al. 1994). The transcription factor *NtBBF1* (*Nicotiana tabacum* rol B domain B factor 1) is highly involved in the regulation of *rol B* gene, and it appears that the expression of *rol B* and *NtBBF1* is much similar and unlike *rol B* the endogenous *TF NtBBF1* is found to be non-sensitive to auxin (Baumann et al. 1999). One other protein, called *RBF1* (Rol binding factor 1), is shown to bind to *rol B* promoter domain and stimulates the expression pattern in various non-meristem root cells. De Paolis observed that for the induction of auxin- and meristem-specific expression, the binding between DNA binding with one finger (Dof) domain of endogenous transcription factor *NtBBF1* (*Nicotiana tabacum rol B* domain B factor 1) and cis regulatory element is important in *Nicotiana* (De Paolis et al. 1996). The binding site is found to be highly conserved between different *rol B* sequences and *Ri* plasmids (Handayani et al. 2005). In *Nicotiana*, HRs and protoplasts have shown to have much sensitivity towards auxin than non-transformed cultured tissues (Spano et al. 1998; Maurel et al. 1994; Shen et al. 1988, 1990).

Apart, the domain of *rol B* promoter actively binds with a protein *RBF1* responsible for the expression in various root cells; however, the concentrations of *RBF1* protein are not found to be significantly different in transgenic and non-transgenic plants (Filetici et al. 1997). The pRiA4 restricted to plasma membrane region having tyrosine phosphatase activity encodes *rol B* protein and can interfere in the auxin signalling pathway by altering the phosphatase/kinase cascade during signalling (Filippini et al. 1996). However, it has been hypothesised that the CX5R, a conserved motif present in pRiA4 *rol B*, is highly responsible for tyrosine phosphatase activity (Lemcke and Schmülling 1998b). It may be possible that the rol B protein stimulates a signal via tyrosine phosphatase activity in auxin perception and leads to increase in the concentration of auxins in the cultured tissues. The

increment in the auxin concentrations to higher folds stimulates undifferentiated cells to differentiate and subsequently results in the organogenesis depending upon the local auxin present in the environment and most probably in root formation. On contrary, *rol B* gene in mikimopine strain 1724 is nuclear localised. It was observed that tobacco protein *Nt*-14-3ωII binds with the pRi1724 *rol B* protein (Moriuchi et al. 2004), but no such nuclear localisation sequence is present on either *Nt*-14-3-ωII or pRi1724 *rol B* protein, suggesting that these may take part in the nuclear import of the protein. The ratio of endogenous versus exogenous level of plant growth regulators, physiological state of the host plant and competence of an individual cell determine the overall fate of the organ to be produced and have said that roots are the most frequent adventitious organ, therefore resulted in root formation.

Rol C: *Rol C* has shown its significant role in the formation of shoot and contains an open reading frame of 540 bp in size and has been involved in encoding a cytokinin-β-glucosidase. Among all other rol genes, *rol C* as a single gene results in the improvement of horticultural and ornamental traits in host plant and hence, most advantageous for floriculture technology. Insertion of *rol C* gene also results in the phenotypical changes, viz. altered leaf morphology and reduction in seed production and in apical dominance also. In addition, plants with *rol C* gene display stunted growth with bushy branches of lateral shoot followed by more number of flowers which are reduced in size; the plants have also shown to have better rooting capacity.

R*ol C* protein affects the phenotype of the whole plant and these characteristics are almost common to all species. The altered architecture includes a small size of the regenerated plant along with reduced intermodal length, decreased heights of stems, apical dominance and male sterility but increased number of flowers with reduced size. *Rol C* gene also offers many advantageous prospects and may result in altered morphology with difference in colour, size and shape of leaves. Other phenotypical alterations have been observed like stunted growth with bushy appearance of lateral branches and wrinkled leaves as this characteristic affected the plant morphology and overall texture and has increased its value in terms of ornamental/horticultural plant. The difference in the size, morphology and texture of leaves, flowers, internodes, stems, etc. totally depends upon the integration and expression of *rol C* gene. It is not easy to control the integration of *rol C* gene and its site-specific localisation, copy number and change in the expression level throughout the complexed genome. But the expression could be managed by using specific promoters.

As stated earlier, *rol C* gene acts on the cytokinin beta-glucosidase enzyme that could lead to the enhanced level of cytokinin in the host plant and hence the altered morphology. Likewise, gel permeation study has shown the enhanced activity of carbohydrate isoforms in transformed ginseng cells. Moreover, transformation by *rol C* gene has shown significant increase in the bioactivity level of 1,3-p-D-glucanase and D- and P-galactosidase in transformed tissues when compared to non-transformed cells. These observations could be the resultant of high copy number, length of the promoter and the quantity of mRNA expression in transformed species.

Rol D: R*ol D* has an open reading frame of about 1032 bp and known to encode functional protein having sequence of approximately 344 amino acids. The functional protein encoded by the *rol D* gene is found to have sequence homology with an enzyme, i.e. ornithine cyclodeaminase. The *rol D* gene is also known to be involved in the proline production at the stage of flowering and sometimes also behaves as an osmoprotectant during stress in plants. Surprisingly, *rol D* strongly accelerates the induction of flowering both in cultured tissues and tobacco plants. The role and consequences of *rol D* have always been a topic of discussion (Table 18.1).

Table 18.1 Rol gene functions, localisation and expression

Gene	Type of protein and localisation	Probable phenotypes and expression	Involvement/function
Rol A	Non-integral membrane associated	Expressed in plant as well as in bacteria	Involved in the reduction of hormonal concentration in the transgenic plants
	Small protein with molecular mass of approx. 11.4 kDa with high isoelectric point, i.e. 11.2	Expression may be regulated by auxin	Prevents cell elongation through diffusible factor
		Enhanced rooting efficiency of the plant, shortening of internodes, altered leave phenotype and causing wrinkling and curling of leaves	Transmit via grafting experiments may interfere with protein degradation metabolism
			Can block gibberellin biosynthesis
Rol B	Restricted to plasma membrane but may transport to nucleus	Induced by auxin	Involved in the stimulation of newly formed meristems
		Necrosis and callus, increased the level of IAA, hydrolyse indoxyl glucosides	
		Formation of adventitious roots along with modified shoots	
		Can be inhibited by glyco-glucuronides	
		Resulted in necrosis and altered phenotype of leaves, i.e. shape and also increased in flower size	
		RBF1 and NtBBF1 TF bind to the promoter region	
	Tyrosine phosphatase activity	Interacts with Nt14-3-3ωII	Alter auxin sensitivity/ perception via interference with signal transduction pathway

(continued)

Table 18.1 (continued)

Gene	Type of protein and localisation	Probable phenotypes and expression	Involvement/function
Rol C	Cell autonomous cytosolic/ cytoplasmic	Variegated leaves with sharp edges, mosaics pattern	Auxin synthesis, conjugation of auxin to lysine/ornithine
	Cleaves cytokinin oligosaccharin or glucosides	Reduction in size of epidermal cell of internodal region, thus resulted in dwarfism	Involves in formation of root and cell proliferation
			Promotes cell division via sucrose
		Induced by sucrose	Reduces size of leaf cells
		Potential of inducing HRs is generally high in comparison to untransformed plants	
		Promoter interacts with plant nuclear proteins	
Rol D	Incapable of inducing root formation on its own, i.e. individual rol D cannot induce HRs	Results in extensive flowering and rooting efficiency decreased	Causes alteration in the hormone balance in plant tissues and hence involved in induction of flowering
	Found in TL-DNA region of agropine Ri plasmid	Functionally associated with proline metabolism and as the proline functions as an osmoprotectant, it has shown to be involved in flowering	Rol D is known to show sequence similarity with enzyme ornithine cyclodeaminase (OCD), which converts ornithine into proline, so it may have a role in the proline metabolism; proline is thought to be involved in the production of hydroxyproline-rich glycoproteins (involved in cell division)
		The activity of rol D promoter decreases due to increase in the concentration of auxin levels above the threshold	Involved in stress conditions as it determines the fate of meristem also
		Intensive production of proliferative axillary buds	Have well-documented role in later stages of meristem formation

18.2.1 Rol Genes: Individual and Synergistic Effect of Togetherness (Rol A + B + C + D)

An important aspect whether the Rol genes show synergistic or individual effect or results in the feedback inhibition and cause inhibitory effect on morphology and root induction of the host plant.

Rol A, *B*, and *C* genes (T-DNA) have significant synergistic effect in root induction, because each Rol gene is regulated by its own promoter and expressed independently in inducting HRs. The HR induced from CAMV35S-*rol C* was found to be highly branched and abundant, while the HR under the expression of CAMV35S-*rol A* was poorly developed with wrinkled roots. Also, HRs induced from CAMV35S-*rol B* were found to be much unbranched and have thick phenotype. It corroborates with the earlier results that *rol B* was found to be prominent in inducing HRs followed by *rol A* and *rol C* in transgenic tobacco when inoculated on MS_0 medium.

The Rol genes have been widely used to improve the floral traits like flower colour, plant shape, aroma, etc. Among all the rol genes, *rol C* has been extensively used by horticulturalists in order to improve the plant yield, flower trait and molecular regulation of flowering pattern. *Rol C* has been considered as one of the promising genes to study the protein interactions and different physiological and morphological effects to improve floricultural traits (Casanova et al. 2005). The regenerated tobacco plant with *rol A*, *B*, *C*, and *D* has shown to have altered apical dominance; as a result transgenic tobacco has bushy morphology with roots showing plagiotropic effects. However, the same plants also exhibited various abnormal phenotypes such as stunted growth, reduction in leaves size, reduced intermodal length and flowers with longer pistils (Sinkar et al. 1988). Moreover, the transgenic tomato having *rol A*, *B*, and *C* was found to produce normal flowers with small size, but decreased pollen viability (Van Altvorst et al. 1992). Similarly, transgenic tomato owing *rol A*, *B*, and *C* produced comparatively small-sized flowers and less seed also (Schmülling et al. 1988). The significant achievements of Rol genes (individual or together) are shown in Table 18.2.

18.2.2 Modulation of Metabolic Profiles and Accumulation of Pharmacologically Active Compounds with the Integration of *rol* Gene

Updating knowledge in the field of *Agrobacterium* and plant interaction has gained much applause in recent years. Hairy root culture is found to exhibit the similar/identical or often higher capability to synthesise secondary metabolite as compared to that of non-transformed roots. HR culture is often found to synthesise novel metabolites which are not found in untransformed tissues (Banerjee et al. 1995). The discovery and function of the rol genes are found to be specific and involved in biosynthesis of secondary metabolites in families like Araliaceae, Solanaceae, Rosaceae, Rubiaceae, Vitaceae, etc. (Kiselev et al. 2007; Bulgakov 2008). In many cases it has been found that the individual rol gene is enough to overcome the impotency of cultured plant tissue to produce the adequate amount of secondary metabolites (Kiselev et al. 2007). The characteristics related to the *rol* gene

Table 18.2 Overview of studies for elucidation of functional role of Rol genes in HRs (individual or combined)

Rol gene	Species of the plant	Improved trait	References
Rol A, B, C	*Ginkgo biloba*	Terpenoid production in cell cultures	Ayadi and Guiller (2003)
	Vinca minor	Increase in alkaloid level and vincamine and overexpression of tryptophan decarboxylase and strictosidine synthase	Verma et al. (2014)
	Papaver somniferum	Enhanced production of alkaloids (codeine, morphine and sanguinarine)	Bonhomme et al. (2004)
	Rubia cordifolia	Increased in Ca^{2+}-dependent protein kinase activity in callus cultures	Veremeichik et al. (2012)
	Catharanthus roseus	Enhanced synthesis of terpenoid indole alkaloid	Hong et al. (2006)
	Atropa belladonna	75-fold increase in production of tropane alkaloids	Bonhomme et al. (2000b)
	Beta vulgaris	Higher biomass	Thimmaraju et al. (2008)
Rol B, C	*Rubia cordifolia*	Enhances the synthesis of anthraquinone in callus cultures	Bulgakov et al. (2003a)
	Trifolium alexandrinum L.	Influenced the growth capacity of HR vigorously	Tanaka et al. (2001)
	Catharanthus roseus	Higher indole alkaloid production	Palazon et al. (1998b)
	Rubia cordifolia	Suppress the formation of ROS and enhancement of stress tolerance	Bulgakov et al. (2008a)
Rol C	*Atropa belladonna*	Enhancement in the production of tropane alkaloids	Bonhomme et al. (2000a)
	Maackia amurensis	Resulted in higher accumulation of six flavonoids (daidzein, calycosin, formononetin, 4′-O-β-glucopyranosyldaidzin, maackiain and 6′-O-malonyl-3-O-β-D-glucopyranosylmaackiain)	Grishchenko et al. (2013)
	R. cordifolia	Stable increase in anthraquinone content up to 1.3–4.3 times that of non-transformed cells	Bulgakov et al. (2002) and Shkryl et al. (2008)
	Vitis amurensis	Stimulates phenylalanine ammonia-lyase (PAL) and stilbene synthase STS gene resulted in accumulation of higher content of resveratrol	Dubrovina et al. (2010)
	Panax ginseng	Increased activity of CDPK gene was reported in somatic embryos of panax ginseng	Kiselev et al. (2009b)

(continued)

Table 18.2 (continued)

Rol gene	Species of the plant	Improved trait	References
	Cynara cardunculus var. *altilis DC*	Increased in the production of caffeoylquinic acid	Vereshchagina et al. (2014)
	Panax ginseng	Pg WUS expression in callus cultures	Kiselev et al. (2009c)
	Panax ginseng	Increased concentrations of ginsenoside	Bulgakov et al. (1998)
	Beta vulgaris	Production of betalain in HR	Thimmaraju et al. (2008)
	N. tabacum	Production of nicotine	Palazon et al. (1998a)
	N. langsdorffii	Confers high resistance to chromium (Cr VI), changes in sugar and phenolics content	Bubba et al. (2013)
	R. cordifolia	Accumulation of anthraquinone up to 15-fold increase in concentration than untransformed tissue	Shkryl et al. (2008)
	Vitis amurensis	Production of resveratrol more than 100-fold than untransformed tissue	Kiselev et al. (2007)
Rol B	*N. tabacum*	Accumulation of nicotine	Palazon et al. (1998a)
	R. cordifolia	Accumulation of anthraquinone up to 2.8 times higher in concentration than untransformed	Shkryl et al. (2008)
Rol A	*Camptotheca acuminata*	Camptothecin and 10-hydroxycamptothecin accumulation	Lorence et al. (2004)

expression and its mechanism of action stimulated researchers to investigate the effect on secondary metabolism. The degree of production of secondary metabolites depends on the type of secondary metabolites and plant species which could vary from 2- to 300-fold (Bulgakov et al. 2013).

The effect and expression of *rol* genes were illustrated with different groups of secondary metabolites, viz. pyridine alkaloids, tropane alkaloids, etc. in plant system. The role of *rol* genes involved in biosynthetic pathways seems to be highly efficient and remarkably stable for a long time. Genetic transformation that proceeds via single *Agrobacterium rhizogenes*-derived *rol* gene could serve as an efficient platform for modulation of biosynthetic pathways in plants as well as in cultured cells. However, it is well documented that *rol* genes are often involved in transcriptional activation of plant defence mechanisms, although the mechanism or underplaying actions are still in doubt. *Agrobacterium* oncogenes (*rol A*, *B*, *C*) have shown to be active modulators in plant's biosynthetic pathways, cell differentiation,

growth and development. Earlier reported studies have shown that *rol* genes (*A*, *B*, *C*, *D*) are potential activators and capable to overexpress the content of important secondary metabolites individually. *Rol B* gene can stimulate the production of stilbenes (Kiselev et al. 2009a) and anthraquinone (Bulgakov et al. 2002, 2003a, b) in transformed plant cell culture. The *rol C* gene alone increases the production of pyridine alkaloids (Palazon et al. 1998a), indole alkaloids (Palazon et al. 1998b), tropane alkaloids (Bonhomme et al. 2000a), anthraquinone (Bulgakov et al. 2002, 2003a, b) and ginsenosides (Bulgakov et al. 1998). Similarly, Palazon has observed the significant effect of *rol A* gene on the production of nicotine (Palazon et al. 1997a, b). It was noticed that, when the HR from transgenic tobacco was assessed for auxin sensitivity test versus root induction, the *rol* genes (*rol A*, *B*, *C*, *D*) were shown to be highly sensitive to auxin. Protoplast of transformed tobacco and *L. japonicus* has been assessed for auxin sensitivity by measuring membrane polarisation technique, and it has been shown that Rol genes of transformed tobacco and TL-DNA region of *L. japonicus* were highly sensitive, i.e. about 100–1000-fold to various auxin concentrations, respectively. Apart, it was also observed that the activity and expression of rol genes were highly influenced with the alteration in polyamine biosynthesis.

The influence of *rol* genes on secondary metabolite accumulation was also studied in *Rubia cordifolia*. They investigated the influence of rol genes in combination and individually and have observed that rol genes act as positive regulators for synthesis of anthraquinones. R*ol A* genes have already proved its remarkable role as an activator of secondary metabolism. In addition, *rol B* gene appears to be the most authentic stimulator. The role of *rol C* gene varies with different aspects (Bulgakov et al. 2008). Earlier reports have established a healthy relation between the variation in production of secondary metabolites and insertion of *rol* genes via inducing HR. Furthermore the change in the content and phenotype of HR could be easily correlated with the type of *rol* genes and the extent to which polypeptide encoded by *rol C* gene is activated (Palazon et al. 1998b). Interestingly in *N. tabacum*, the HR growth kinetics and the production of nicotine in HR/regenerants were much higher after the insertion of *rol A*, *B*, and *C* genes than *rol C* gene alone. Also, the levels of polypeptide encoded by Rol C gene can easily be correlated with nicotine accumulation, as observed by immunoassay studies Palazon et al. (1998b). The effect of rol genes in the biosynthesis of secondary metabolites was also studied by Bonhomme and coworkers (Bonhomme et al. 2000b). The experimentation was carried out in such a way that HRs were induced in duplicates in which the first was transformed by *rol A*, *B*, and *C* and *NPT-II* genes and the other transformed with *A. tumefaciens* harbouring *NPT-II* and *rol C* gene in *Atropa belladonna* species. The content of two pharmacological active ingredients (scopolamine and hyoscyamine) was measured to evaluate the role of *rol C* gene in the biosynthesis of tropane alkaloids. After 3–4 weeks of culture, the integration of *rol A*, *B*, and *C* gene was found to be highly efficient, i.e. 75-fold increment was observed in the HR growth kinetics. However, *rol C* gene alone has shown only 17-fold higher in HR growth rate in tropane

alkaloid production in *A. belladonna* HR cultures. The significant correlation between tropane alkaloids and *rol C* was also observed by many workers so far (Bonhomme et al. 2000a, b; Pinol et al. 1996), ginsenoside biosynthesis (Bulgakov et al. 1998) and *Catharanthus roseus* alkaloids (Palazon et al. 1998b). The overall role of rol genes is illustrated in Table 18.2.

18.2.3 Expression and Effects of Rol Genes in Phenotype and Physiology of HR/Regenerated Plants

Agrobacterium rhizogenes owing rol genes integrated in TL-DNA region of *Ri* plasmid are potentially active to induce HRs in competent host plant. These transformants when regenerated into plants have been considered equally important too because of its economic value. However, a lot of work has been done but there are certain lacunas in relation to the role of action/activity and localisation of either individual or in group of rol genes (*A*, *B*, *C*). The regenerants via HRs owing TL-DNA are associated with the problems like delayed flowering and relatively less accumulation of conjugated and free polyamines. This can be correlated with the study conducted in male sterile tobacco that expresses *rol A* along with decreased accumulation of polyamine conjugates (Sun et al. 1991).

18.2.4 Expression of Rol A Gene

The presence of *rol A* gene is ubiquitous in on all Ri plasmid. However, the studies suggested that the N-terminal of the protein is highly conserved in *A. rhizogenes* strains. *Rol A* gene is supposed to be transcribed in the phloem cells and highly accumulated in the stems than roots and leaves (Carneiro and Vilaine 1993; Sinkar et al. 1988).

The expression of *rol A* genes is found variable in different transformed plants. The Nicotiana plant expressing *rol A* gene was stunted and bushy in appearance having dark green wrinkled leaves and abnormal flowers (Carneiro and Vilaine 1993; Schmülling et al. 1988). The expression of the *rol A* gene is interlinked with plant hormone like *rol A* which is responsible for reduction in the concentration of cytokinins, abscisic acid, auxins and gibberellins in transgenic tobacco, although the decrease in the concentrations of these PGRs strictly depends on the type of tissue and different developmental stages of the host plant (Dehio et al. 1993). Similar kinds of phenotypes were also shown by inhibitors of GA formation; however, when these plants were treated with GA, the resultant does not show the restoration of *rol A* gene (Dehio et al. 1993). The blocked activity of GA-20 oxidase complex may be responsible for this type of phenotypes; likewise, the concentrations of precursors like GA-53 and GA-19 have increased with simultaneous decrease in the concentrations of GA-1 and GA-20 (Moritz and Schmulling 1998).

18.2.5 Expression of Rol B Gene

The *rol B* oncogene is supposed to be the most essential and sufficient for the induction of HRs; however, it does not have any role in the biosynthesis of hormones (Britton et al. 2008). *Rol B* alone when integrated in the host plant produces highly branched and fast growing roots with ageotropic property (Altamura 2004). Earlier, the expression of *rol B* gene was specific to induce roots, but *rol B* induces the production of new meristems and subsequently leads to organogenesis (Altamura 2004). *Rol B* is present in all parts with weaker expression in pericycle, shoot, root phloem parenchyma and flower meristems (Altamura et al. 1991). Expression of *rol B* gene is highly regulated in the transformants showing altered shoot phenotypes like variation in leaf structure, heterostyly, necrosis pattern on leaves, increased in size of flower, etc. (Schmülling et al. 1989). The *rol B* gene is found to be approximately 60 % similar in all Ri plasmids of different strains (Meyer et al. 2000). The expression of *rol B* gene is highly associated with the auxin biosynthesis as transformed protoplast and HR in *Nicotiana* were shown to be highly sensitive than non-transformed tissues (Maurel et al. 1994; Shen et al. 1990; Spano et al. 1998). *Rol B* is supposed to interfere in the auxin synthesis pathway and can alter the expression of auxin (Maurel et al. 1994). This hypothesis is supported by an experiment during which expression of auxin can be blocked by raising the antibodies against auxin-binding protein and observed that if *rol B* is expressed then a relatively higher concentration of antibodies is required to alter the auxin response. From these results it interpreted that the expression of the *rol B* gene either may enhance the auxin activity or may stimulate the response of auxin-binding proteins (Venis et al. 1992). Also, *Rol B* gene has been suggested in increasing the sensitivity of *rol B* to auxin in transformed carrot discs and kalanchoe leaves. The transformed tissues do not show any response to auxin, when it is incorporated exogenously in medium (White et al. 1985; Spena et al. 1987). Unlike in carrot, the presence of *rol B* gene alone is not sufficient to produce root as the presence of auxin provided by T_R region of T-DNA of aux gene has shown to play a significant role in inducing roots other than *rol B* gene alone (Cardarelli et al. 1987b; Capone et al. 1989a).

18.2.6 Expression of Rol C Gene

The *rol C* gene is found on all *Ri* plasmids. However, *rol C* protein shares similarity of approximately 65 % in amino acid sequence (Meyer et al. 2000). The transformed plant owing *rol C* gene is phenotypically short with lanceolate leaves, showing reduced apical dominance and early flowering having small flowers with low pollen production (Schmülling et al. 1988). In addition, the transformants owing 35S:*rol C* display male sterility with pale green leaves (Schmülling et al. 1988). The phenotype of the regenerated plants owing 35S-*rol C* gene has short internodal length with sterile male flowers. However, the addition of GA_3 (exogenously) could store intermodal length, but no effect was observed in male sterility (Schmulling et al. 1993).

Apart, the transformants with *rol C* gene display stunted height with reduction in size of epidermal cell inside the internodal region (Oono et al. 1990). *Rol C* alone is not found sufficient to induce HR, but though produces more roots than untransformed plant, but less number of roots, with the plants owing all *rol* (*A*, *B, C*) genes (Palazon et al. 1998a, b). Also, the *rol C*-positive seedlings were found to be highly sensitive to cytokinin, with higher tolerance to abscisic acid, gibberellins and auxins (Schmulling et al. 1993). In addition, enhancement in accumulation in water-soluble polyamine conjugates has been seen with concomitant reduction in the production of ethylene in the flowers (Martin-Tanguy et al. 1993).

The role, function and biochemical activity of *rol C* is still a question till date with no authentic results. However, many attempts have been made to reveal how the *rol C* gene can interfere with plant growth and development. Estruch et al. (1991a) have made an observation during an in vitro activity assay in which recombinant *rol C* was used and demonstrated that the protein has β-glycosidic activity and is able to cleave the inactive cytokinin glycosides that subsequently release active and free cytokinins (Estruch et al. 1991a). In addition to measure the level of free cytokinins in various tobacco cultivars, it was observed that the decrease in level is a resultant of reduced metabolism in cytokinins synthesis (Nilsson et al. 1993). Unlike the results in which *rol C* gene expressed under a tetracycline-inducible promoter and was not able to release free cytokinins in the tissue due to non-hydrolysis cytokine glucosides (Faiss et al. 1996). However, these results are in accord with the facts that *rol C* is localised in cytoplasm and therefore is unavailable in the conjugated cytokinin glycosides present in the vacuole (Nilsson and Olsson 1997; Faiss et al. 1996). Apart from the hypothesised facts, it has also been suggested that oligosaccharins are also thought to influence plant growth and development (Faiss et al. 1996). The change in the gibberellin content has influenced on *rol C* gene in transformed plants with increment and reduction in the concentrations of GA19 and GA1, respectively (Nilsson et al. 1993; Schmulling et al. 1993). The transformant with *rol C* along with T_L-DNA region has shown its prominent expression in leaves. However, if the *rol C* gene is present alone in the host plant, then highest expression can be seen in roots (Leach and Aoyagi 1991; Oono et al. 1990; Sugaya et al. 1989). On contrary, higher expression was observed in leaves, while weaker expression was restricted to internodes (Nilsson et al. 1993). The *rol C* gene is found to be highly expressed in root protophloem initial cells and followed by phloem companion cells (Guivarch et al. 1996b). In addition, the sucrose metabolism is found to be highly associated with *rol C*, and the expression of *rol C* can be induced by supplementing sucrose in the cultured cells (Nilsson et al. 1996). The interlinking connection between *rol C* and sucrose metabolism is due to the overlapping similarity in sucrose-responsive sequence in *rol C* promoter (Yokoyama et al. 1994). The overlapped sequence is able to control *rol C* expression in phloem cells (described and identified by Sugaya and Uchimiya 1992). Sucrose is known to provide carbon source which promotes cell division and root initiation in PTC experiments. Based on which it was hypothesized that sucrose may act as a substrate on which the *rol C* works (Nilsson and Olsson 1997). Having this much of knowledge

about *rol C* gene, still there is a need to explore the mechanism and effect as an individual or in combination with other rol genes.

18.2.7 Expression of Rol D Gene

The *rol D* gene is found in the TL-DNA of *Agropine* strain and associated with the conversion of ornithine to proline (Maurizio et al. 2001), but the exact function is still unknown till date. The presence of *rol D* gene alone is not sufficient for root induction (Mauro et al. 1996). The expression of *rol D* gene depends on the developmental stage of the tissue, but not found to be tissue specific. The expression can be seen in each organ in adult plant; however, it has never been observed in apical meristems. It was also observed that the expression of *rol D* decreases on ageing plant with no expression at senescence stage (Trovato et al. 1997). The transgenic tobacco owing *rol D* gene is shown to induce early flowering with large number of flowers and reduction in roots (Mauro et al. 1996). The flowers are high in number but showed heterostyly and inhibit self-fertilisation; however, if the flowers were manually selfed, then plants produce viable seeds (Mauro et al. 1996). Likewise in *rol B* gene, *rol D* also has Dof-binding promoter which takes part in auxin induction. The *rol D* gene resulted in meristem formation at later stages of development in plants and could probably be involved in determining the fate of newly generated meristem (Altamura 2004).

The *rol D* gene is localised in the cytosol and shows a sequence similarity to OCD (ornithine cyclodeaminase), enzyme responsible for conversion of ornithine to proline (Trovato et al. 2001). Since proline is an osmoprotectant and is produced during stress condition, therefore it may be possible that during flowering, higher level of proline was found in the flowers (Trovato et al. 2001). The synthesis of proline may enhance the biosynthesis of cell wall components like glycoprotein that are hydroxyproline rich involved in the cell division and extension (Trovato et al. 2001). An elucidation of functional role of Rol genes has been done (Table 18.3).

18.3 Conclusions

This chapter focuses on the potential of Rol genes A, B and C (combined or individual) on transgenic technology that has already been proven fruitful in efficiently enhancing the various aspects of plant architecture/framework like photosynthetic assimilation, phenotype and genotype to enhance the quality and yield of the desired product for the production of elite clones. As well, the cyclic development of root meristems and root tips opens opportunities for fundamental studies of the Rol gene and promoter expression. Apart, the present scenario reveals that Rol A, B, and C in combination and Rol C individual have been studied in HR for phytochemical accumulation and in developing a plant with desired trait also. However, the combined

Table 18.3 Overview of studies for elucidation of functional role of Rol genes in transformed plants (individual or combined)

Rol genes	Plants	Phenotype	References
Rol A, B, C	*Kalanchoe blossfeldiana*	Postharvest performance, flower longevity due to increased ethylene tolerance	Christensen and Muller (2009)
	Hypericum perforatum	Altered in size, hairy root syndrome, altered secondary metabolism, reduced fertility, etc.	Komarovska et al. (2010)
	Gentiana sp.	Stunted plants; short internodes (except in one species); reduced apical dominant; branched stems; elliptical, rolled and wrinkled leaves; increased rooting; enhanced and early flowering	Hosokawa et al. (1997)
	Rudbeckia salicariifolia	Wrinkled leaves, highly lateral branches of roots and reduction in flower size	Daimon and Mii (1995)
	Tobacco, *kalanchoe*	Regenerants shown the formation of advanced hairy root syndrome	Spena et al. (1987)
	Pelargonium species	More internodes, more lateral branches, more leaves, shorter stature, leaves are dentate and dark in colour, reduction in flowering, enhanced concentrations in essential oil	Pellegrineschi and Davolio- Mariani (1996) and Pellegrineschi et al. (1994)
	Ipomoea trichocarpa	Abundant root, smaller and wrinkled leaves, reduced stem length, decrease in no. of flowers, delay in flowering	Otani et al. (1996)
	Datura sanguinea, *D. arborea*	Reduced plant height, increased internode number, short internode, dark colour smaller flowering when flowering was not inhibited, increased no. of leaves with small size	Giovannini et al. (1997)
	Nierembergia scoparia	Reduced internode length, dwarf, narrower and small leaves, normal fertility and increased efficiency of rooting	Godo et al. (1997)
	Antirrhinum majus	Dwarfness, highly branched stems with decreased apical dominance, short internodes, poor root system, delayed flowering with reduced fertility or sometimes sterile	Hoshino and Mii (1998), Handa (1992a), and Senior et al. (1995)
	Lilium longiflorum	Stunted height, short internodal length, less no. of flowers which are small in size, decreased pollen viability	Mercuri et al. (2003b)
	Limonium sp.	Dwarfness, reduced internodal length, small wrinkled and curly leaves, reduced apical dominance, early flowering with smaller size and reduced pollen viability	Mercuri et al. (2001, 2003a)
	Hypericum perforatum	Altered phenotype, increased no. and density of dark and translucent glands, reduced fertility, alteration in secondary metabolites pathway, retarded gametophytes (male + female)	Komarovska et al. (2010)
	Eustoma grandiflorum	Stunted plant height, no. of internodes increased, decrease in internode length, more lateral branches, wrinkled and small leaves, changed shape of corolla, reduction in fertility in some species	Handa et al. (1995), Giovannini et al. (1996), and Handa (1992b)

(continued)

Table 18.3 (continued)

Rol genes	Plants	Phenotype	References
Rol A	*Tobacco*	Enhances the activity of β-glucuronidase enzyme to 50-fold high	Barros et al. (2003)
	Tobacco	Wrinkled phenotype	Sinkar et al. (1988)
	N. tabacum	Accumulation of nicotine	Palazon et al. (1998a)
Rol B	*Rosa hybrid*	Decrease in formation of lateral shoots	Vander salm et al. (1996, 1997)
Rol A, B	*Rosa hybrid*	Plant exhibits erect habit with reduced branches, modified flowering, increase in no. of flowers	Allavena et al. (2000)
Rol C	*Dianthus caryophyllus*	Dwarfness, increased lateral branches, increased in flowers bearing stems, smaller flowers lie at single plane	Zuker et al. (2001) and Ovadis et al. (1999)
	Osteospermum ecklonis	Plant with erect habit, leaves are pale green in colour, flowering enhanced, small flower with reduction in fertility or maybe sterile in nature also	Allavena et al. (2000)
	Rosa hybrida	Stunted plant height, lateral branching increased, leaves are wrinkled and chlorotic, poor rooting ability, less no. of flowers with reduced fertility and large no. of thorns were observed	Souq et al. (1996)
	Salpiglossis sinuata	Leaves are narrow, dwarfness, small flowers, flower that are male sterile	Lee et al. (1996)
	Pelargonium x domesticum	Reduced plant height, reduction in surface area of leaves, petals and flowers, early flowering	Boase et al. (2004)
	Petunia axillaris X (*P. axillaris X P. hybrida*)	Phenotypically dwarf plant, reduced intermodal length, variable leaf size (either reduced or increased) advanced flowering, altered fertility (male or female, both)	Winefield et al. (1999)
	N. langsdorffii	Confers high resistance to chromium (Cr VI), changes in sugar and phenolics content	Del Bubba et al. (2013)

effect of Rol B and C has not been studied till date as per our knowledge goes; so it could be proven as an area of great interest, particularly in relation to novel phytochemical accumulation or desired secondary metabolite production. In addition, it could easily be demonstrated that Rol D individually as well as in combination with other Rol genes has not been exploited so far; the manipulations in number and copy of integration of rol genes (A, B, C, D) either individually or combined will no doubt match the laborious and time-consuming techniques of molecular biology to study the secondary metabolite pathways and biosynthetic routes operating *in planta*.

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Part IV

Crop Improvement

Synthesis of Silver Nanoparticles from Plants and Their Applications

19

Asra Parveen and Srinath Rao

Abstract

Nanobiotechnology defines applications of biosystems to produce novel functional materials. Production of metal nanoparticles is increasing to develop innovative technologies. There are various techniques to characterize the synthesized silver nanoparticles. UV-Vis spectroscopy is used for absorbance pattern, X-ray diffraction reveals crystalline nature with FCC geometry with mean particle size, Fourier transform infrared spectroscopy is for chemical compositions associated with NPs, and transmission electron microscopy is used to determine the shape of the NPs. AgNPs are one of the important materials having wide applications in optoelectronic devices, biosensors, and catalysis. Application of silver nanoparticles depends on the different charges, chemical composition, size, and shape. There are various methods to synthesize AgNPs. Keeping in view of synthesis and applications of AgNPs, a brief study of available literature for biosynthesis method and practices of silver nanoparticles has been reviewed.

Keywords

Nanobiotechnology • Metal NPs • Characterization techniques • Silver nanoparticles • Applications

A. Parveen • S. Rao (✉)
Plant Tissue Culture and Genetic Engineering Laboratory, Department of Botany, Gulbarga University, Kalburgi 585106, Karnataka, India
e-mail: srinathraomm@gmail.com

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19.1 Introduction

Bionanoscience and bionanotechnology is an interdisciplinary area of research that works with the integration of chemistry, materials science, biology, medicine, and engineering. However, bionanotechnology has appeared as a combination of biotechnology and nanotechnology to develop biological synthesized nanomaterial as environmentally friendly. Nowadays, the use of nanoparticles is gaining because of their distinct biological, optical, chemical, and mechanical properties. Similarly, nanobiotechnology follows the principles and techniques of nanoscale and realizes the transformation of biosystems (living or nonliving). Nanotechnology industry has increasing market value of many billions of US dollars (Aitken et al. 2006) and would grow up to 3 trillion US dollars by 2018 (Global Industry Analysts 2008; Woodrow Wilson 2009). Nanoparticles have two broad groups, namely, organic and inorganic NPs. Organic nanoparticles include carbon nanoparticles (fullerenes), and inorganic nanoparticles include magnetic, noble metal (gold and silver) NPs, and semiconductor NPs (titanium dioxide and zinc oxide). The Lycurgus Cup of the fourth century AD (Fig. 19.1) is a good example which appears green in reflected and red in transmitted light due to the presence of colloidal gold and silver (Turkevich 1985). The color changes as the cup contains 50 and 100 nanometers of AuNPs and AgNPs in the glass.

Nanotechnology is gaining importance since the twenty-first century to find cheap, convenient, and safer methods for the production comparing to physical and chemical methods. Basically, there are two techniques for the production of nanoparticles: (1) the top-down approach and (2) the bottom-up approach (Fig. 19.2). In top-down approach, the larger materials are broken down using ultrafine grinders, lasers, and vaporization followed by cooling (Senapati et al. 2005); the top-down approach depends on the laws of quantum mechanics. In bottom-up approach, the small molecules get rearranged to form nanostructures with unique properties

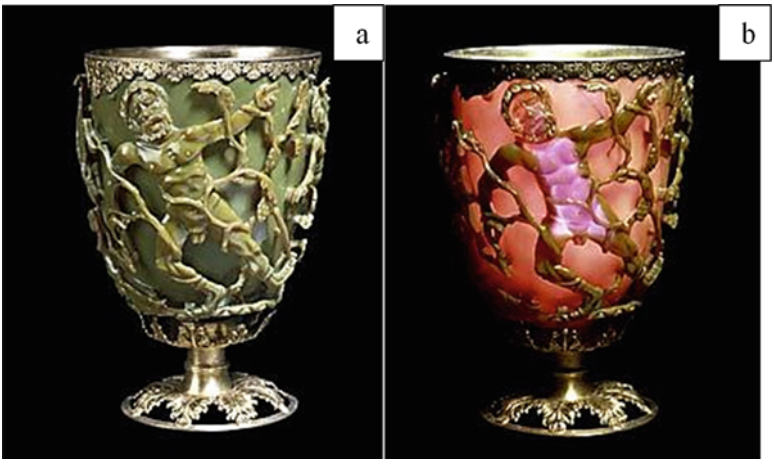

Fig. 19.1 Lycurgus Cup (**a**) reflected light (**b**) transmitted (www.theguardian.com)

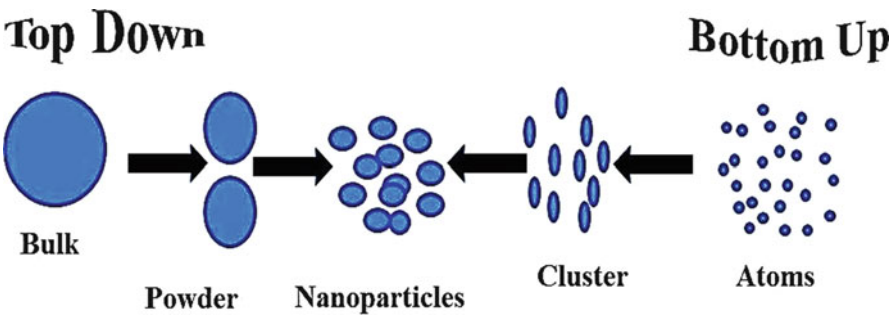

Fig. 19.2 Schematic representation of the building up of nanostructures

where NPs "build up" atom by atom by self-organizing or self-assembly (Seeman and Belcher 2002).

Since the work of Faraday, researchers have developed different approaches for the synthesis of colloidal noble metal nanoparticles by physical, chemical, and biological methods. Physical and chemical methods used for the synthesis of MNPs are sol-gel (Shukla and Seal 1999), laser ablation (Mafune et al. 2001), chemical reduction (Chaki et al. 2002), solvothermal (Rosemary and Pradeep 2003) and ion sputtering method (Raffi et al. 2007), etc. Although these methods produce pure, well-defined nanoparticles but are quite expensive, the methods used are actually dangerous for the environment (Kumar and Yadav 2009). Nanoparticles are the building blocks that consist of few hundred atoms to millions of atoms with properties (chemical, biological electrical, mechanical, and optical). Biosynthesized NPs are unlike from the bulk material and have remarkable applications in various fields (Fig. 19.3).

The plant-based biosynthesized metal nanoparticles (gold and silver nanoparticles) have received much attention as alternative method to chemical and physical methods. Metal nanoparticles produced using plant extracts are cost effective therefore economically used as valuable alternative for the bulk production. The biosynthesized nanoparticles additionally remain constant than the chemical method (Duran et al. 2010), where plant extracts act as both reducing and capping agents in the synthesis. Silver, gold, platinum, and palladium are the most studied NPs; Ag and Au nanoparticles are widely used in biomedical field.

19.2 Biosynthesis of Metal Nanoparticles from Plants

Medicinal plants have been the richest bioresource used in medicine, nutraceuticals, food supplements, and chemical entities for synthetic drugs (Ncube et al. 2008). The phytochemicals are not only important in medicines but also aid in the reduction and stabilization of metal nanoparticles playing dual function in the synthesis. Green synthesis of NPs is inexpensive and environmentally friendly and can easily synthesize in large scale which does not require toxic chemicals, energy, pressure, and temperature. Various organisms (unicellular and multicellular), i.e., bacteria,

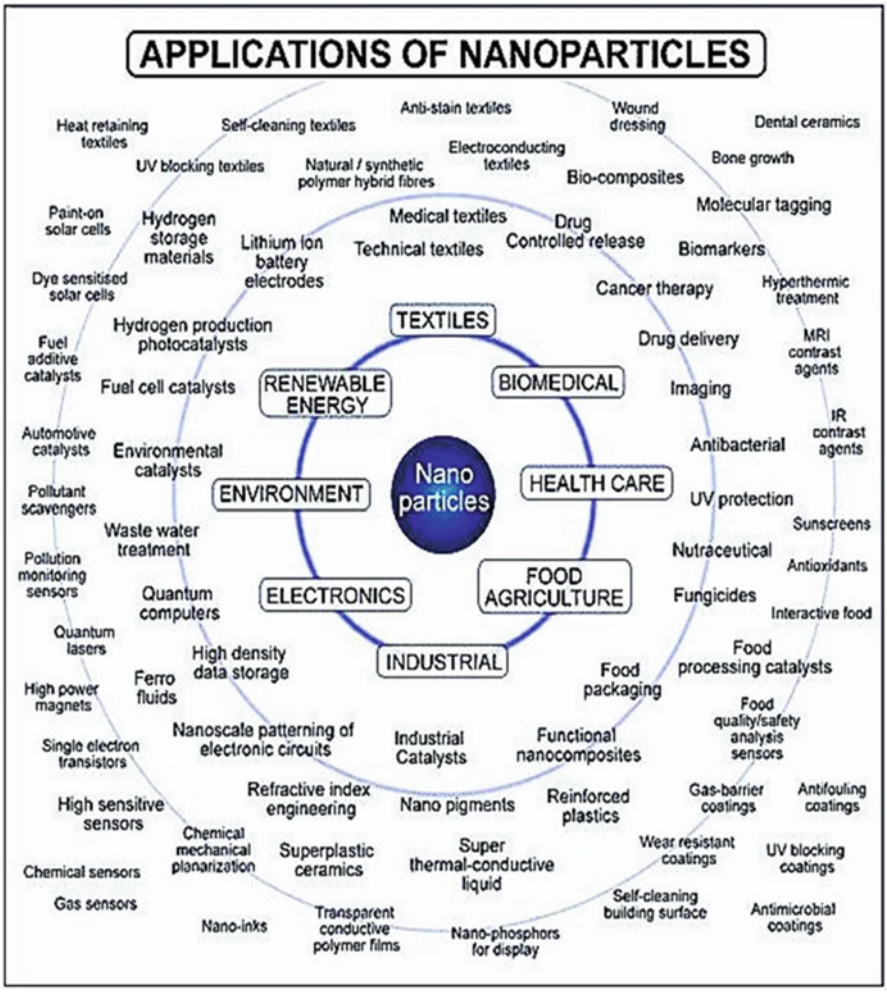

Fig. 19.3 Nanotechnology in different fields (http://www.deakin.edu.au)

fungi, and yeast, have been used to synthesize inorganic nanomaterials extracellularly and intracellularly (Mann 1996; Kumar et al. 1995; Ahmad et al. 2005). The rapid extracellular synthesis of AgNPs and AuNPs using leaf extracts of numerous plants has been reported like *Aloe vera* (Chandran et al. 2006), *Spinacia oleracea* and *Lactuca sativa* (Kanchana et al. 2011), *Chenopodium album* (Dwivedi and Gopal 2010), and *Cassia alata* (Gaddam et al. 2014). Most of the plant material contains various water-soluble anionic components such as thiocyanate, nitrate, chlorides, sulfates, starches, tannins, saponins, terpenoids, polypeptide, and lectins (Darout et al. 2000). The biosynthesis of nanoparticles by plant material (fresh and dry) takes place at room temperature and requires less energy results in both intracellular (inside living cells) and extracellular (outside the cells). There is increase in the use of different medicinal plants for the nanoparticle synthesis (Table 19.1).

Table 19.1 List of few plants used in the biosynthesis of AgNPs

Sl. No.	Plants used	Biomolecules involved	Nanoparticles	Size (nm)	References
1	*Datura metel L.*	Plastohydroquinone or plastrocohydroquinol	Ag	16–40 nm	Kesharwani et al. (2009)
2	*Mentha piperita L.*	Menthol	Ag, Au	90 nm, 150 nm	Ali et al. (2011)
3	*Zingiber officinale* Rosc.	Alkaloids, flavonoids	Ag, Au	10 nm	Singh et al. (2011)
4	*Citrullus colocynthis L.*	Polyphenols	Ag	31 nm	Satyavani et al. (2011)
5	*Achyranthes aspera* L.	Polyols	Ag	20–30 nm	Daniel et al. (2011)
6	*Desmodium triflorum* (*L.*)	Water-soluble antioxidative agents like ascorbic acids	Ag	5–20 nm	Ahmad et al. (2011)
7	*Andrographis paniculata Nees.*	Hydroxyflavones, catechins	Ag	28 nm	Sulochana et al. (2012)
8	*Astragalus gummifer*	Proteins	Ag	13.1 ± 1.0 nm	Kora and Arunachala (2012)
9	*Coleus aromaticus Lour.*	Flavonoids	Ag	40–50 nm	Vanaja and Annadurai (2012)
10	*Dioscorea bulbifera L.*	Polyphenols or flavonoids	Ag	8–20 nm	Ghosh et al. (2012)
11	*Dioscorea oppositifolia L.*	Polyphenols	Ag	14 nm	Maheswari et al. (2012)
12	*Glycyrrhiza glabra L.*	Flavonoids, terpenoids, thiamine	Ag	20 nm	Dinesh et al. (2012)
13	*Hydrilla verticillata*	Proteins	Ag	65.55 nm	Sable et al. (2012)
14	*Lantana camara L.*	Carbohydrates, glycosides, and flavonoids	Ag	12.55 nm	Sivakumar et al. (2012)
15	*Averrhoa bilimbi*	Amines, alcohol	Ag, Au	50–175 nm	Isaac et al. (2013)
16	*Capparis zeylanica*	Amines, phenolic compounds	Ag	50–90 nm	Saranyaadevi et al. (2014)
17	*Terminalia arjuna*	Phenol/carboxylic group	Ag	3–50 nm	Ahmed and Ikram (2015)

Nanotechnology has evolved rapidly to develop new techniques in synthesis and characterization (Sharma et al. 2009). Sau and Rogach (2010) stated the earlier methods used for the synthesis result in lesser quantities with poor morphology. Synthesis of nanoparticles with poor morphology. Birla et al. (2009) reported the use of toxic chemicals and higher temperatures for synthesis process were toxic to

the environment. Since then biological synthesis of nanoparticles captured attention by using bacteria (Shahverdi et al. 2009), fungi (Govender et al. 2009), actinomycetes (Ahmad et al. 2003), algae (Chakraborty et al. 2009), etc. Mukherjee et al. (2008) started a greener approach for the synthesis which was environmentally friendly as no toxic chemicals, temperature, and pressure are required. Hence, bioresearchers also focused on the synthesis of bionanoparticles instead of using chemically or physically synthesized nanoparticles (Thakkar et al. 2010). The yield of different sizes and shape of nanoparticles depends on the type of chemical, physical, and biological agents used. The biological means used for the production of nanoparticles are generally microbes (Gajbhiye et al. 2009) and plants (Jha et al. 2009; Javad et al. 2014) which results in both extracellular and intracellular synthesis (Shaligram et al. 2009). Physical and chemical synthesis requires reducing and stabilizing agents (sodium borohydride, sodium citrate, and alcohols) which are mostly toxic and flammable with low production rate (Bar et al. 2009). The reduction rate of metal ions is faster at room temperature using biological agents and is a low-cost, nontoxic, and less labor-intensive technique. The exact mechanism of nanoparticle synthesis using biological agents is still in confusion as different bioagents react differently with metal ions. The biologically synthesized AgNPs have been in used silica-coated Ag nanowires and electric circuits (Kvistek and Prucek 2005). Plant-based nanoparticle synthesis is advantageous over other biological methods (microbial) as the synthesis reaction rate is very high and does not require specific conditions to grow the microbes Kumar and Yadav (2009).

Several plants have been used in biosynthesis of silver nanoparticle. Shankar et al. (2003) explained geranium leaf extract in rapid synthesis of stable and crystalline AgNPs (16–40 nm). Shankar et al. (2004) reported the extracellular synthesis of pure metallic silver, gold, and bimetallic Au/Ag nanoparticles from *Azadirachta indica* leaf broth. Ankamwar et al. (2005) reported that *Emblica officinalis* fruit extract leads to rapid reduction of the Ag ions in stable AgNPs. However, Chandran et al. (2006) controlled the shape and size of AgNPs with *Aloe vera* leaf extract as reducing agent. The volume of extract and temperature used during the reaction led in characteristic nanoparticles. Huang et al. (2007) reported the use of *Cinnamomum camphora* leaf for the synthesis of AgNPs (55–80 nm). The shape of nanoparticles differs due to biomolecules present in leaf extracts. They may be polyol and water-soluble heterocyclic components responsible for the reduction of silver ions. Li et al. (2007) identified 3 nm protein moieties capped the silver nanoparticles that aid in the reduction and capping of AgNPs.

Udayasoorian et al. (2011) have reported AgNP synthesis from leaf extract of *Cassia auriculata* under shaking conditions with 20–40-nm particle size. Velavan et al. (2012) investigated strong antioxidant activity of silver nanoparticles synthesized from *Cassia auriculata* flower extract. Amaladhas et al. (2012) reported sennosides, a water-soluble constituent in *Cassia angustifolia* leaf extract reacted as both reducing and capping agents in the synthesis of AgNPs. Gaddam et al. (2014) reported biofabrication of AgNPs from *Cassia alata* leaf extract and its antimicrobial activity. There are reports where aqueous callus from different plants has been used as reducing agent for the synthesis of AgNPs like *Sesuvium portulacastrum* L. (Nabikhan et al. 2010). Reddy et al. (2015) used aqueous callus extract of *Centella*

Fig. 19.4 Visual observation of silver nitrate after reduction (AgNPs) at different concentrations of leaf extracts (Parveen et al. 2012)

asiatica for the synthesis of AgNPs and reported strong inhibitory activity against *B. subtilis*, *S. aureus*, *E. coli*, and *P. aeruginosa*.

19.2.1 Size and Shape

Various factors govern the size and shape of the bionanoparticles like reaction temperature (Rai et al. 2006), concentrations of the leaf broth (Song et al. 2010), reaction time (Li et al. 2007), etc. The reaction temperature increases the reaction rate where most Ag ions consumed to form nuclei (Song and Kim 2009).

19.2.2 Characterization of Nanoparticles

Kumar and Yadav (2009) explained characterization techniques of nanoparticles. The first step to characterize AgNPs is by visual observation (Fig. 19.4). The nanoparticles from different salts show characteristic peaks at different absorptions monitored by UV-Vis spectroscopy. Silver nanoparticles have an absorption peak between 400 and 450 nm. The increase in characteristic peak with increased reaction time and concentration of plant extracts used indicates the formation of nanoparticles. UV-Vis spectra show characteristic peaks of the surface plasmon resonance (SPR) in nanosized particles.

X-ray diffraction (XRD) technique characterizes the metallic nature of nanoparticles. The wavelength is equivalent to the size of the atom used for analyzing the structural organization of atoms and molecules. The energetic X-rays penetrate through the nanomaterial giving detailed information of the bulk structure (Putnam et al. 2007). For very small crystallite sizes, signals in XRD are broadened, a phenomenon described by the Scherrer. Fourier transform infrared (FTIR)

Table 19.2 Different techniques for characterization of nanoparticles

Techniques	Measures
Ultraviolet-visible spectroscopy (UV-Vis)	Absorption spectrum, optical characterization
Transmission electron microscopy (TEM), scanning transmission electron microscopy (STEM), high-resolution TEM (HRTEM)	Particle size and characterization
Scanning electron microscopy (SEM)	Particle size and characterization
Atomic force microscopy (AFM)	Particle size and characterization
Dynamic light scattering, photon correlation spectroscopy (PCS)	Average particle size, size distribution, and stability of nanoparticles
X-ray diffraction (XRD)	Average particle size for a bulk sample
Energy-dispersive X-ray spectroscopy	Elemental analysis
Fourier transform infrared spectroscopy (FTIR)	Spectral data

spectroscopy is used to determine biomolecules of plants associated with nanoparticles. Microscopic techniques such as scanning electron microscopy (SEM), transmission electron microscopy (TEM), and atomic force microscopy are mainly used to study morphology of nanoparticles. Some of the commonly used characterization techniques are given (Table 19.2).

19.2.3 Characterization of Associated Biomolecules

According to Iravani (2011), the reduction of metal NPs occurred by the biomolecules present in the plant extracts like enzymes, proteins, vitamins, polysaccharides, etc. Nanoparticles interact with biomolecules, and adsorption of proteins occurs on the surface of nanoparticles forming nanoparticle protein complexes (NP-PC) which makes NPs biocompatible. These active biomolecules control the composition, size, and shape of bionanoparticles (Xie et al. 2007). Proteins isolated from green alga played dual function in the ions reduction, and controlled the characteristics like size and shape of gold ions (Chandran et al. 2006; Kaur et al. 2009). Plants have a broad range of metabolites that aids in the reduction faster than microbial synthesis. Parveen and Rao (2014a) considered plant biomolecules/proteins are responsible for the reduction and have dual function in biosynthesis and stabilization of nanoparticles (Fig. 19.5).

19.3 Applications

Due to numerous applications of silver nanoparticles, future aspects of synthesis and its applications are becoming significant in many fields such as energy, health care, environment, agriculture, etc. Ag ions and AgNPs are highly toxic to the

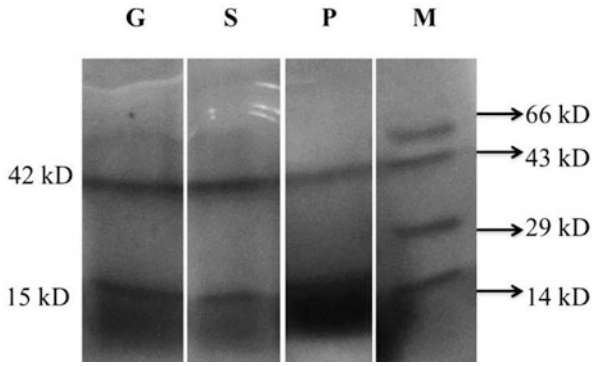

Fig. 19.5 SDS-PAGE analysis of NP-associated proteins (Parveen and Rao 2014a)

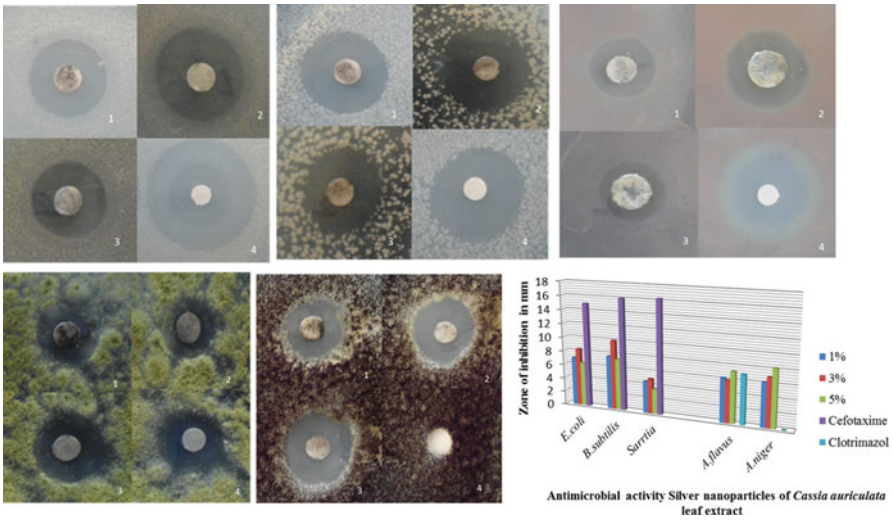

Fig. 19.6 Antimicrobial activity of Ag nanoparticles against (**a**) *E. coli*, (**b**) *B. subtilis*, (**c**) *Serratia* spp., (**d**) *A. flavus*, (**e**) *A. niger* (Parveen et al. 2012)

microorganisms (e.g., viruses, bacteria, fungi) (Wen et al. 2007). Silver nanoparticles have inhibitory and bactericidal properties as it was an excellent antibacterial agent used from history. Parveen et al. 2012 have reported substantial antibacterial and antifungal impact in vitro by using biosynthesized AgNPs at very low concentrations (Fig. 19.6). Matsumura et al. (2003) suggested that silver ions interact with phosphorus moieties in DNA inactivating DNA replication and react with sulfur-containing proteins to inhibit enzyme functions.

Nowadays, AgNPs are incorporated in various consumer goods like cosmetics, toothpastes, surface cleaners, detergents, antimicrobial paints, toys, home appliances, shoe insoles, brooms, automotive upholstery, textiles, and food storage containers (Thomas et al. 2007; Amendola et al. 2007; Navaladian et al. 2008; Fernandez

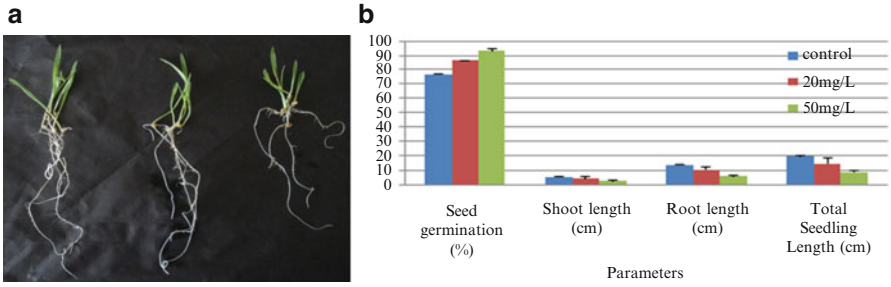

Fig. 19.7 Effect of AgNPs on seed germination and seedling growth of *Pennisetum glaucum* (Parveen and Rao 2014b)

et al. 2008). Many commercial products such as T-shirts, socks, sportswear, and fabrics are incorporated with functionalized AgNPs, which is useful in the medical field to avoid high risks of contamination associated with surgical suits (Benn and Westerhoff 2008). Some of the products and devices containing silver such as catheters, prostheses, vascular grafts, and wound dressings are being used in the medical field (Furno et al. 2004). Likewise, AgNPs have also been used by food packaging industry in the packaging materials for the preservation of food for longer duration. Researchers have reported sonochemical coating technique for preparation of materials from the colloidal AgNPs using ultrasonication (Samberg et al. 2011).

The metallic NPs have surface plasmon resonance (SPR) properties used in making sensors like real-time searching of membrane transport in living microbial cells (Xu et al. 2004), nitro-explosives (Marcia 2006), immunoassay labeling (Morones and Frey 2007), DNA sequence detection (Jacob et al. 2008), fibrinogens in human plasma (Zhi Liang et al. 2007), glucose sensor in medical diagnostics (Mishra et al. 2007), biolabeling, optical imaging of cancer (Wiley et al. 2007), and herbicide detection (Dubas and Pimpan 2008).

Silver nanoparticles have tremendous electronic applications in optics, data storage devices, battery-based intercalation materials, high-density recording devices, integrated circuits (IC), and capacitors (Navaladian et al. 2008; Kim et al. 2007; Deshmukh and Composto 2007). Silver nanoparticles and silver nanocomposites have been used as catalyst in CO oxidation (Liu et al. 2005), p-nitrophenol reduction to p-aminophenol (Fernandez et al. 2008), and gaseous acetaldehyde photodegradation (Hamal and Klabunde 2007).

Nanoagriculture evolves with the ambition to impart beneficial effects on the crop plants (Kotegooda and Munaweera 2011). Nanoparticles serve as "magic bullets" holding herbicides, chemicals (fungicides, insecticides, etc.), and genes (Nair et al. 2010). Nanoparticles can be utilized to improve disease resistance, nutrient utilization, and enhanced growth in crop plants. Parveen and Rao (2014b) reported that biosynthesized AgNPs have enhanced the seed germination in *pearl millet*, but the growth of seedlings was affected (Fig. 19.7), whereas the same concentration of AuNPs was found beneficial (Parveen et al. 2016). Parveen and Rao (2014c) reported (anticancer activity of biosynthesized AuNPs and AgNPs on human

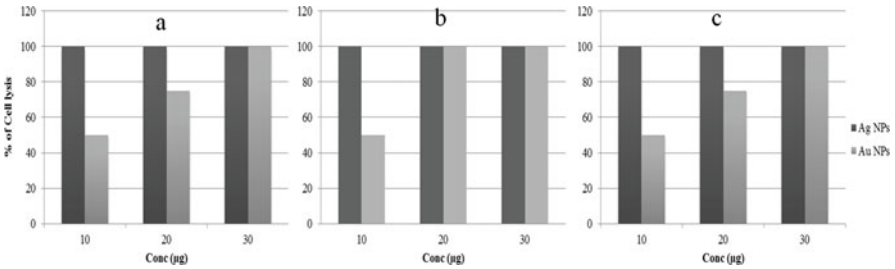

Fig. 19.8 Comparative study (showing anticancer effect of AuNPs and AgNPs synthesized using *Cassia auriculata* leaf extract: (**a**) A549, (**b**) LNCap-FGC, (**c**) MDA-MB (Parveen and Rao 2014c)

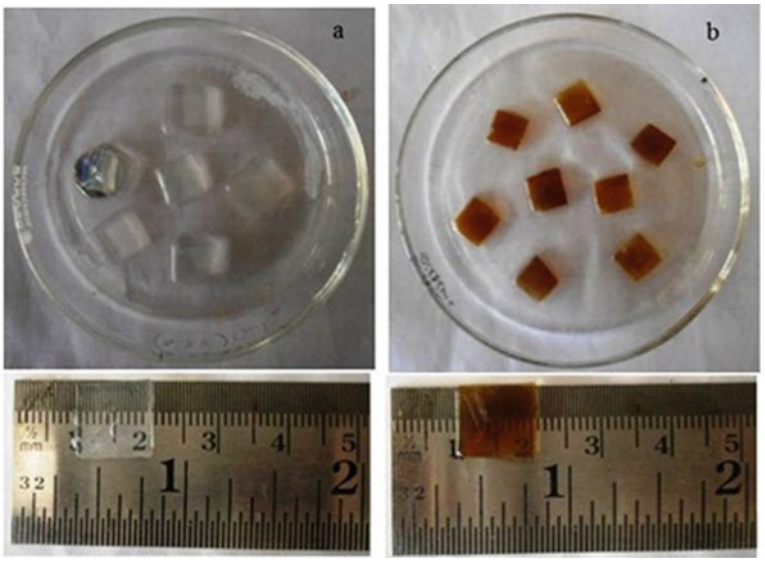

Fig. 19.9 Preparation of silver bionanocomposite (Ag-BNC) (Parveen et al. 2015)

carcinoma cells lines by MTT assay and found AgNPs were more toxic than AuNPs (Fig. 19.8). Parveen et al. (2015) have incorporated metal NPs to prepare bionanocomposites which have differential applications as antimicrobial and can be utilized in preparation of wound dressing, biosensor, and electronics (Figs. 19.9 and 19.10).

19.4 Conclusion

Bionanotechnology serves as an important research area for the fabrication and use of metal nanoparticles. Various physical and chemical methods were designed for the production of nanoparticles, but the complications of using these methods led the researchers to explore for alternative, safe, and easy methods. A number of

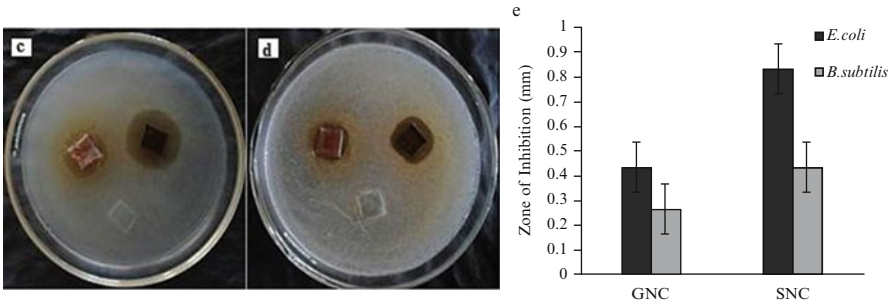

Fig. 19.10 Antibacterial activity of Au-BNC and Ag-BNC on *E. coli* and *B. subtilis* (Parveen et al. 2015)

plants have been exploited for the synthesis of silver nanoparticles. The nanoparticles were characterized by various analytical techniques, such as UV-Vis, FTIR, XRD, TEM, SEM and zeta potential measurements, etc. Plant-mediated nanoparticle synthesis is environmentally friendly, nontoxic, easier, and cheaper for bulk production of NPs. However, the exact mechanism of bionanoparticle synthesis is still under study. Silver nanoparticles have tremendous applications and can be utilized in electronics, catalysis, biology, pharmaceutical, and medical diagnosis.

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Biotechnological Approaches for the Improvement and Conservation of *Alnus glutinosa* (L.) Gaertner

20

Mª del Carmen San José, Laura V. Janeiro, Mª Teresa Martínez, Silvia Valladares, Mª José Cernadas, Raquel Montenegro, and Elena Corredoira

Abstract

European alder [*Alnus glutinosa* (L.) Gaertner], also called black alder or European black alder, is a medium-sized tree that is widely distributed throughout Europe. In the few decades, black alder populations have declined drastically, partly as a result of deforestation and the disappearance of riparian habitats but mainly because of alder blight disease, caused by *Phytophthora alni*. In order to protect these important genetic resources, the existing conservation methods must be improved and new tools developed.

Biotechnological methods, particularly in vitro or tissue culture methods, could be beneficial for the large-scale multiplication, improvement and conservation of the species. Axillary shoot proliferation from cultured meristems is the most frequently used method of micropropagation, as it provides genetic stability. Axillary shoot multiplication has been achieved in several species of the genus *Alnus*. Although most of these reports refer to material of juvenile origin, such as seedlings or young trees, there have been few reports regarding the propagation of mature trees. Plantlets from mature trees of *A. glutinosa* are obtained and are viable for reintroduction to the natural habitat of the species. The explants used for in vitro multiplication can be stored at 4 °C under dim lighting for up to 18 months. The stored material can be successfully recovered and shows good

M.d.C. San José (✉) • M.T. Martínez • M.J. Cernadas • R. Montenegro • E. Corredoira
Instituto de Investigaciones Agrobiológicas de Galicia, CSIC,
Avenida de Vigo s/n., 15705 Santiago de Compostela, Spain
e-mail: sanjose@iiag.csic.es

L.V. Janeiro
INLUDES, Diputación Provincial de Lugo, Ronda da Muralla 140, 27004 Lugo, Spain

S. Valladares
Fundación Promiva, Ctra. M-501. Vía de servicio de Villaviciosa
de Odón a Boadilla del Monte. Villaviciosa de Odón, 28670 Madrid, Spain

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growth that develops into shoots that are morphologically similar to those of non-stored controls. This technique reduces maintenance requirements and the risk of genetic alterations.

Somatic embryogenesis is considered the most efficient in vitro procedure for mass propagation of plants. This technique has been successfully applied to immature zygotic embryos of black alder. This protocol may help to enhance the propagation of *A. glutinosa* and other alder species and could also provide a regeneration system for future use in genetic manipulation for introducing genes that confer resistance to pathogens such as *P. alni*.

In vitro shoot tips and embryogenic cultures can be successfully cryopreserved by vitrification, indicating that long-term storage of black alder germplasm may be possible. The material could then be safely stored while field tests are undertaken.

Keywords
Adventitious shoots • Axillary shoots • Black alder • Cryopreservation • Germplasm conservation • Micropropagation • Somatic embryogenesis

20.1 Introduction

The genus *Alnus*, a member of the family Betulaceae, comprises some 30 species of monoecious trees and shrubs. They are distributed throughout the world, and very few of them reach a large size.

20.1.1 General Features

Alnus, the Latin name classically used to refer to various species of alder, is derived from the Indo-Germanic root el-ol-, meaning shiny (particularly in reference to red and brown colours) and refers to the fact that the alder wood turns reddish orange after being cut. The wood was said to have protective properties, and the young branches were used to make whistles to attract spirits from the air. The species name *glutinosa* (sticky) refers to the sticky shoots and young leaves (López 2014).

There are four alder species native to Europe: the common alder (*A. glutinosa*), the grey alder (*A. incana*), the Italian alder (*A. cordata*) and the green alder (*A. viridis*). The type species is *A. glutinosa* (L.) Gaertner. The common, black or European alder (*A. glutinosa*) is of considerable landscape value along waterways and plays a vital role in riparian ecosystems. Members of the genus *Alnus* are generally pioneer species that are able to colonise bare, open ground rapidly and are highly tolerant of wet sites.

The black alder is a medium-sized tree that does not usually grow taller than 20 m, although specimens of more than 30 m are known. It is typically a component of mixed broadleaved forest, although it represents less than 1 % of the forest cover in many countries, because the most suitable sites have been converted to agricultural land. The

tree has a shallow root system, with some deep roots that can reach water in sites where the water table is low. The surface roots have brownish-yellow nodules that house actinomycete bacteria (*Frankia alni*) that are capable of fixing atmospheric nitrogen. The nitrogen thus fixed, at rates of 60–400 kg h^{-1} $year^{-1}$, is available to both the host tree and to the environment. The trunk is straight, reaches up to 1 m in diameter, and the bark is dark brown in adult trees and reddish brown in young ones. The crown of young trees is almost pyramidal and later becomes rounder or irregular in shape, with open branches. The leaves (4–10 cm) are simple, broad, with a well-developed petiole, dark green on the upper surface and paler green on the undersurface. They are round, elliptic or obovate, with a double-serrated margin and a rounded or slightly indented tip. The alternately arranged leaves appear in April or May, and when young they are sticky (as are the branchlets). The tree flowers between February and April, and the fruits appear in autumn. The empty cones remain on the tree until the following spring and are a distinctive feature of the alder tree in winter (López 2014).

20.1.2 Distribution and Propagation

Alder inhabits a large part of Europe, North and Northeast Asia (penetrating Asia Minor and the Caucasus) and Northwest Africa. The species was introduced to North America in the colonial era (Rehder 1940), and it has since become naturalised throughout the Northeastern United States and Maritime region of Canada. It is somewhat flexible as regards climate conditions and is well developed in cold temperate and warm temperate climates. It also tolerates intense cold winters and is known to resist minimum temperatures of −40 °C outside the growth season but is not resistant to late spring frosts.

The species is found at elevations ranging from almost sea level to 1,700 m, on the banks of rivers, streams and watercourses, as it requires soil that is almost permanently damp. Indeed, it is not very tolerant of summer drought. It prefers siliceous soil and can grow in poor soils due to the capacity of the roots to fix nitrogen from the atmosphere.

Alder shows a good capacity to regenerate from seeds and by sprouting from stumps but not from roots. Alder matures at between 3 and 30 years of age, depending on the ecotype and the stand conditions. The mature trees produce plentiful seeds every 3 or 4 years which are dispersed by wind and water. Germination rates are highly variable, ranging from 10 to 90 %, depending on the crop year and the stand. Growth is rapid, with yields of around 9–11 m^3/ha year. The species is moderately long lived, surviving for around 100–120 years (Claessens et al. 2010).

Although conservation of the alder germplasm is possible by cold stratification and cryopreservation of seeds (Chmielarz 2010), storage of this material is of little interest, because seeds are genetically heterogeneous. Vegetative propagation of *A. glutinosa* from cuttings taken from juvenile specimens is possible using standard horticultural techniques (Périnet and Lalonde 1983); however, propagation from cuttings obtained from mature trees is difficult (Claessens et al. 2010).

20.1.3 *Phytophthora* Disease of Alder

Black alder populations have declined drastically throughout Europe in recent years, partly as a result of deforestation and the disappearance of characteristic riparian habitats but mainly because of alder blight disease caused by *Phytophthora alni* and subspecies (Brasier et al. 2004). At the beginning of the 1990s, a new disease, which was given the names alder root and collar rot, caused the loss of large numbers of alder trees. The disease has been described in several European countries and has had a destructive impact in some areas, such as Southeast England, Northeast France, Bavaria and Northern Spain (Gibbs 1995; Gibbs et al. 1999; Streito et al. 2002; Jung and Blaschke 2004; Tuset et al. 2006). All European alder species and red alder (*A. rubra*) are highly susceptible to the pathogen, although *A. glutinosa* is the most susceptible. In 2001, the North American Plant Protection Organization (NAPPO) published an emerging pest alert (in its Phytosanitary Alert System) regarding alder *Phytophthora*, before the fungal causal agent had been described. In 2004, Brasier and colleagues formally described the causal agent as *P. alni* and considered that it may be a hybrid between *P. cambivora* and *P. fragariae* (a pathogen of strawberry) (Brasier et al. 1999). There are three subspecies: a standard hybrid designated *P. alni* subsp. *alni*, a Swedish hybrid called *P. alni* subsp. *uniformis*, and a subspecies designated *P. alni* subsp. *multiformis*, which includes Dutch, German and UK variants. Diseased trees have smaller leaves than usual, which are yellow, disperse and often fall prematurely. The branches die, and flowering and fruiting are intensified. The most characteristic symptom in affected trees is necrosis in the internal zone of the cortex, which produces large cankers at the level of the root and trunk (Gibbs et al. 1999).

The alder, along with elm, is currently one of the most seriously threatened species in European ecosystems. Given the important role that the species plays in stabilising riverbanks, purifying water and controlling water temperature as well as in the biodiversity of terrestrial and aquatic habitats, its disappearance would seriously affect the stability of fluvial ecosystems. Recognition of the importance of this species and of the danger represented by spread of the disease, which cannot yet be controlled, requires the development of new strategies of conservation of at least the most representative examples.

20.1.4 Economic Importance and Uses

Despite its relative scarcity in forests, black alder shows a good potential for timber production. Nevertheless, the production of high-quality black alder timber is only possible on sites that correspond closely to its autecological optimum, as with other valuable broadleaf species. In these conditions, alder grows as rapidly as ash, maple and cherry, and the timber can fetch high prices due to its desirable characteristics. Moreover, the species contributes greatly to riverine ecosystems and to the services they provide. It contributes to biodiversity by providing habitats for specific flora and fauna, both on the tree itself and in the flooded root system. It assists in water

filtration and purification in waterlogged soils, and the root system helps to control floods and stabilise riverbanks (Claessens et al. 2010).

Alder is suitable for repopulating poor soils and flooded areas, whereas other tree species require open, sunny sites. It has been widely used in gardening as an ornamental plant, as well as for windbreaks. It was also one of the first species used in Europe to stabilise dunes.

The timber is very light in colour immediately after felling but then acquires a reddish or brownish-orange tone. It decomposes readily on contact with air, but is very resistant when submerged, and is valuable for making posts and foundation piles for hydraulic constructions and in the past was used for houses constructed in water, as in Amsterdam and Venice. It is easy to work with and to stain or apply other surface treatments and is therefore suitable for imitating other high-quality timbers (ebony, mahogany and cherry). At present its main application is for making plywood and industrial items, as well as for artisanal work. It is also used to produce high-quality charcoal. Alder was also commonly used in home remedies for various ailments. The bark is very rich in tannins (19%) and contains the fatty acids palmitic and stearic acid. Products made from the bark have astringent, anti-inflammatory, decongestant and antipyretic properties and have been used as substitutes for quinine and as antidiarrhoeals (Alvárez et al. 2000).

Woody plants (especially black alder) are also potentially useful for accumulating and extracting heavy metals from polluted watercourse banks. The success of this application and of obtaining specimens tolerant/resistant to alder diseases would require regeneration of whole plants from cultured somatic tissues under selected conditions (Bajji et al. 2013).

Since the 1990s, biotechnology has provided new means of propagating and conserving the species, including in vitro tissue culture. Micropropagation methods could be beneficial for the large-scale multiplication, improvement and conservation of this species.

This chapter presents a review of micropropagation studies carried out on *A. glutinosa*, by culture of axillary buds and differentiation of adventitious buds and somatic embryogenesis, as well as of the conservation methods used (slow growth and cryopreservation) for mid- and long-term maintenance of the material.

20.2 Micropropagation

Germplasm preservation plays an important role in the maintenance of biodiversity and prevention of genetic erosion. The traditional method of conserving phytogenetic resources is in situ conservation (parks or natural reserves) (Iriondo 2001). Although efficient, this method is costly, and there is a high associated risk of loss of trees due to environmental factors and pests (Engelmann 2004). Ex situ conservation is an alternative that has been internationally recognised as important (UNEP 2002) and is being used by numerous organisations for biodiversity conservation (Engelmann 2011). Ex situ conservation techniques can be used effectively to complement in situ methods and represent the only option for the conservation of

recalcitrant species or species in danger of extinction and for material from elite or genetically transformed genotypes (Sarasan et al. 2006). As there are not yet any effective methods for controlling *Phytophthora* disease of black alder (Webber et al. 2004; Jung and Blaschke 2004), conservation of the species in its natural habitat does not appear feasible, and biotechnological procedures such as micropropagation techniques provide the only means of cloning specimens selected for their tolerance/resistance to disease. Micropropagation protocols are also a prerequisite for cryopreservation and genetic transformation methods, which can complement conventional breeding programmes. Regeneration of black alder plants by in vitro culture has been achieved by multiplication of axillary buds, adventitious shoot regeneration and somatic embryogenesis (Table 20.1).

20.2.1 Axillary Shoot Multiplication

Axillary shoot multiplication from cultured meristems is the method most frequently used for micropropagation as it provides genetic stability and is easily attained in many plant species (George et al. 2008). In black alder, this in vitro culture technique enables short- to medium-term ex situ storage of valuable genotypes and massive production of the clonal plant stock to restore areas devastated by disease. The first studies involving micropropagation of alder by axillary shoot multiplication date from the 1980s, when the importance of actinorhizal host plants in forestry was first recognised (Tremblay et al. 1986; Périnet and Tremblay 1987). In these studies, micropropagation of several species of the genus *Alnus*, including *A. glutinosa*, was achieved, although most of them used material of juvenile origin, such as seedlings or young trees, and therefore of unknown value. Two decades later, when European alder populations were seriously damaged by disease, the first studies with material of adult origin were carried out (Table 20.1).

20.2.1.1 Material of Juvenile Origin

Brown (1980) mentions an unpublished study on the in vitro regeneration of plants from seedling cultures of *A. glutinosa* and *A. rubra*; however, the first report of in vitro propagation of alder is attributed to Garton et al. (1981), who used material from 2-month-old plants. Aseptically germinated seedlings were used by Read et al. (1982) and Périnet and Lalonde (1983), as well as 2-year-old plants grown in the field by Tremblay and Lalonde (1984) and Périnet and Tremblay (1987).

Two mineral salt formulations were successfully used for the micropropagation of black alder from juvenile material: Woody plant medium (WPM; Lloyd and McCown 1980) and Murashige and Skoog medium (MS; Murashige and Skoog 1962). Read et al. (1982) reported the WPM to be better than MS medium for inducing shoot proliferation with less callus formation. Subsequently, Tremblay and Lalonde (1984) compared MS (Blaydes 1966) and WP media and observed good growth of *A. glutinosa* in the WP and MS media. Other mineral salt formulations such as B5 (Gamborg et al. 1968) Nagata and Takebe (1971) and Crone's solution (Lalonde 1979) were not beneficial and were sometimes even deleterious, to the

Table 20.1 Summary of studies involving in vitro biotechnological methods applied to black alder *Alnus glutinosa* (L.) Gaertner

Material used for explants	Results	Basal medium and carbohydrate source	Plant growth regulators	Reference
Juvenile (2-month-old)	Axillary shoot proliferation	WPM + sucrose	BA (1 μM)	Garton et al. (1981)
	Rooting	Ex vitro	–	
Juvenile (seedlings)	Axillary shoot proliferation	WPM + sucrose	BA (0.1–1 μM)	Read et al. (1982)
	Rooting	Ex vitro	–	
Juvenile (seedlings)	Axillary shoot proliferation	MS + sucrose	BA (0.5–5 μM)	Périnet and Lalonde (1983)
	Adventitious shoot	MS + sucrose	BA (0.5–5 μM)	
	Rooting	½ MS + sucrose	IBA (1 μM)	
Juvenile (2-year-old)	Axillary shoot proliferation	MS + sucrose	BA (2.5 μM)	Tremblay and Lalonde (1984)
	Rooting	½ MS + sucrose/ glucose	IBA (1 μM)	
Juvenile (seedlings and 2-year-old)	Axillary shoot proliferation	MS + sucrose	BA (2.5 μM)	Périnet and Tremblay (1987)
	Rooting	½ MS + sucrose	IBA (1 μM)	
Juvenile (seedlings) /mature (18-year-old)	Axillary shoot proliferation	WPM	Z (–)[a]	Naujocks et al. (2009)
	Rooting	½ WPM	IBA (–)[a] + NAA (–)[a]	
Mature (sexually mature)[a]	Axillary shoot proliferation	WPM + glucose	BA (1–5 μM) + TIBA (0.1–3 μM)	Lall et al. (2005)
	Rooting	½ WPM + glucose		
Mature (25–30-year-old)	Axillary shoot proliferation	WPM + glucose	BA (0.44 μM)+IAA (2.85 μM)+Z (2.28 μM)	San José et al. (2013)
	Rooting	½ WPM + glucose	IBA (0.49 μM)	
Juvenile (seeds and seedlings)	Adventitious shoots	WPMm + glucose	TDZ (1–10 μM); 2ip (5–50 μM); BA (1–5 μM)	Bajji et al. (2013)

(continued)

Table 20.1 (continued)

Material used for explants	Results	Basal medium and carbohydrate source	Plant growth regulators	Reference
Juvenile (immature ZE)	Somatic embryogenesis	MS + sucrose	BA (2.22 μM) + 2,4-D (0.9–9.05 μM)	Corredoira et al. (2013)
	Plant regeneration	WPM + glucose	BA (0.44 μM) + Z (0.46 μM)	
Mature (25–30-year-old)	Slow-growth conservation (4 °C)	WPM + glucose	BA (0.44 μM)+IAA (2.85 μM)	San José et al. (2015a)
Mature (25–30-year-old)	In vitro-grown shoot tips	Vitrification	–	San José et al. (2014)
	Cryopreservation			
Juvenile (somatic embryos initiated from immature ZE)	Somatic embryos	Vitrification	–	San José et al. (2015b)
	Cryopreservation			

Abbreviations for basal media: *MS* Murashige and Skoog medium (Murashige and Skoog 1962), *½ MS* MS half-strength macronutrients, *WPM* Woody plant medium (Lloyd and McCown 1980), *½ WPM*, WPM half-strength macronutrients, *WPMm* WPM modified

[a]Not specified. *ZE* zygotic embryos

growth of black alder in comparison with the MS medium (Tremblay and Lalonde 1984). MS salts used at two or four times strength induced complete necrosis within 3 weeks, whereas half-strength MS salts rapidly resulted in deficiency symptoms (Tremblay and Lalonde 1984).

The sugar requirement of alders during culture varies between species and between genotypes at the intraspecific level and plays a particularly important role in the development of alder tissue cultures during the multiplication stage (Tremblay et al. 1986). Tremblay and Lalonde (1984) observed that 87 mM sucrose (3 % w/v) was optimal for *A. glutinosa*, whereas all other species (seven species were tested) grew better in glucose. With small variations (58.3–87.5 mM, 2–3 % w/v), this sugar has been used for black alder propagation using juvenile material (Table 20.1). However, in a subsequent study, Welander et al. (1989) compared the effect of different carbon sources (sucrose, glucose and fructose) on shoot multiplication of black alder (although these authors did not indicate the age of the source of their explants) and found that 88 mM of glucose was the most effective carbon source in *A. glutinosa*. These authors suggested that the differences might be explained by clonal diversity.

Different types and concentrations of cytokinins have been tested with the aim of optimising the in vitro multiplication of black alder, with benzyladenine (BA) being the most widely used (Table 20.1). Périnet and Lalonde (1983) compared BA (0.5–25 μM) and N^6-(Δ^2-isopentenyl) adenine (2ip) (5–80 μM) and found that 1 μM BA produced the best results. Read et al. (1982) used the same concentration of BA to obtain multiple shoot formation. Tremblay and Lalonde (1984) and Périnet and Tremblay (1987) used a higher concentration of BA (2.5 μM), although in both studies a decrease in the concentration of BA favoured shoot elongation. Auxins have not generally been used during the multiplication stage. The presence of auxin in combination with cytokinin was reported to be deleterious for inducing callus on leaves and shoots during initiation and multiplication steps (Read et al. 1982; Périnet and Lalonde 1983; Tremblay and Lalonde 1984).

Rooting of shoots produced in vitro has been successfully achieved both in vitro and ex vitro (Table 20.1). Garton et al. (1981) applied ex vitro methods to *A. glutinosa* microshoots and observed that 100 % of the shoots rooted within 3 weeks in Jiffy mix. Read et al. (1982) used a similar system and achieved rooting rates of between 95 and 100 %. In vitro rooting was achieved with MS medium with half-strength nutrients and supplemented with indole-3-butyric acid (IBA) (1 μM) (Table 20.1). Périnet and Lalonde (1983) achieved 100 % rooting success, even in the absence of IBA, although the addition of 1 μM auxin to the rooting medium increased the number of roots per shoot and reduced the time required for 100 % rooting from 28 days for the auxin-free medium to 14–21 days for auxin-supplemented medium. Tremblay et al. (1986) confirmed that in vitro-propagated microshoots of *Alnus* species can be rapidly rooted, independently of the technique used (mist bed or in vitro), permitting rapid large-scale multiplication of alders.

Acclimatisation of the plants obtained in vitro was successful, independently of the type of substrate used. The growth of the in vitro-propagated plantlets under

greenhouse conditions was even faster than growth of plantlets of the same species obtained from seedlings (Tremblay et al. 1986).

20.2.1.2 Material of Adult Origin

Efficient methods are required for the clonal propagation of *A. glutinosa* that have been selected at a mature stage. This is because it is difficult to predict the characteristics of mature trees from juvenile plants and also because most individuals that might be selected for their tolerance/resistance to disease caused by *Phytophthora* are already adult trees. Despite the technological advances that have been made over the last few years, there are few reports on the propagation of mature *A. glutinosa* trees (Table 20.1). Lall et al. (2005) established a protocol for multiplying adult material of black alder, by using shoot tips taken from fruit-bearing branches of a sexually mature tree. Culture media for the introduction and shoot multiplication comprised WPM with BA (1–5 μM), and incorporation of 2,3,5-tri-iodobenzoic acid (2,3,5-TIBA) (0.1–3 μM), an auxin transport inhibitor, was required to minimise callus and root formation during the multiplication stage. These authors consider that the high auxin content of this species is an adaptation to its natural habitat, which suffers from frequent flooding. Naujoks et al. (2009) established in vitro clones from material of juvenile origin (from 6 months to 1 year old) and ten clones of adult material (about 18 years old) for studies of resistance to black alder decline. The multiplication medium (WPM) was supplemented with zeatin (Z), although the authors do not report the concentrations used or the results of establishment or in vitro multiplication trials. San José et al. (2013) successfully established three of the five genotypes selected, by using material from adult trees (20–30 years) selected for their size and because they did not show symptoms of the disease (Fig. 20.1a). The cuttings were forced to shoot in a phytotron, and the shoots that developed were used to establish the cultures (Fig. 20.1b). The best results for both establishment and multiplication were obtained by supplementing the WPM with 0.44 μM BA and 2.85 μM of indole-3-acetic acid (IAA). The authors indicate the need to carry out periodic transfer (every 3 weeks) of the shoots until the end of the subculture period of 9 weeks and that in the final subculture, 2.28 μM Z was added to the medium to favour shoot elongation (Fig. 20.1c). The results of this study showed that the performance of shoot cultures of mature *A. glutinosa* was affected by the type of sugar in the culture medium and that glucose (111 mM) was better than sucrose for inducing shoot development. Lall et al. (2005) also used glucose (167 mM) as a carbon source in the proliferation of axillary shoots established from an adult tree.

As with material of juvenile origin, shoots established from mature material rooted easily, even without auxin treatment. Lall et al. (2005) observed successful rooting of shoots in medium without auxin. IBA and NAA were used by Naujocks et al. (2009), although, in both studies, the authors did not provide the corresponding data. San José et al. (2013) achieved high rooting percentages (76.7 %) even with medium without auxin (Fig. 20.1d). However, inclusion of IBA (0.49 μM) in the medium for 2–3 days increased the rooting rate (90 %), number of roots per rooted shoots (from 1.9 to 3.0) and length of the roots (26.4–35.7 mm). In addition, in the shoots treated with auxin, 63 % of the roots developed lateral roots, whereas

Fig. 20.1 Micropropagation and conservation of *Alnus glutinosa*. (**a**) Black alder mature trees established in vitro. (**b**) Forced flushing of branch segments in the growth chamber. (**c**) Axillary shoots following 9 weeks of culture on proliferation medium. (**d**) Adventitious root development on shoots treated with 0.49 μM IBA. (**e**) Acclimatised plantlets of black alder after 6 months in the greenhouse. (**f**) Somatic embryos developed from the upper surface of a cotyledon excised from a zygotic embryo. (**g**) Secondary somatic embryos after 6 weeks on proliferation medium. (**h**) Shoots developing from successfully cryopreserved shoot tips. (**i**) Formation of a new somatic embryo from cryopreserved embryo clumps

in the control only 43 % of the roots developed lateral roots, which is important for the establishment of root architecture in higher plants. San José et al. (2012) carried out an anatomical study of the development of adventitious roots in black alder shoots grown in rooting medium, with or without IBA. These authors found that addition of auxin to the medium accelerated the formation of meristemoids and the appearance of roots. These were visible 5–6 days after the start of the treatment, whereas in the control, without IBA, root emergence was delayed until day 8.

Rooted plants were successfully transferred to ex vitro conditions, with survival rates of more than 90 % (Fig. 20.1e). In addition to comprising a safe germplasm bank that provides material for subsequent transformation studies aimed at

improving resistance of the trees to *P. alni*, the plants obtained in vitro can be transferred to their natural environment and be used to reforest areas devastated by the disease.

20.2.2 Adventitious Shoot Propagation

Formation of numerous adventitious buds was reported in nodal regions of black alder shoots in MS medium supplemented with 0.5–5 μM BA (Périnet and Lalonde 1983). The in vitro regeneration ability of black alder was subsequently studied by Bajji et al. (2013) with mature seeds and juvenile explants (hypocotyl segments, cotyledonary nodes and cotyledons) from 2-week-old in vitro seedlings. Mature seeds were cultured in modified WPM (WPMm) in the presence of different concentrations of thidiazuron (TDZ) (0–10 μM) for 2–4 weeks under light conditions. The explants derived from seedlings grown in vitro were cultured in the same medium (WPMm) supplemented with different concentrations of 2ip (0–50 μM) and BA (1–5 μM) for 2–6 weeks in the dark. For all explant types tested, the best responses in terms of regeneration frequency, and especially the number of buds/shoots per regenerated explant, were obtained when the explants were precultured for 2 weeks. When mature seeds were used as explants, addition of 1 μM TDZ induced the formation of more buds/shoots. When juvenile explants were used, cotyledon was the most responsive explant, and organogenesis was observed in all media and induction periods with a significant effect of BA compared to 2ip. All explants (100 %) produced adventitious buds after culture for 2 weeks in the dark in medium supplemented with 1–3 μM BA. Rooting and acclimatisation of these shoots were not considered in this study. As the authors note, the aim of these studies was to develop a protocol for regenerating adventitious shoots from mature explants (e.g. leaves or internodes) based on the procedures (in vitro selection or genetic transformation) used to improve the potential use of woody plants (especially black alder) for accumulating and extracting heavy metals from the banks of polluted watercourses.

20.2.3 Somatic Embryogenesis

Somatic embryogenesis is a powerful tool for improvement of forest trees as it is considered to be the most appropriate means of in vitro regeneration of woody plants (Vieitez et al. 2012). To date, only one study of somatic embryogenesis involving members of the genus *Alnus* has been published (Corredoira et al. 2013), despite the importance of the genus. These authors described the induction of somatic embryos from immature zygotic embryos of black alder. The study findings show that there is a short period during the development of the zygotic embryo when it is possible to establish embryogenic cultures. Somatic embryos were

initiated from zygotic embryos collected 1–3 weeks post-anthesis (WPA), i.e. when they were at globular or early cotyledonary stage and 0.5–1 mm in length. The induction frequency (16.6 %) and the mean number of somatic embryos (4.5 embryos/explant) were highest after culture of zygotic embryos, collected at 3 WPA, on MS medium supplemented with 0.9 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.22 μMBA. No embryos were induced on medium supplemented with only BA. Initial somatic embryos developed indirectly from callus tissue formed on the surface of the zygotic embryos (Fig. 20.1f). These results illustrate a feature widely seen in plant tissue culture, i.e. that the developmental/physiological status of the tissue used to initiate a culture may influence the subsequent potential of that culture. Embryogenic competence was maintained by secondary embryogenesis, which was affected by explant type, plant growth regulators and genotype. Repetitive embryogenesis occurred after culture of small groups of whole somatic embryos or isolated cotyledon explants on proliferation medium consisting of half-strength MS medium with 0.44 μM BA (Fig. 20.1g). Histological analysis of isolated cotyledon explants showed that secondary embryos generated directly from differentiated embryogenic cells formed on the surface layers of cotyledons. Somatic embryos were observed at successive stages of development, including cotyledonary-stage embryos with shoot and root meristems. For plantlet conversion, somatic embryos were transferred to maturation medium supplemented with 3 % maltose, followed by 6 weeks of culture in germination medium, consisting of WPM supplemented with 0.44 μM BA and 0.46 μM Z. Under these conditions, the plant conversion rate was 8 % with a germination rate of 20 %. This protocol appears promising for mass propagation, conservation and genetic transformation of black alder.

We have carried out initial experiments aimed at inducing somatic embryogenesis from leaves and apices of shoots obtained from selected adult trees and maintained in vitro. Murashige and Skoog medium supplemented with different concentrations of BA (2 μM), picloram (10–40 μM), NAA (5.4–21.6 μM) and 2,4-D (4.5–9 μM) was used, but so far the results have been unsuccessful.

20.3 Germplasm Conservation

The use of in vitro tools can yield additional backup collections and provide alternative means of propagation and conservation of species (Reed et al. 2011). However, micropropagation requires periodic transfer of cultures to fresh medium and the inclusion of plant growth regulators, as well as organic and inorganic components in the culture media. These requirements increase the cost of the conservation techniques, and more importantly, they increase the risk of somaclonal variation, and therefore the genetic fidelity of the stored germplasm is not ensured. These risks can be decreased using other unconventional conservation methods such as slow growth for the medium-term storage and cryopreservation for long-term storage (Ashmore 1997; Reed et al. 2011).

20.3.1 Medium-Term Storage

Reduced growth is one of the main techniques used for preservation of genetic diversity. The technique is routinely used for medium-term conservation of numerous species of both temperate and tropical origin, including crop plants such as potato, *Musa*, yam and cassava (Ashmore 1997; Engelmann 1999, 2011). The main advantages are the maintenance of disease-free cultures, reduced risk of genetic alterations, lower maintenance costs and the need for less space than with other techniques. The use of this technique lengthened the period of subculture considerably, without affecting either the viability of the explants or their capacity for regrowth on transfer to standard conditions (Lambardi and De Carlo 2003). Reduced growth can be achieved by decreasing the temperature and/or the light intensity, using growth retardants, omitting carbon sources or increasing the osmolarity of the medium (Engelmann 1997). Of these options, temperature reduction yields the best survival of the material and is therefore the most frequently used method, usually carried out under low lighting or in darkness (Engelmann 2011). These techniques are clearly useful due to their flexibility, simplicity and practicality, and they are the most direct way of restricting the growth and development of explants in vitro (Engelmann 1997; Turner et al. 2001).

20.3.1.1 In Vitro-Grown Shoot Tips

A simple method was developed for the medium-term storage of in vitro-grown shoot tips of *A. glutinosa* established from trees aged 20–30 years (San José et al. 2015a). In this study, shoot apex and nodal segments excised from 9-week-old in vitro-grown shoots, derived from three clones of adult material, were used as explants. Two pre-storage treatments were used: Day 0, when the explants were placed in cold cabinets at 2–4 °C, immediately after subculture, and day 10, when the explants were placed in cold cabinets 10 days after subculture. After cold storage for 3–24 months, the explants were removed from the cold cabinets and transferred to fresh proliferation medium under standard growth conditions. Under these conditions, a high percentage (75–87 %) of cultures remained viable after 18 months, with no significant differences as regards the type of explant or pre-storage period. The stored material was successfully recovered and multiplied, showing good growth and developed into normal shoots that were morphologically similar to those of non-stored controls. Cold storage is a safe, inexpensive method for conserving plant germplasm and considerably reduces the cost of micropropagation, which is high for alder, as in vitro cultures must be transferred to fresh medium every 3 weeks.

The histological study of these shoots, stored in the cold for a total of 24 months, showed that the main change was the accumulation of starch granules in cells of the shoot apex, as well as in cells located close to the vascular bundles, after 3 months. As the duration of cold storage increased, the number and size of the starch granules decreased, but cell plasmolysis and the content of lipid droplets increased. Cold damage to the shoots was widespread after 24 months at 4 °C.

20.3.2 Long-Term Storage

Advances in biotechnology have generated opportunities for the conservation of genetic resources, and the use and maintenance of plant materials at cryogenic temperatures (cryopreservation) now represent a suitable option for long-term storage. Cryopreservation allows the conservation of cells, tissues and organs derived from in vitro culture (such as shoot tips, callus cultures and somatic embryos) in liquid nitrogen (Reed 2008). Cryopreservation in liquid nitrogen (LN; −196 °C) is currently considered the most valuable method for the long-term preservation of biological material. The main advantages of cryopreservation are its simplicity and applicability to a wide range of genotypes (Pence 2014). Theoretically, plant material can be stored indefinitely at this low temperature without any changes being observed. In alder, cryopreservation has been used for the long-term conservation of orthodox seeds (Chmielarz 2010), shoot tips derived from in vitro culture of adult tree material (San José et al. 2014) and somatic embryos (San José et al. 2015b). The vitrification method has been used in the past few years. This involves the dehydration of samples with highly concentrated vitrification solutions followed by rapid cooling (Sakai 2000). Vitrification refers to the physical process of transition of an aqueous solution into an amorphous, glassy state during ultrarapid freezing. This is increasingly the method of choice for long-term preservation of plant tissues.

20.3.2.1 In Vitro-Grown Shoot Tips

An effective two-stage cryopreservation process has been developed for the long-term conservation of apices derived from in vitro culture of adult tree material (San José et al. 2014). Shoot tips (0.5–2 mm in length) excised from 3- to 9-week-old shoots were precultured in hormone-free WPM supplemented with 0.2–0.3 M sucrose for 2–3 days at 4 °C in the dark and then treated with a mixture of 2 M glycerol plus 0.4 M sucrose (loading solution (LS); Matsumoto et al. 1994), for 20 min at 25 °C. Osmoprotected shoot tips were first dehydrated with 50 % plant vitrification solution 2 (PVS2; 30 % glycerol, 15 % ethylene glycol, 15 % dimethyl sulphoxide and 0.4 M sucrose; Sakai et al. 1990), before being placed in 100 % PVS2 for 30–90 min at 0°Cor 25 °C. Shoot tips were plunged in liquid nitrogen (LN) for at least 2 h. The cryovials were removed from LN, rapidly rewarmed in a water bath, at 40 °C for 2 min, and washed twice, for 10 min at 25 °C, with 1.2 M sucrose solution, before being transferred to WPM supplemented with 2.22 μM BA, 2.85 μM IAA, 0.92 μM Z, 111 mM glucose and 6 g/l Difco Bacto agar. This protocol for the cryopreservation of in vitro-grown shoot tips was successfully applied to three alder genotypes derived from adult trees, with recovery rates higher than 50 % (Fig. 20.1h). The best results were obtained with 0.5–1 mm shoot tips excised from 6-week-old shoots precultured in hormone-free WPM for 2 days at 4 °C, before being treated with the loading solution and dehydrated with 50 % PVS2 solution for 30 min at 0 °C and 100 % PVS2 solution for another 30 min at 0 °C. This technique enables conservation of selected alder specimens, and therefore if trees disappear as a result of disease or natural disasters, identical copies could be used to replace sample specimens or for reforestation purposes.

20.3.2.2 Somatic Embryos

San José et al. (2015b) have developed a reproducible protocol for cryopreservation of black alder somatic embryos using a vitrification method. Somatic embryos clumps (1–2 mm) were precultured in hormone-free MS medium half-strength macronutrients, supplemented with 0.3 M sucrose for 3 days at 4°C or 25 °C and then treated with LS for 20 min at 25 °C. Osmoprotected somatic embryos were dehydrated using plant vitrification solution 2 (PVS2). The effect of incubating the cultures for different times with PVS2 (0–90 min) at 0°C or 25 °C was evaluated. The embryos were plunged directly in LN for at least 2 h. Following rapid thawing in a water bath at 40 °C for 2 min, the somatic embryos were transferred onto MS1/2 supplemented with 0.44 μM BA, 87.5 mM sucrose and 6 g/l vitro agar. The best results were obtained when the somatic embryos were precultured in medium with 0.3 M sucrose for 3 days at 25 °C before being dehydrated with PVS2 solution for 60 min at 0 °C. Recovery of vitrified somatic embryos was higher than 90 % in both embryogenic lines tested (Fig. 20.1i). In order to check the maintenance of the germination ability, somatic embryos recovered from cryopreservation were proliferated and matured before plantlets were obtained by germination. After 2 months on germination medium, plantlets with no morphological abnormalities were produced. Cryopreservation did not affect the plant regeneration potential of *A. glutinosa* through somatic embryogenesis. The ploidy stability of the regenerated material was assessed by flow cytometry. Analysis of DNA ploidy stability showed no significant differences between the control; PVS2-treated, cryopreserved somatic embryos; and the plantlets developed from cryopreserved somatic embryos.

These findings indicate the potential usefulness of long-term storage of alder embryo cultures, as well as the valuable transgenic cell lines in liquid nitrogen.

20.4 Conclusions and Future Prospects

Although the ecological value of alders is well known, these trees have not often been the focus of forest protection concerns (Cech 1998). However, the damage caused by *P. alni*, especially to *A. glutinosa*, has led to the need to develop new methods for the rapid multiplication of selected genotypes and for obtaining genetically improved material. During recent years, there has been increased interest in mass propagation of woody plants and the use of biotechnological approaches. Of the biotechnological methods available, micropropagation methods are becoming increasingly important for use in the genetic transformation and cryopreservation of desirable selected lines of black alder (Park 2002). The present chapter illustrates the various approaches used for micropropagation, adventitious shoot regeneration, somatic embryogenesis and in vitro conservation of black alder germplasm.

To date, successful axillary shoot proliferation has been developed in black alder, for use with juvenile material, and also selected mature trees. Shoot multiplication

culture appears to be the most promising method for immediate application, to accelerate progress in breeding and massive production of alders, in order to produce selected specimens that could be used to restore areas affected by disease.

Regeneration of plants via the formation of adventitious buds from mature seeds and juvenile explants has been successfully developed in black alder. This method can still be used to capture somaclonal variation, obtain chimeral modifications and apply selection and mutagenic pressures due to the adventitious nature of regeneration. Development of this technique with material of adult origin will be particularly useful for producing improved trees.

Somatic embryogenesis is an ideal regeneration system for genetic transformation. Regeneration of plants from genetically transformed cells is a key step in developing a protocol for the genetic transformation of black alder. In this context, a protocol has been developed for inducing somatic embryogenesis from immature zygotic embryos of alder. Somatic embryos of black alder survived and regenerated whole plants. However, further research is required to refine the process of somatic embryogenesis. Establishment of embryogenic cultures from explants derived from mature black alder trees is one of the major research goals that should be achieved in the near future. Combining genetic studies with somatic embryogenesis and genetic transformation is probably the best way of accelerating the improvement processes in this species.

Reduced growth and cryopreservation have been successfully applied to germplasm conservation in *A. glutinosa*, thus favouring the secure maintenance of the genotypes selected during field trials. In the past few years, considerable progress has been made in the area of cryopreservation of woody plant germplasm. Cryopreservation ensures safe, long-term conservation of genetic resources of plant species with recalcitrant seeds, of vegetatively propagated species and of biotechnologically produced material such as somatic embryos, cell lines and genetically transformed material. In vitro-grown shoot tips and somatic embryos have been shown to survive, and plants successfully regenerate after cryopreservation, without any phenotypic abnormalities. These are positive findings in relation to the conservation of black alder genetic resources.

Although transgenic technology is of enormous potential application in *A. glutinosa*, transgenic plants have not yet been developed. The genus *Alnus* is geographically, ecologically and taxonomically diverse, and the vast genetic variability could be taken advantage of in a genetic improvement programme. Conventional tree breeding programmes for genetic amelioration of alder involve selection of particular traits, hybridisation and propagation of selected genotypes (Tremblay et al. 1986). Biotechnological methods can facilitate these processes and rapidly yield improved plants. Future efforts should aim to transform the material by using genetic constructs that would confer resistance to pathogens such as *Phytophthora alni*.

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Isolated Microspore Culture and Its Applications in Plant Breeding and Genetics

21

Mehran E. Shariatpanahi and Behzad Ahmadi

Abstract

Isolated microspore culture (IMC) represents a unique system of single cell reprogramming in plants wherein a haploid male gametophyte, the microspore, switches its default gametophytic developmental pathway toward embryogenesis by specific stress treatment. The application of a stress treatment(s) is necessary for efficient embryogenesis induction. Depending on species, microspores are often induced by cold and heat shock, osmotic stress, starvation, anti-microtubular agents, stress hormones, antibiotics, or polyamines. This technique (IMC) is likely to remain as a well-known method in plant breeding since it allows for the rapid production of completely homozygous lines while, in the context of developmental biology, it allows for in vitro embryogenesis to be explored in greater detail. Isolated microspores also represent ideal recipients for several gene transfer techniques including electroporation, microprojectile bombardment, and *Agrobacterium*-mediated transformation. IMC is also extensively used for genetic studies, i.e., studying inheritance of quantitative traits, quantitative trait loci (QTL) mapping, and genomics and gene identification, for mutation and selection and also used for producing reversible male-sterile lines. Male sterility avoids the labor costs of manual emasculation and serves as a molecular strategy for transgene containment by preventing pollen release to the environment. Combination of this technique with doubled haploid (DH) production leads to an innovative environmentally friendly breeding technology. In addition, the usefulness of DHs for reverse breeding program, an applied plant breeding technique introduced to directly produce parental lines for any hybrid plant, is also generally discussed.

M.E. Shariatpanahi (✉) • B. Ahmadi
Agricultural Biotechnology Research Institute of Iran (ABRII), Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran
e-mail: mehran.shariatpanahi@abrii.ac.ir

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Doubled haploid • Microspore embryogenesis • Stress • Breeding • Genetics

21.1 Introduction

Haploid is a general term describing sporophytes with single set of parental chromosomes. Haploid and DH plant production technique offers an excellent system to speed up plant breeding programs, genetic analysis, physiological studies, gene transformation, QTL mapping, and reverse breeding programs (Liu et al. 2005; Wijnker et al. 2012; Brew-Appiah et al. 2013; Ilyas et al. 2014).

Haploids occur rarely in nature and therefore of limited practical value. To be useful, they must be produced in large numbers. Haploids/DHs are mainly produced via: (1) parthenogenesis, (2) wide hybridization followed by chromosome elimination, and (3) androgenesis/gynogenesis (Asif 2013; Mishra and Goswami 2014). Parthenogenesis is a type of asexual reproduction wherein haploid plants develop from unfertilized egg cells (Bohanec 2009). Elimination of one parental genome after fertilizing the ovule by pollen of another species can occur in intraspecific, interspecific, intergeneric, or more distant hybrids leading to haploid plant formation of only one parent (Dunwell 2010). The DH production method used in barley (*Hordeum vulgare* L.) is a typical example of selective chromosome elimination in the cross between *H. vulgare* and diploid *H. bulbosum*, so that the chromosomes of *H. bulbosum* will be finally eliminated (Jauhar 2003). Gynogenesis, haploid regeneration from un-pollinated female gametophytes, is another method for haploid production that has been used in many agronomically important species such as sugar beet and onion (Portemer et al. 2015). Nowadays androgenesis, regeneration of haploid or DH plants through the anther or isolated microspore culture, has attracted the interests of many researchers and become the method of choice for haploid production in a wide variety of species.

Microspores, immature pollen grains, are produced within the anthers (microsporangia or pollen sacs) of the flower. Microsporogenesis, the process of formation of haploid unicellular microspores, commences with dividing microsporocytes (pollen mother cells or meiocytes) by meiosis into the four haploid microspores. Under normal conditions (in vivo), microspores are programmed to develop into the mature pollen grains in a process called "microgametogenesis" (Ma 2005). However, by manipulating the environment of the gametic cells under specific in vitro conditions, it is possible to divert the default developmental pathway toward embryogenesis giving rise to haploid plants rather than mature pollen grains (Shariatpanahi et al. 2006).

The first successful regeneration of haploid plants through the in vitro cultured anthers was reported by Guha and Maheshwari (1964) in *Datura innoxia*. Soon later, androgenic haploid plants were achieved by Bourgin and Nitsch (1967) from cultured anthers of *Nicotiana sylvestris* and *N. tabacum*. During the past four decades, many improved methods have been developed to increase the efficiency of

haploid production from cultured anthers and isolated microspores in various species (Ferrie and Caswell 2011).

DH production through the IMC technique drastically reduces the time period needed to develop new cultivars (Germana 2006). It generally takes 10–15 years to release a new cultivar through the conventional breeding programs, i.e., the pedigree or bulk methods in self-pollinating crops, which includes selfing and subsequent selection. On the other hand, regeneration of haploid plants followed by chromosome doubling and selection of superior lines reduces the time frame by 3–4 years. In cross-pollinating heterozygous crops, which often express a high degree of self-incompatibility and inbreeding depression, IMC is a rapid method to produce homozygous lines that can further be used in the development of synthetic varieties or hybrids (Immonen and Anttila 1996). Because microspore-derived DH plants are genetically fixed and stable (true breeding lines), they can be replicated in trials allowing better estimates of within and between line variation and also improve the selection efficiency (Collard et al. 2005). In addition, the phenotype of DH plants is not masked by dominance effects, and traits encoded by recessive genes can be easily identified (Szarejko and Forster 2007; Ferrie and Möllers 2010). IMC also provides tremendous potential for studying quantitative inheritance. By using F2-derived DH population, genetic parameters and number of segregating genes can be estimated. Also, a smaller population of DH plants is needed for screening desirable recombinants than would be the case for conventional diploid populations (Martinez et al. 2005; Hussain et al. 2012). DH populations are available for DNA extraction and mapping 1.5 years after the initial crosses, i.e., almost as quick as an F2 or BC1 population and definitely much faster than a pedigree inbred or single seed descent population. DHs can be re-grown and distributed in seed form so that it is comfortable to screen with many markers. Map construction from a DH population derived from the F1 of a cross is relatively simple because the expected segregation is that of a backcross (Forster and Thomas 2003). DH populations are of favor in the QTL identification as multisite replicated trials can be grown 3 years after the first cross (Forster and Thomas 2003; Collard et al. 2005).

In this chapter, common stresses used for microspore embryogenesis induction are generally discussed. Then, the latest progresses in the induction and regulation of microspore embryogenesis and also its applications in plant breeding programs and genetic studies have been argued in a greater detail.

21.2 Induction of Microspore Embryogenesis

Application of a stress(s) is necessary to induce microspore embryogenesis. The stresses required to switch the default developmental pathway of microspores toward embryogenesis vary with the species and even within a species and among its genotypes or varieties also. Nevertheless, several inducing factors have been identified that positively influence the efficiency of microspore embryogenesis in diverse species (reviewed by Shariatpanahi et al. 2006). Heat shock is usually conducted at 30–37 °C for a varying duration from few hours up to several days,

whereas cold treatment is carried out at 4–10 °C from few days up to several weeks. It has been indicated that heat treatment significantly affects the expression of Rho-GTPases (ROP) in isolated microspores of *B. napus* which is involved in the cytoskeletal rearrangement (Chan and Pauls 2007). Heat shock influences microtubule distribution, leading to more symmetrical division in microspores, and blocks further gametophytic development (Dubas et al. 2011). Heat shock not only induces microspore embryogenesis but also accelerates the process of embryo formation (Ahmadi et al. 2012b). Heat/cold treatment also affects auxin distribution and therefore the fate of embryogenesis either via or without suspensor formation (Prem et al. 2012a; Dubas et al. 2014). Auxin mainly localizes in a polar way already in the uninucleate microspores subjected to the mild (1 day at 32 °C) or without (18 °C) heat treatment, which is essential for initiation of suspensor-bearing MDEs. While microspores are treated to a prolonged heat (32 °C for 5 days), auxin polarization arranges at a suspensor-free few-celled embryos (Dubas et al. 2011, 2014). Culture of isolated microspores in the induction medium containing non-metabolizable carbon sources, e.g., in mannitol or polyethylene glycol, has also proved to be useful in many species (Muñoz-Amatriaín et al. 2006), hot pepper (Kim et al. 2008), eggplant (Bal et al. 2009), wheat (Ayed et al. 2010), and oil palm (Indrianto et al. 2014). Transcriptome analysis of barley microspores revealed that 4-day mannitol treatment substantially affected the expression of 2673 genes. Upregulation of transcription factors related to stress responses and changes in developmental pathway of treated microspores toward embryogenesis took place during the pretreatment (Muñoz-Amatriaín et al. 2006). In addition, colchicine, a microtubule-depolymerizing agent, has been used as a stress pretreatment for embryogenesis induction in various species (Soriano et al. 2007; Klima et al. 2008; Dubas et al. 2010; Islam 2010; Li et al. 2012). Application of anti-microtubular agents, e.g., colchicine, cytochalasin D, trifluralin, or oryzalin, leads to cytoskeletal rearrangement which plays a key role in determining the developmental fate, since the disruption of these networks triggers or is sufficient to induce gametic embryogenesis in the absence of a stress treatment (Soriano et al. 2013). Recently, several novel inducers have been reported to switch gametophytic developmental pathway of microspores toward embryogenesis which are discussed below.

21.2.1 Antibiotics

Microbial contamination is a serious and often unavoidable problem in the field of microspore culture. Contaminations introduced to in vitro cultures can be exogenous or endogenous originating from explants' surface, intracellular spaces within the plant tissues, or poor aseptic conditions during manipulation in laminar flow hoods. Endogenous bacterial contamination has long been considered as one of the major bottlenecks in androgenesis induction in vegetables such as *Capsicum annuum* L. which drastically losses the number of isolated cultures (Lantos et al. 2012; Asif et al. 2013b). Such contaminations may be substantially reduced or eliminated during the in vitro stages of plant tissue culture using antimicrobial treatments such

as antibiotics. In the *Agrobacterium*-mediated gene transformation, it is also essential to use antibiotic for eliminating the remained *A. tumefaciens* cells after cocultivation. Despite their common and successful application to minimize bacterial growth in the cell and tissue cultures, antibiotics are shown to retard/inhibit or stimulate explant growth and development. Their role(s) in affecting the developmental events is not well understood, but it has been assumed that the antibiotics mimic plant hormones since some of them possess an auxin-like activity (Qin et al. 2011). The number of MDE formation from wheat isolated microspores decreased with increasing level of Timentin in the induction medium (Brew-Appiah et al. 2013). 100–400 mg l^{-1} Timentin was recognized as the optimal concentration so that a reasonable number of MDEs were produced while their regeneration efficiency was retained. Optimal antibiotic agent, on the one hand, should provide reliable defense for bacterial infections and, on the other hand, keep microspores intact and viable. Reported by Asif et al. (2013b), 24 bacterial isolates were strongly inhibited using vancomycin and cefotaxime (both 100 mg l^{-1}) in microspore culture of triticale and wheat. In addition, microspore embryogenesis induction medium fortified with the same antibiotic treatments resulted in increased embryo-like structure (ELS) formation, green and albino plant production in triticale. In wheat, significant genotype-dependant effect of cefotaxime was noticed for ELS, green and albino plant regeneration. Vancomycin, on the other hand, was not advantageous to MDE formation so that it significantly reduced ELS number and green plant regeneration in all wheat genotypes tested (Asif et al. 2013b). Exogenously applied cefotaxime (50 mg l^{-1}) and vancomycin (100 mg l^{-1}) for 24 h and 48 h, respectively, profoundly enhanced microspore embryogenesis induction in *B. napus*. Higher levels and durations were detrimental so that normal plant regeneration substantially decreased and resulted in callusing (Ahmadi et al. 2014).

21.2.2 Antioxidants

Isolation and stress treatment of microspores cause oxidative damage to the cells in the form of lipid peroxidation, protein oxidation, nucleic acid damage, and appearance of apoptotic-like bodies, all eventually lead to cell death and thus decrease in viability of cultured microspores (Żur et al. 2009; Rodríguez-Serrano et al. 2011; Sharma et al. 2012). Despite having a quick harmful effect on microspore viability, the increase in reactive oxygen species (ROS) production has been reported to be favorable to microspore embryogenesis induction if firmly adjusted (Hoseini et al. 2014; Żur et al. 2014) so that treatment of barley (*H. vulgare* L.) microspores with ROS scavengers resulted in decreased cell death during early stages, but later reduced the total number of MDEs produced (Rodríguez-Serrano et al. 2011). In plant cells, the precise equilibrium between ROS generation and decomposition depends on the activity of enzymatic and nonenzymatic components of the antioxidative system. Major ROS-scavenging enzymes include superoxide dismutase (SOD), peroxidase (POX), and catalase (CAT), whereas ascorbic acid, glutathione, proline, α-tocopherol, carotenoids, and flavonoids belong to the most important

nonenzymatic antioxidants (Gill and Tuteja 2010). Reported by Żur et al. (2009), induction medium, as the major inducer of microspore embryogenesis in triticale, had a temperature-dependant manner effect on the activity of antioxidative enzymes, i.e., SOD, POX, and CAT, so that the activity of all enzymes under low-temperature (5 °C) regime was similar as in the control subjected only with the stress connected with the establishment of in vitro culture. By contrast, the type of induction medium exhibited drastic effect on the activity of all abovementioned enzymes when cultures were incubated at higher-temperature (32 °C) regime (Żur et al. 2009). Low-temperature pretreatment (3 weeks at 4 °C) of freshly excised triticale tillers significantly increased the activity of enzymatic antioxidants in the anthers of responsive DH lines when compared with the recalcitrant ones (Żur et al. 2014). Principal component analysis (PCA) revealed that the activity of POX, CAT, and SOD was positively correlated with the efficiency of embryogenesis (Żur et al. 2014). Antioxidative enzymes play important roles in microspore embryogenesis induction which cannot be replaced even by highly active nonenzymatic antioxidants (Żur et al. 2014). The ability to sustain antioxidative enzyme activity under inductive stresses is also an important factor needed for highly effective microspore embryogenesis induction, allowing for the generation of signals initiating microspore reprogramming and simultaneously protecting the cells from further toxic effects of ROS accumulation (Żur et al. 2014). Plastids and mitochondria are the major target/production sites of ROS during abiotic stresses. Highly increased levels of ROS in plastids not only decrease the viability of microspores but also are correlated with albinism, as albino plants are deprived of chlorophyll (Asif et al. 2013a). Exogenously applied mitochondrial or plastid antioxidants, e.g., glutathione in the induction medium, significantly increased embryogenesis and green plant regeneration in contrast to the untreated cultures in wheat and triticale (Asif et al. 2013a). Glutathione and ascorbate are nonenzymatic antioxidants that detoxify and scavenge free radicals and regulate the redox cellular state. Synergistic effect of ascorbate and glutathione has been observed in microspore embryogenesis of *B. napus* in a dose-dependent manner. Microspore embryogenesis was enhanced using 10 mg l^{-1} glutathione in combination with 5 or 10 mg l^{-1} ascorbic acid. However, abnormal embryos were observed when glutathione level was increased by 100 mg l^{-1} (Hoseini et al. 2014). Ascorbate and glutathione are also involved in the plantlet regeneration from MDEs. Buthionine sulfoximine (BSO) treatment which affects glutathione and ascorbate metabolism produced lower ascorbate levels in the regenerated MDEs and activated expression of meristem-specific genes including *ZWILLE*, *SHOOTMERISTEMLESS*, and *ARGONAUTE 1* (Stasolla et al. 2008). Expression of these genes is required for shoot meristem formation and correct embryo patterning via activating phytohormones, e.g., cytokine biosynthesis (Scofield et al. 2013). The enhancing effect of α-tocopherol as intercellular and intracellular physiological antioxidant which maintains homeostasis of labile metabolites in the cells was also reported in isolated microspore culture of *B. napus* (Hoseini et al. 2014). However, the effect of α-tocopherol on microspore embryogenesis follows a temperature-dependant manner so that it was more effective on microspore embryogenesis at 30 °C in comparison with those cultures treated at 32.5 °C.

21.2.3 Phytohormones

Phytohormones also known as "plant regulators" including auxins, cytokinins, gibberellic acid, brassinosteroids, ethylene, abscisic acid, jasmonic acid, and salicylic acid are chemical messengers that coordinate cellular activities, i.e., cell division and differentiation. Last decade has witnessed astonishing progresses in the induction and regulation of MDE formation using these phytohormones. Current achievements in this area are discussed below.

21.2.3.1 Auxins

Auxins have cardinal roles in coordination of many growth and behavioral processes and are essential for plant body development. Auxins also have an outstanding role(s) during the somatic/gametic and zygotic embryogenesis. HPLC analysis of endogenous auxin level revealed that auxin content was increased about 100-fold in microspore-derived proembryos when compared with the auxin content detected at the single cell level (Dubas et al. 2014; Rodríguez-Sanz et al. 2014). Such high auxin levels could characterize dividing cells being stimulated to grow and divide. Differential distribution of the plant hormone auxin is also important and prerequisite for normal MDE formation. Using the reporter β-glucuronidase (*GUS*) and the green fluorescent protein (GFP) markers under control of synthetic auxin-responsive DR5 or DR5rev promoters, Dubas et al. (2014) observed polar auxin distribution after exine rupture, which lasted at the late globular stage under the prolonged heat stress condition. This polarity is not only the effect of heat stress but also reflects genome-regulated pattern of *B. napus* MDE development. The apical-basal embryogenic axe formation can be probably marked by such polar pattern of reporter gene activity (Dubas et al. 2014). Treatments with the inhibitor of the polar auxin transport 1-N-naphthylphthalamic acid (NPA) or high doses of antiauxin *p*-chlorophenoxyisobutyric acid (PCIB, chemical compound that competes with auxin for binding sites) affected embryogenesis initiation and further MDE development in contrast to untreated cultures. Neither new MDEs nor development of embryo-like structures were detected in the treated cultures, whereas in the embryogenic cultures without NPA and PCIB, embryogenesis was normally initiated and progressed (Ahmadi et al. 2012a; Rodríguez-Sanz et al. 2014).

MDEs are mainly formed via two major routes: a zygotic-like pathway characterized by initial suspensor formation followed by embryo proper formation and the second route characterized by initially unorganized embryos lacking a suspensor (Dubas et al. 2014; Soriano et al. 2013). The pattern of embryogenic development is highly based on the intensity and distribution of the plant hormone auxins during the embryogenesis process (Dubas et al. 2014). Polar auxin transport is needed for embryo proper specification from the suspensor in the zygotic-like pathway. Tracking this pathway revealed higher auxin concentration in one pole of initially inducted uninucleate microspores. After asymmetric transverse division, higher auxin activity was noted in the basal cell, recognized as the place of auxin biosynthesis from which the hormone is transported to the apical cell. After a series of basal cell transverse divisions and suspensor formation, directional auxin flow takes

place from the suspensor cells to apical cells. As the proembryo progresses, auxin mainly accumulates at the places where the cotyledon primordium and provascular strands began to form. On the contrary, in the suspensor cells except for the region of the hypophysis, auxin activity decreases. In the suspensor-lacking pathway, on the other hand, no auxin polarity was observed in uninucleate microspores and after symmetrical divisions. When a multicellular structure was released from the exine, auxin was observed at the only one pole. This polar auxin distribution lasted up to the late dermatogen stage. Such polar pattern of auxin distribution probably marks the apical-basal embryogenic axe formation (Dubas et al. 2014).

21.2.3.2 Stress Hormones

Microspores under certain stress conditions in vitro can switch their developmental programmed pathway toward embryogenesis. Stress hormones are produced by plants in response to a number of environmental stresses, including some that are used to induce androgenesis. Applying low-temperature pretreatment to excised inflorescences of triticale led to intensive ABA accumulation in comparison with untreated tillers (Żur et al. 2012).

However, there has been no correlation between ABA concentration and androgenesis induction efficiency, whereas negative correlation was seen between regeneration ability and concentration of ABA collected from cold-treated tillers. Monitoring changes in plasma membrane fluidity and ABA content associated with embryogenesis induction in *B. napus* microspores revealed that plasma membrane remodeling followed by inductive stress (heat shock at 32 °C for 24 h) is coincided with changes in ABA content in both microspores and induction medium (Dubas et al. 2013). Heat shock causes microspores' plasma membrane more rigid and increases ABA level in the induced microspores. However, heat treatment also increased ABA content in microspores of nonresponsive cultivars which had no clear-cut impact on androgenesis induction efficiency, suggesting a more complex mechanism of initiation process. On the contrary, promotive effects of stress hormones, i.e., ABA, salicylic acid, and jasmonic acid, were observed when exogenously applied to the induction medium (Ahmadi et al. 2014). ABA at 0.5 mg l^{-1} for 12 h enhanced microspore embryogenesis by about threefold compared with untreated cultures. High embryogenesis frequency was also observed when cultures were exposed to 1.0 mg l^{-1} jasmonic acid for 12 h. However, microspore embryogenesis and plantlet regeneration significantly decreased as jasmonic acid level was increased. SA treatment (0.2 and 0.5 mg l^{-1}) during the 6 h exposure also caused a profound increase in the number of embryos generated relative to untreated cultures, but treatment at longer durations decreased MDE formation, so that 2.0 and 5.0 mg l^{-1} SA for 12 and 24 h completely inhibited embryogenesis induction. Nevertheless, initial sporophytic divisions were observed in the treated microspore at those toxic levels, but all failed to proceed further into the fully developed MDEs (Ahmadi et al. 2014).

21.3 Applications of Microspore Embryogenesis

21.3.1 Gene Transformation

Microspores contain gametic number (n) of chromosomes. Both the haploid chromosome number and embryogenic capability exhibit microspores, one of the most interesting cells to be stably transformed. Based on the developmental stage of microspores, transforming procedures can be classified into two major categories, gametophytic and sporophytic (Resch and Touraev 2011). The gametophytic route includes (i) transforming mature pollen wherein a recombinant DNA is incorporated into the pollen genome before pollination or introduced to stigma before/after pollination and (ii) transformations based on microspore maturation where the foreign DNA is delivered into the microspores, cultured and matured under in vitro condition, and then used for pollination to achieve transformants (also known as male germ line transformation). The sporophytic route is based on transforming embryogenic microspores, wherein the default gametophytic fate of isolated microspores is shifted toward sporophytic pathway to produce MDEs instead of mature pollen grains (Brew-Appiah et al. 2013). Once transgenes are incorporated into the haploid genome of targeted cells prior to S phase (DNA synthesis) and genome doubling, the DHs may become completely homozygous for the transgenes. Thus, IMC not only provides an ideal system for gene transformation but also is amenable for transgene to be readily selected under in vitro conditions. Isolated microspores also provide an excellent system to avoid hemizygousity. On the contrary, identification of homozygous transgene(s) derived from somatic (diploid) targets requires the assessment of two additional segregating generations bred from hemizygous transformants (Hansel et al. 2012). Nowadays, many researchers have recognized the great benefit(s) that a functional microspore transformation system would offer and tried to transform microspores in diverse species using eletroporation, microprojectile bombardment, and *Agrobacterium tumefaciens*-mediated methods demonstrating stable integration of DNA.

Electroporation has been reported to be an efficient tool for foreign DNA delivery into the plant cells. The first report of electroporation-mediated delivery of DNA to the microspores arose from detection of *chloramphenicol acetyltransferase* expression in maize microspores by Fennell and Hauptmann (1992). Soon after, the delivery of *β-glucuronidase* (*GUS*) to the microspores of *B. napus* (Jardinaut et al. 1993) and maize (Jardinaut et al. 1995) was reported. Obert et al. (2004) conducted an experiment to optimize the electroporation medium, concentration of donor DNA, voltage, and pulse duration in order to gain a reproducible protocol for maize microspores. Taken together, they found that the highest amount of *GUS*-expressing microspores was achieved when a combination of 200 mg ml^{-1} DNA, three pulses with frequency of 0.1 Hz, and field 400 V/cm during 20 ms was applied (Obert et al. 2004).

Ingram et al. (1999) transformed MDEs obtained from in vitro cultured anthers of wheat using microprojectile bombardment. They found that paper supports during the bombardment significantly improve the efficiency of transformation so that

bombardment of MDEs on semiliquid regeneration medium without a filter paper on the surface of the medium led to a 45 % reduction in the mean number of GUS foci/MDE at 1-day post-bombardment. The presence of semiliquid medium over the MDEs could act as a protective film, with its surface tension reducing the impact of gold particles (Ingram et al. 1999). Type and concentration of carbohydrate in the culture medium also affect the expression of transgene. Significantly longer *GUS* expression in the transformed MDEs was noted in the presence of high amounts of maltose (0.4 M) in the culture medium. High osmoticum medium is thought to protect tissues during bombardment by reducing cell turgor, causing plasmolysis. This leads to reduced leakage of cell contents following bombardment. High osmoticum medium may also induce membrane changes, leading to increased cell tolerance to microprojectile impact (Clapham et al. 1995; Ingram et al. 1999). Transgenic plants could be produced by the direct delivery of plasmid DNA into the isolated microspores using high-velocity microprojectiles (Yao et al. 1997; Nehlin et al. 2000). Higher rate of transformation was observed when 48 h incubated microspores were used as the targets. Size of gold particles is another determining factor which severely affects the efficiency of transformation. Bombardment of incubated microspores with particle sizes of 1.5–3.0 μm resulted in a four- to sixfold increase in *GUS* expression compared with particles of 1.0 μm (Nehlin et al. 2000). Drastic decline in viability of microspores was observed within the first week of culture which could adversely affect the efficiency of process. Renovation of culture medium 24 h, 48 h, or 72 h after initial culture showed beneficial effects on microspore viability (Nehlin et al. 2000). Working on isolated microspores of wheat, Mentewab et al. (1999) and Folling and Olesen (2001) observed transient expression of marker genes, but transgenic wheat plants were not obtained, probably due to the inefficient microspore regeneration protocol in the experiments. Less than 100 MDEs were produced from isolated microspores of several spikes in the controls. The efficiency is likely to be further reduced with the introduction of genes by microprojectile bombardment since microspores are very sensitive to physical damage. The success in microspore transformation fairly relies on the effectiveness of microspore regeneration protocol. Thus, a highly efficient microspore culture system is required as a basis for transformation experiments.

Agrobacterium-mediated transformation is in general a much more efficient way of introducing foreign genes into some species, e.g., *Brassicas*, than microprojectile bombardment or other techniques (Takahata et al. 2005). A comparison between *Agrobacterium*- and biolistic-based transformation methods in *H. vulgare* highlighted some of the advantages of the *Agrobacterium* system such as higher efficiency of transformation, lower copy number of transgene(s), and more stable inheritance with fewer rates of the transgene(s) silencing (Travella et al. 2005). Being potentially useful recipient of foreign genes, Cegielska-Taras et al. (2008) transformed *B. napus* MDEs using *A. tumefaciens* strains EHA105 and LBA4404, both carrying the binary vector pKGIB containing the *uidA* gene and the *bar* gene for resistance to phosphinothricin as a selectable marker. Transformed MDEs expressed *GUS* and regenerated plants exhibited resistance to herbicide Basta. Since the physical barrier imposed by the thick microspore wall constitutes an obstacle for

efficient transforming of isolated microspores, Abdollahi et al. (2009) implemented additional methods to overcome this drawback. They obtained the best result through the co-transformation by microspore bombardment with DNA-coated microprojectile particles, followed by *A. tumefaciens* infection, a process called "agrolistic." The "agrolistic" method combines the benefits of the *Agrobacterium* transformation system with that of the biolistic DNA delivery method (Ziemienowicz et al. 2012). Although profound progresses in gene transformation methods based on IMC technique have been made in the last decades, there is still demand for further investment and improvement in both model and crop plant species.

Nevertheless microspore transformation is widely used to avoid hemizygousity; surprisingly this system has confronted with the regeneration of hemizygous transgenics in some cases (Shim et al. 2009; Brew-Appiah et al. 2013). This dilemma appears when microspores targeted to transformation have progressed into the G2 stage or going into mitosis becoming binucleate (Shim et al. 2009). The type of microspore pretreatment has a great influence of rate of hemizygous regeneration. Mannitol with cold treatment for 4 days and mannitol treatment for 7 days at 28 °C followed by biolistic bombardment led to a few hemizygous transgenic plants in barley. Conversely, a cold pretreatment for 28 days, which slows but does not arrest the cell cycle, led to a few homozygous transgenics using biolistic bombardment. Reported by Shim et al. (2009), microspores in cold plus mannitol pretreatment for 4 days were held in the G1 and S cell cycle stages during pretreatment, while in the cold pretreatment for 28 days, they progressed slowly into the G2 stage or some going into the mitosis. It has been hypothesized that inducing a transgene(s) at the G1 or early prior to S phase, following pretreatment, should produce homozygous transformants, whereas introduction at the G2 phase should lead to regeneration of hemizygous transgenic plants (Shim et al. 2009).

21.3.2 Induced Mutation and Selection

Plant breeding programs are based on creating genetic variation, selection, evaluation, and multiplication of desired genotypes. Microspore culture provides an ideal method for creating variation via induced mutation (Seyis et al. 2014). Any DNA alteration imposed to haploid cells would be homozygous in the DH plants facilitating the identification of recessive mutants. On the other hand, recessive mutations with deleterious effects cannot be recovered using this method (Ferrie and Möllers 2010). The new genetic variability from cultured haploid cells will be increased by the application of physical, chemical mutagens, T-DNA insertion mutagenesis (Jung and An 2013), AC/DS transposon mutagenesis (Wang et al. 2013), and site-directed mutation using transcription activator-like effector nucleases (TALENs, Gurushidze et al. 2014). Induced mutation and selection has been extensively used to improve yield, quality, disease, and pest resistance in crops such as *Brassicas*, wheat, rice, barley, cotton, peanuts, and beans, which are seed propagated (Barro et al. 2002; Seyis et al. 2014). Many selected mutants have been released as cultivars; several others have been used as parents in the basic breeding programs, i.e.,

pedigree of some of the leading cultivars. The release of high-yielding and short-height barley mutants as varieties "Diamant" and "Golden Promise" have had a major impact on the brewing industry in Europe. These mutants have been used as the parents of many leading barely cultivars released in Europe (reviewed by Ahloowalia and Maluszynski 2001).

Availability of refined protocols for MDE production and manipulation of haploid tissues in *Brassicas* has attracted the attention of breeders to induce mutation and in vitro selection in this responsive species. Thanks to rapid generation of large MDE haploid populations, IMC has become a useful tool for mutation and selection. Polsoni et al. (1987) accomplished a successful rapid isolation of oilseed rape (*B. napus* L.) microspores by mechanical homogenization (maceration) of whole fluorescence with floral buds no longer than 4.5 mm, also designated as large-scale microspore culture. With this method, millions of microspores can be isolated in each trial, resulting in a final yield of several hundred MDEs within 1 month, so they outlined the efficiency of this technique for induced mutation and in vitro screening (Polsoni et al. 1987). Soon later, Swanson et al. (1988) selected mutant DH lines which were tolerant to chlorsulfuron herbicide about 10–1000 times more than the corresponding plants. Imidazolinone-tolerant canola DH plants were also obtained with five to ten times improved tolerance to the field-recommended rates of Pursuit and Scepter (Swanson et al. 1989). Ultraviolet (UV) light has been widely used as a physical mutagenic agent. DH plants of *B. napus* regenerated from microspores exposed to UV irradiation exhibited changes in the level of resistance to herbicides, pathogens, abiotic stresses, and saturated fatty acid profile. Irradiation of *B. napus*-isolated microspores led to regeneration of DH plants exhibiting increased resistance to the chlorsulfuron and *Alternaria brassicicola*, suggesting the generation of novel heritable resistance to this herbicide and pathogen (Ahmad et al. 1991). Also, *B. campestris* DH plants resistant to soft rot disease were produced by UV irradiation (Feng-Ian and Takahata 1999). EMS, as a chemical mutagen, has also been utilized to construct mutant library. From a population of nearly 400 DH plants derived following EMS treatment, nine lines were identified that exhibited promising useful changes in erucic acid content in the seed oil (Barro et al. 2001). Isolated microspores of *B. napus* were exposed to EMS mutagen and then screened on the culture medium supplemented with oxalic acid as a selection agent of *Sclerotinia sclerotiorum* (Liu et al. 2005). Of the 54 DH lines produced, two DH lines of resistant mutants exhibited greater resistance when compared with donor lines and the resistant control. In addition, induced mutation followed by in vitro selection in the media containing trans-4-hydroxy-l-proline as a selecting agent resulted in high cold tolerance in the regenerated DH plants of *B. napus* (McClinchey and Kott 2008; Janska et al. 2010). Furthermore, EMS treatment efficiently affected the fatty acid profile of regenerated mutant DH plants (Ferrie et al. 2008). Fatty acid analysis of *B. napus* DH lines indicated a range of 5.0–7.7 % in saturated fatty acid proportions. In *B. juncea*, saturate proportions ranged from 5.4 to 9.5 %, and of the 7000 *B. rapa* lines that were analyzed, 197 lines exhibited raised oleic acid (>55 %), 69 lines showed reduced linolenic acid content (<8 %), and 157 lines had low saturated fatty acid proportions (<5 %), when compared with the parental lines (Ferrie et al.

2008). Male-sterile DH lines with cytoplasmic inheritance or dominant genetic male sterility were obtained using ^{60}Co γ-ray irradiation which provides valuable resources for developing male sterility in *Brassicas* (Huang et al. 2014). Instead of treating isolated microspores, Prem et al. (2012b) subjected germinating seeds to EMS and ENU in order to obtain mutant donor plants. Irrespective of genotype, isolated microspores cultured from ENU-treated donor plants produced no MDE. In addition, the response of mutant plants derived from EMS mutagen was about 100 times lower than nontreated donor plants. They concluded that instead of having detrimental effects on efficiency of MDE production, valuable mutants can be recovered from mutated donor plants (Prem et al. 2012b). All together, chemical mutagens have several advantages over physical ones. For instance, the rate of mutagenesis is much higher and the variety of point mutations produced is vast, but majority of mutants created by chemical mutagenesis have indicated to exhibit adverse agronomic traits (Han et al. 2007; Xu et al. 2010).

21.3.3 Quantitative Trait Loci (QTL) Mapping and Marker-Assisted Selection (MAS)

DH populations suggest tremendous material for constructing genetic linkage maps and QTL analyses. The major advantage of DHs over other commonly used populations such as F2 and backcross is that they produce completely homozygous lines which can be easily multiplied without any genetic alteration occurring and thus facilitates conducting replicated trials across different locations and years for various agronomic traits (Collard et al. 2005). In addition, DH populations allow for assignment of components of environmental variation, which can be valuable for analysis of physiological effects (Pink et al. 2008). To date, the QTLs responsible for many important agronomic traits including seed yield and oil content (Lionneton et al. 2002; Chen et al. 2010; Cloutier et al. 2011; Yang et al. 2012), glucosinolate accumulation (Lou et al. 2008), chlorophyll content (Ge et al. 2012), bolting time (Ajisaka et al. 2001; Nishioka et al. 2005; Yang et al. 2007; Chen et al. 2010), and resistance to clubroot (Suwabe et al. 2003), blackleg (Yu et al. 2005, Yu et al. 2008), *Verticillium longisporum* (Rygulla et al. 2008), downy mildew (Yu et al. 2009), *Sclerotinia* stem rot (Wu et al. 2013), and Turnip mosaic virus (Zhang et al. 2008; Jin et al. 2014) have been surveyed and analyzed with the molecular map constructed from DH populations resulting from IMC technique.

The construction of linkage maps and QTL analysis is often costly and time-consuming; therefore, any alternative system(s) that can save time and expenses would be highly appreciated, especially if resources are limited. One "short-cut" method used to identify markers that tag QTLs is bulked segregation analysis (BSA, Collard et al. 2005). In BSA, a population is screened for a trait, and individuals at the two extreme ends of the distribution formed into two contrasting bulks. The DNA samples of two pools or "bulks" are then combined. By making DNA bulks, all loci are randomized, except for the region containing the gene of interest. Polymorphic markers, after screening two bulks, may indicate marker(s) that are

linked to a gene or QTL of desired trait (Collard et al. 2005). This technique is dependent upon accurate phenotyping, and DH populations have a particular advantage here in that they can be repeatedly tested. Although DH populations proved to be valuable segregant population for QTL analyses, they may not be applicable to outbreeding crops where inbreeding depression can cause nonrandom changes in gene frequency and loss of vigor of the lines (Collard and Mackill 2008).

The markers that have preliminary identified genetic mapping studies are also quite suitable for MAS. Most characters of economic importance are quantitative traits which are affected by environment and numerous loci throughout the genome with individually small effects and thus exhibit low heritability. In such situations, selection is often postponed until the lines become more homozygous in later generations (F5 or F6). Visual assessment for agronomic traits is carried out for resistance to stresses, as well as laboratory tests for quality or other traits to select superiors. The homozygous breeding lines can be harvested in bulk and assessed in replicated trials. The entire process takes considerable amount of time (often 5–10 years) and cost (Collard and Mackill 2008). In order to improve efficiency of selection, integrating molecular genetics with artificial selection known as MAS has been arose (Pink et al. 2008). DNA markers used for MAS should be highly reliable (reproducible), codominant in inheritance, relatively simple and cheap to be used, and generally highly polymorphic. Once tightly linked markers that reliably predict a trait phenotype have been identified, they may be used for MAS. For many agronomic traits, homozygous and heterozygous plants cannot be distinguished by conventional phenotypic screening. This technique allows breeders to select individuals based on their genotypes and thus accelerate the breeding process (Collard et al. 2005; Collard and Mackill 2008).

21.3.4 Reversible Male Sterility

F1 hybrid cultivars often represent high heterosis and therefore of great commercial value and the protection of breeder's rights (Birchler et al. 2003; Ribarits et al. 2007). Commercial production of hybrids is only feasible if a reliable and cost-effective pollination control system is available. Various procedures have been reported to block self-pollination of the female flowers such as mechanical removal of anthers or male flowers, application of male-specific gametocides, or application of male sterility (Parez-Prat and van Lookeren Compagne 2002).

Male sterility, as an efficient system for hybrid production, avoids the manual emasculation labor and serves as a molecular strategy for transgene containment by preventing pollen release to the environment, but most crops lack a naturally occurring system (Parez-Prat and van Lookeren Compagne 2002; Ribarits et al. 2007; Toppino et al. 2011). In addition, the achievement of complete male sterility in the female parent and the restored fertility in F1 hybrids is the major bottleneck in the commercial hybrid seed production.

Thanks to recent progresses in the area of metabolic engineering in pathways of microgametogenesis, biotechnology has added new possibilities to obtain reversible

male-sterile plants (Ribarits et al. 2007; Toppino et al. 2011). It has been indicated that glutamine plays an essential role during pollen development, as isolated and in vitro cultured microspores are unable to develop into the functional pollen grains in a medium lacking glutamine (Ribarits et al. 2007). Transformation of *Nicotiana tabacum* L. with mutated tobacco *glutamine synthetase* genes, which synthesize glutamine from glutamate, fused to the tapetum- and microspore-specific promoters caused pollen abortion closed to the first pollen mitosis and thus resulted in male sterility. Completely homozygous male-sterile lines were rapidly produced by microspore embryogenesis technique. Furthermore, foliage sprays with glutamine and in vitro maturation restored the fertility of male-sterile lines (Ribarits et al. 2007). Using this technique, F1 hybrid seed production technology can be applied in many commercially important crops.

21.3.5 Reverse Breeding

Reverse breeding is a novel breeding technique which allows for production of new hybrid plant varieties in a much shorter time frame and ambient numbers compared to conventional plant breeding techniques (forward breeding). The term "reverse breeding" was originally introduced to describe a technique in plant cell cultures, where homozygous lines are produced from heterozygous parent lines (Dirks et al. 2009; Wijnker et al. 2012). Here, the term "reverse breeding" includes the earlier proposed usage but goes beyond the original definition by widening the methods used to produce homozygous lines (Palmgren et al. 2014).

Generally, reverse breeding comprises two essential steps: (i) the suppression of crossover based in a selected hybrid plant (chosen for its elite quality) and (ii) regeneration of DHs from microspores containing nonrecombinant chromosomes. The knockdown of gene expression involved in homologous recombination, essential for reverse breeding, can be achieved by targeting genes using RNA interference (RNAi) or siRNAs, resulting in predominantly posttranscriptional gene silencing. Alternatively, dominant-negative mutations of the target gene can be used (Dirks et al. 2009). Silencing *DISRUPTED MEIOTIC cDNA1* (*DMC1*) gene resulted in nonrecombined parental chromosome segregation during meiosis in *A. thaliana* gametes (Wijnker et al. 2012). The protein encoded by *DMC1* is essential for meiotic homologous recombination which plays a central role in generating diversity of genetic information and also is essential for the reductional segregation of chromosomes that must occur in order to produce gametes during the sexual reproduction (Cloud et al. 2012). The second step in reverse breeding is to convert microspores, carrying nonrecombined chromosomes, into the DH plants through the IMC technique, as an efficient method, or other techniques depending on the plant species (Forster et al. 2007). From the regenerated DHs, complementary parents can be screened for reconstituting the initial heterozygote (Dirks et al. 2009). It seems that many plant breeding enterprises would benefit from this novel technique to deal with market demands and develop new varieties that are more sustainable or productive in a wide range of crops, including fruits and vegetables in the near future.

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Indirect Somatic Embryogenesis and Plantlet Development from Mature Seed Embryo Explants of *Bambusa arundinacea* (Retz.) Wild

22

P. Venkatachalam and K. Kalaiarasi

Abstract

Efficient plant production via indirect somatic embryogenesis was established by using mature seed-derived embryo explants of *Bambusa arundinacea*. The present investigation demonstrates an optimized method for somatic embryogenesis using various auxins and cytokinins. The seed-derived embryos as explants were cultured on MS medium containing 1.0 mg/L each of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyl amino purine (BAP) to induce embryogenic callus, and the maximum percent of embryogenic callus induction obtained was (85 %) with compact and nodular structure. Proliferated embryogenic callus was transferred onto MS medium fortified with 2,4-D (1.0 mg/L) and α- naphthalene acetic acid (NAA) (1.0 mg/L) in combination with different doses of BAP and/or kinetin (KIN) (0.5–4.0 mg/L) for somatic embryo formation and maturation. Somatic embryos developed were rapidly multiplied upon frequent subculture onto fresh medium to attain maturation. The highest percent of embryo maturation (94 %) as well as germination of somatic embryos (25.1 %) was noticed on MS medium containing the combination of 2,4-D+NAA+BAP (1.0 mg/L each). The matured embryos were germinated into full plantlets which were transferred into paper cups initially. The acclimatized plantlets were hardened successfully in the pots containing soil under greenhouse conditions where about 90 % of the plants were survived. Histological investigations confirmed the initiation of embryos during the somatic embryogenesis process. Therefore, indirect somatic embryogenesis is an alternate promising tool for high-frequency plant regeneration. In this investigation, a reliable plant regeneration protocol via somatic embryogenesis has been developed, and it could be more suitable for commercial scale micropropagation of *Bambusa arundinacea* plants in the near future.

P. Venkatachalam (✉) • K. Kalaiarasi
Plant Genetic Engineering and Molecular Biology Lab, Department of Biotechnology, School of Biosciences, Periyar Palkalai Nagar, Periyar University, Salem 636011, TN, India
e-mail: pvenkat67@yahoo.com

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Keywords
Bamboo • *Bambusa arundinacea* • Somatic embryogenesis • Large-scale propagation

Abbreviations

2,4,D	2,4-Dichlorophenoxyacetic acid
BAP	6-Benzyl amino purine
FAA	Formalin acetic acid
KIN	Kinetin/6-furfurylaminopurine
MS	Murashige and skoog
NAA	α-Naphthalene acetic acid
SE	Somatic embryogenesis
SEM	Scanning electron microscope

22.1 Introduction

Bamboos are hollow and woody plants and monocotyledonous perennial grass belonging to the family Poaceae and grow in tropical and subtropical regions, with prominent knots and having rapid growth, which can reach up to 40 m in height. They cover about 14 million hectares in the world containing about 1200 species belonging to 70 genera (CBTC 2008). For various uses, the requirement of bamboo is estimated at 26.69 million tonnes, but the supply of bamboo is 13.47 million tonnes only against the current demand (Haque 2009). This has prompted to adopt biotechnological tools such as in vitro propagation for large-scale uniform seedling production. Due to the long and erratic flowering cycle (30–45 years) in *Bambusa arundinacea*, plant propagation via seeds has hindered the commercial cultivation of this important species. Furthermore, other factors were also greatly restricting the large-scale plant production of this species via conventional methods (Saxena and Bhojwani 1993). Therefore, plant tissue culture is one of the fast and reliable plant propagation techniques for large-scale multiplication of bamboo plants in short duration. Bamboo shoots are being used as an important vegetable due to the presence of low in fat, high fiber content and various nutrients such as amino acids, vitamins, flavonoids, phenolic compounds, trace elements, and steroids (Nirmala et al. 2011). Muniappan and Sundararaj (2003) demonstrated the presence of flavonoids and glycosides and traces of alkaloids and phytosterols in the methanolic extracts of bamboo, and these compounds are being currently used for various applications such as antiulcer and anti-inflammatory activity. It has been reported that various parts of *Bambusa arundinacea* plants contain high medicinal properties for several applications including antidiabetic potential (Macharla 2011),

antibacterial activity (Zhang et al. 2010a), anti-inflammatory and protective effects (Hong et al. 2010). The ethanolic extracts of the root part of *Bambusa arundinacea* were endowed with potential antihyperglycemic activity (Jaimik et al. 2011; Sundeep Kumar et al. 2012). Most recently, Kalaiarasi et al. (2015) demonstrated the anticancer activity of bamboo leaf extract-derived biomolecules-coated silver nanoparticles against human prostate cancer (PC3) cell lines.

Somatic embryogenesis is a simple process in which somatic cells can differentiate into individual embryos capable of producing whole plantlet for large-scale identical plant production (Mudoi et al. 2013). In somatic embryogenesis, a single cell can produce the whole plantlet identical to mother plant which is the great advance of this propagation method compare to the organogenesis process (Vasil 1987). For the first time, Mehta et al. (1982) reported the regeneration of plantlets from mature embryos of *Bambusa arundinacea* via somatic embryogenesis. Later, plant regeneration via somatic embryogenesis was established by using zygotic embryo explants from *D. strictus* (Saxena and Dhawan 1999), *Otatea acuminate aztecorum* (Woods et al. 1991), and *D. hamiltonii* (Zhang et al. 2010b). Most of the micropropagation studies in bamboo using seedling explants were used for plant regeneration either through axillary shoot proliferation or somatic embryogenesis (Beena et al. 2012). However, further refinement is required to develop an efficient micropropagation protocol with maximum plant regeneration frequency, to lead a path for successful development of valuable bamboo cultivars. Plant regeneration via somatic embryogenesis could be highly beneficial for large-scale production of bamboo seedlings with commercial quality. Therefore, the present study is mainly focused on establishing a rapid and reproducible method for high-frequency plant production via indirect somatic embryogenesis in *Bambusa arundinacea* using mature seed embryo as initial explants.

22.2 Materials and Methods

22.2.1 Initiation of Bamboo Cultures

Seeds of *Bambusa arundinacea* were purchased from Forestry Network market, Bangalore, and were authentically confirmed as *B. arundinacea* by The Forest College and Research Institute and Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India. Seeds were dehusked by removing the seed coat and were soaked overnight with Bavistin (carbendazim fungicide) to prevent fungal contamination. After soaking, seeds were treated with 10% (v/v) Tween-20 for 15 min and washed with sterile distilled water for three times. Subsequently, the seeds were disinfected with 0.1% (w/v) aqueous mercuric chloride solution for 15 min and then thoroughly washed with sterile distilled water for several times. All the operations were performed under sterile conditions in the laminar air flow hood. Then the sterilized seeds were transferred onto Murashige and Skoog's (1962) medium containing various growth regulators for initiation of embryogenic callus.

22.2.2 Culture Media and Conditions

In the present study, the medium used was based on MS basal salts and used sucrose as a carbon source. For somatic embryo induction, maturation, and plantlet production, different growth hormonal combinations were added into the medium, and the pH of the media was adjusted to 5.8 with 0.1 N NaOH or HCl prior to adding 7.0 g/L agar and autoclaved at 121 °C with 1.05 kg/cm^2 pressure for 20 min. For embryogenic callus induction, cultures were maintained in the dark condition. Further, in vitro cultures for plantlet production were kept at 25 ± 2 °C under a 16/8 h light/dark cycle, and light was provided by using cool white fluorescent tubes with 60 μE m^{-2}s^{-1} intensity.

22.2.3 Initiation of Somatic Embryos

Seed-derived embryos of *Bambusa arundinacea* were used as an initial explant material for embryo induction. Embryogenic callus initiation was observed after 28 days of culture, and it was further transferred onto MS medium containing different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) (0.5–4.0 mg/L) in combination with 1.0 mg/L 6-benzyl amino purine (BAP) for somatic embryo formation. Initiated embryos were dissected out from the embryonic clusters and subcultured onto fresh embryo multiplication medium. The development of embryogenic callus nature was further confirmed by anatomical studies.

22.2.4 Embryo Maturation and Germination into Plantlets

For embryo maturation, MS medium was fortified with different doses of BAP and/or kinetin (KIN) (0.5–4.0 mg/L) in combination with 1.0 mg/ L each of 2,4-D and NAA. The medium was supplemented with 3% (w/v) sucrose as carbon source. While doing subculture, non-embryogenic callus and brown color tissues were carefully taken out, and only greenish embryogenic callus was transferred into fresh media for further growth and development. Fully developed and matured embryos were identified visually and subcultured onto fresh germination medium for development of full plantlets.

22.2.5 Acclimatization of Plantlets into Soil

Germinated embryos with root and shoot systems were subcultured into half-strength MS medium without any growth regulators for further growth and full plantlet development. Well-developed plantlets were initially placed in plastic cups (95 × 60 mm) containing a mixture of sand/soil in 2:1 ratio, and they were covered with polyethylene bags to ensure high humidity and maintained in the controlled environment for 15 days. Subsequently, polyethylene bags were removed and

maintained for additional week under controlled environment for acclimatization. Later, plantlets were transplanted into pots containing soil with manure and kept under greenhouse condition. Acclimatized plantlets were finally established in the field condition.

22.2.6 Histological and SEM Analysis

Histology was performed by fixing the samples overnight in FAA. Somatic embryos at different stages (globular and cotyledonary) were fixed in FAA (formalin: acetic acid) for 24 h. The fixed tissues were dehydrated with 70 % (v/v) ethanol and then dried at room temperature until critical point. The tissues were stained with safranin and viewed under a light microscope (Magnus XLD). For scanning electron microscopic analysis, somatic embryos were used for coating with gold–palladium on a Quick Cool Coater (Sanyu-Denshi, Japan) and examined under scanning electron microscope (JOEL, Japan) and captured images.

22.2.7 Experimental Design and Data Analysis

Experiments were designed using a completely randomized block design (CRBD) and data were collected from three independent experiments with 20 replicates. Data on various parameters such as the frequency of somatic embryo formation, total no of embryos per explant, and embryo germination percentage were recorded and evaluated by analysis of variance (ANOVA) using SAS program. The differences in mean values were compared by Student-Newman-Keuls test at the $P \leq 0.05$ level.

22.3 Results and Discussion

22.3.1 Embryogenic Callus Initiation and Development of Somatic Embryos

The seed-derived embryo explants failed to produce embryogenic callus which cultured on hormone-free medium. They became brownish in color initially and later dried. However, initiation of embryogenic calli from the embryonic scutella of *B. arundinacea* was noticed on MS medium supplemented with different doses of 2,4-D (0.5–4.0 mg/L) along with 1.0 mg/L BAP. As shown in Fig. 22.1a, embryogenic callus formation was recorded on MS medium augmented with 2,4-D, and the percent of embryogenic callus development was increased, increasing the concentrations of 2,4-D up to 2.0 mg/L. Among the combinations tried, 2.0 mg/L 2,4-D and 1.0 mg/L BAP was found to be the best combination for initiation of maximum percent of embryogenic calli with compact and nodular structure with regeneration potential (Tables 22.1 and 22.2). The highest frequency of embryogenic callus

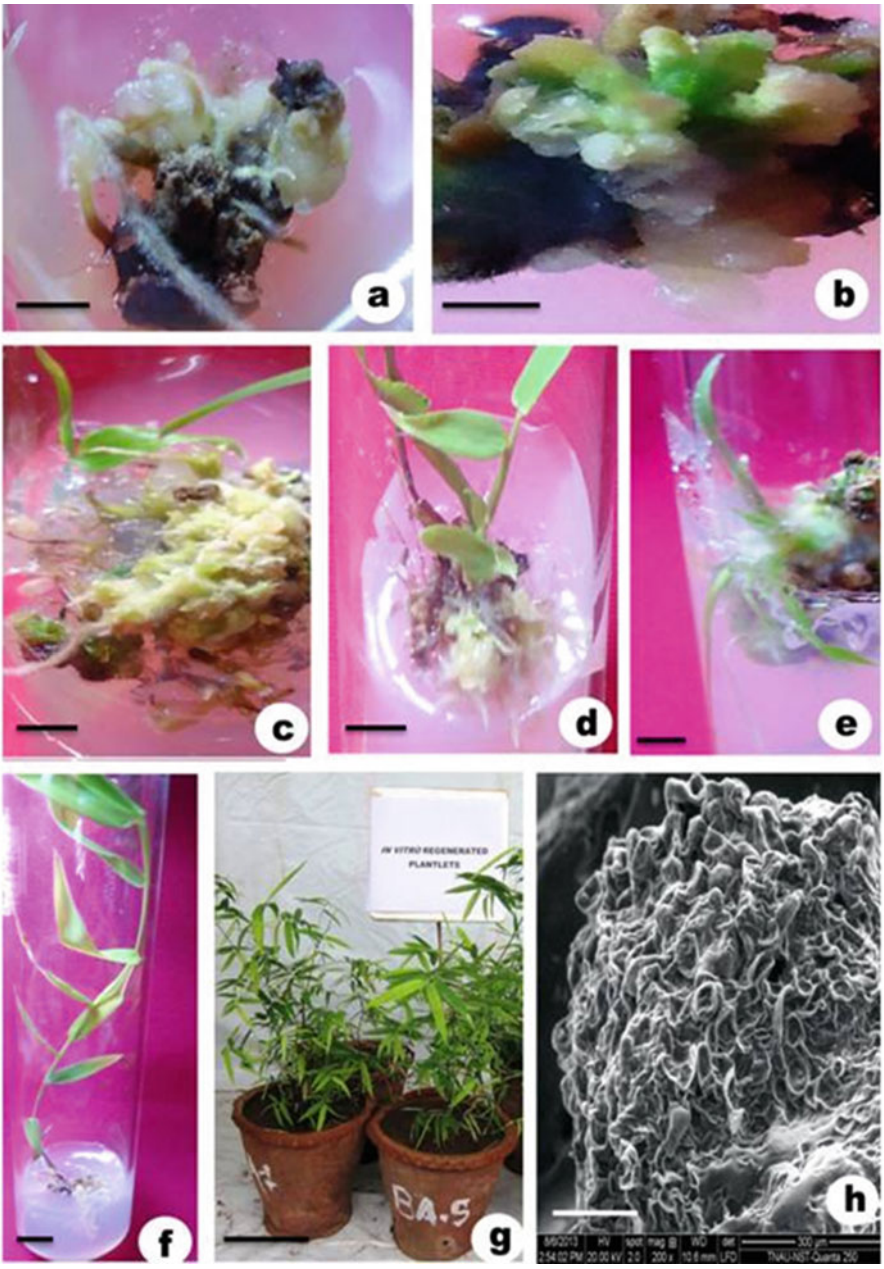

Fig. 22.1 (**a**–**h**) Plant regeneration via somatic embryogenesis in *Bambusa arundinacea*. (**a**) *Yellowish*, granular, and compact callus, (**b**) nodular embryogenic callus obtained during multiplication with buds protruding from callus in cluster, (**c**) maturation of somatic embryos with shoot and root primordia development, (**d**) germination of somatic embryos, (**e**) plantlet development from somatic embryo with shoot and root, (**f**) a plantlet produced via somatic embryogenesis with well-developed shoot and roots, (**g**) acclimatized plants growing in pots under greenhouse condition, and (**h**) scanning electron microscopy image of embryogenic callus (*Bar line* = 1 cm)

Table 22.1 Effect of various concentrations of 2,4-D in combination with 1.0 mg/L BAP on embryogenic callus induction from mature seed-derived embryo explants of *Bambusa arundinacea*

Hormone concentration (mg/L)		Callus induction frequency (%) (Mean±S.E)*	Callus morphology	Rate of callus induction[a]
2,4-D	BAP			
0.5	1.0	69.5 ± 0.56[c]	White, compact, nodular callus	+++
1.0	1.0	85.0 ± 0.24[a]	Creamy yellow, compact, nodular	++++
2.0	1.0	71.4 ± 0.08[b]	Creamy yellow, compact, nodular	++++
3.0	1.0	62.6 ± 0.43[c]	Yellow – brown callus	++
4.0	1.0	57.1 ± 0.67[d]	Yellow – brown callus	++

*Values represent means±standard error. Means followed by same letter within a column are not significantly different at 5 % level

[a]+ indicates that 20 % of callus proliferated vigorously

Table 22.2 Effect of different concentration of two cytokinins (BAP and KIN) along with 2,4-D (1.0 mg/L) and NAA (1.0 mg/L) on maturation of somatic embryos and germination into plantlets

Hormone concentration (mg/L)				Percent of somatic embryo maturation (Mean±S.E)*	Percent of embryo germination (Mean±S.E)*
BAP	KIN	2,4-D	NAA		
0.5	–	1.0	1.0	87.5 ± 0.34[b]	18.2 ± 0.70[c]
1.0	–	1.0	1.0	94.0 ± 0.67[a]	25.1 ± 1.41[a]
2.0	–	1.0	1.0	79.4 ± 0.2[c]	14.4 ± 0.58[c]
3.0	–	1.0	1.0	65.3 ± 0.15[d]	11.8 ± 0.32[d]
4.0	–	1.0	1.0	43.6 ± 0.86[f]	9.1 ± 0.57[d]
–	0.5	1.0	1.0	48.3 ± 0.59[f]	8.06 ± 0.60[e]
–	1.0	1.0	1.0	66.0 ± 1.04[d]	12.17 ± 1.01[d]
–	2.0	1.0	1.0	59.6 ± 1.2[e]	10.8 ± 1.18[d]
–	3.0	1.0	1.0	42.5 ± 0.33[f]	9.5 ± 1.16[d]
–	4.0	1.0	1.0	29.7 ± 0.45[g]	5.0 ± 0.3[e]

*Values represent means±standard error. Means followed by same letter within a column are not significantly different at 5 % level

obtained was 85 % (Fig. 22.1b), and the 2,4-D and BAP combination was not only best for embryogenic callus development but also exhibited regeneration potential when compared with 2,4-D alone. It is noteworthy to mention that 2,4-D at higher concentrations produced non-embryogenic calli with compact in nature. When other auxins tried for somatic embryogenic callus induction, they failed to produce embryogenic callus (data not shown). Results indicated that the embryogenic callus rate was declined at higher concentrations of 2,4-D and became necrotic at later stage. Similar results were also reported earlier by Yeh and Chang (1987). It has been reported that 2,4-D was considered as essential auxin for embryogenic callus

formation in bamboo species (Woods et al. 1991). According to Hu et al. (2011), seed-derived embryo explants had greater potential for embryogenic callus development than young shoot explants in *D. farinosus*. The present results are in agreement with earlier reports. Somatic embryos were obtained when seeds cultured on B5 medium containing 2,4-D (0.5–1.5 mg/L) in *D. strictus* (Rao et al. 1985). High frequency of plant regeneration via somatic embryogenesis was reported using different explants (Sood et al. 2002). Ramanayake and Wanniarachchi (2003) demonstrated that high concentrations of 2,4-D (7.5 mg/L) and NAA (3.0 mg/L) were found to be essential for embryogenic callus formation in *D. giganteus*. Godbole et al. (2002) suggested that development of bamboo plants via somatic embryogenesis is considered as best propagation method for large-scale commercial production of uniform seedlings.

22.4 Embryo Maturation and Germination into Plantlets

In order to produce complete plantlets, it is essential to culture the somatic embryos onto embryo maturation medium for development of root and shoot primordia. To achieve embryo maturation, embryogenic callus was transferred onto a medium containing different levels of BAP and/or KIN (0.5–4.0 mg/L) in combination with auxins (2,4-D and NAA 1.0 mg/L each) for 3 weeks. Among the combinations tested, maximum percent of somatic embryo maturation (94 %) was recorded on a medium containing 1.0 mg/L each of BAP + NAA+ 2,4-D combination (Fig. 22.1c, d), while KIN+NAA+2,4-D (1.0 mg/L each) combination showed 66 % embryo maturation response. Interestingly, BAP was found to be the best cytokinin for highest rate of embryo maturation when compared to the KIN. Upon transfer of greenish coleoptillar stage, somatic embryos onto germination medium produced well-developed shoot axis as well as roots after 28 days of culture (Fig. 22.1e). Isolation of germinated somatic embryos from clumps after development of shoot and root tissue was highly essential because it allowed continuous proliferation and germination of plants from somatic embryos, which could be applied for large-scale propagation (Konan et al. 2007). Well-developed bipolar embryos were transferred onto fresh germination medium containing same growth regulator combinations for embryo germination. Results showed that the BAP+NAA+2,4-D combination was found to be best for maximum percent of embryo germination with well-established root system (25.1 %) followed by KIN+NAA+2,4-D combination (Fig. 22.1f). Of the two cytokinin combinations examined, BAP was found to be best for enhanced rate of somatic embryo germination in *B. arundinacea*. On the other hand, the maturation and germination rate of embryos was slightly decreased at higher doses of cytokinins. It has been reported that BAP showed highest rate of somatic embryo maturation in *B. nutans* by Mehta et al. (2010). According to Saxena and Dhawan (1999), the possible reason for decreased rate of embryo germination is due to physiological immaturity of somatic embryos. However, it is highly essential to refine the embryo germination protocol which is one of the prerequisites for large production of uniform plants via somatic embryogenesis (Venkatachalam et al. 1999).

22.4.1 Acclimatization and Filed Establishment

After germination, plantlets with well-developed root and shoots were gently transferred into fresh half-strength MS medium for further growth (Fig. 22.1f). Healthy plantlets were carefully removed from the culture tubes without damaging root system and washed with sterile water to remove adhered agar traces and transplanted into paper cups containing soil and sand mixture in the ratio of 2:1 and covered with polythene bags. Initially, the plantlets were maintained under controlled environment for acclimatization. After removal of polythene bags, plantlets were established into pots filled with soil and maintained under greenhouse condition (Fig. 22.1g). The survival rate observed was 90%, and the well-rooted plantlets were successfully established in the field condition.

22.4.2 Histological Studies

In order to confirm the embryogenic nature of the callus, histological analysis was carried out by SEM. As expected, the embryogenic cell was differentiated from periphery layer of the callus from parenchyma cells. Histological analysis suggested that somatic embryos had shoot and root meristems as individual structures. Based on histological studies, nodular type of callus was considered as embryogenic nature. Scanning electron microscopy analysis was also carried out to identify the appearance somatic embryo structures. Results indicated that the observed embryos like structures were found to be typical globular stage and bipolar cotyledonary embryos (Fig. 22.1h). The present result is in agreement with earlier report in *Curcuma amada* Roxb. by Soundar Raju et al. (2013). The present plant regeneration protocol might be highly useful for commercial scale propagation of this economically important bamboo species for expansion of its cultivation.

22.5 Conclusions

In summary, this study reports a rapid protocol for plant regeneration via indirect somatic embryogenesis using mature seed-derived embryo explants of *B. arundinacea*. Currently, a simple, rapid, and reproducible plant regeneration system for bamboo was established. Although plant regeneration via somatic embryogenesis in bamboo was reported earlier, the present regeneration method could produce more number of somatic embryos with high plant regeneration rate. Of the two cytokinin combinations used, BAP was identified as best cytokinin for embryo maturation as well as germination into whole plantlets in bamboo. The present regeneration protocol may open up new avenue to produce large-scale bamboo seedlings with uniform nature for commercial cultivation in the near future.

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Part V

Plant Conservation

Micropropagation Technology and Its Applications for Crop Improvement

23

Mohamed A. El-Esawi

Abstract

Micropropagation is one of the current commercial applications of plant tissue culture technologies. It refers to the in vitro clonal propagation of plants from small plant tissues. Micropropagation technology is preferred over the traditional asexual propagation methods. Micropropagation ensures rapid and mass multiplication of genetically identical copies of individual plants, resulting in rejuvenation of old cultivars and quick regeneration of new cultivars resistant to biotic and abiotic stresses. This technology proved to be particularly efficient for orchids and recalcitrant plants. Micropropagation of recalcitrant plants and orchids, such as *Paphiopedilum delenatii*, has been achieved using novel practical methods including wounding technique in combination with liquid culture and stem node culture. Crop improvement using somaclonal variation found in the in vitro cultured cells has also been accomplished, and many somaclonal variants have been released. The focus of this chapter is to discuss and highlight those advances in micropropagation technology as well as somaclonal variants for crop improvement achieved over the recent past years.

23.1 Introduction

Micropropagation is one of the best in vitro commercial applications of plant tissue culture that has become an industrial technology used for in vitro clonal propagation of important horticultural, silvicultural, and medicinal plants from small plant parts. The micropropagation technique is preferred over the traditional clonal

M.A. El-Esawi (✉)
Botany Department, Faculty of Science, Tanta University, 31527 Tanta, Egypt
e-mail: mohamed.elesawi@science.tanta.edu.eg

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propagation methods. Micropropagation ensures true-to-type, rapid, and mass multiplication of genetically identical copies of individual plants in a relatively short time and limited space, resulting in rejuvenation of old varieties and quick regeneration of new varieties resistant to biotic and abiotic stresses. Furthermore, in micropropagation, the rate of multiplication is often much higher, and the micropropagated plants could acquire more new desirable traits, such as increased number of runners in strawberry and bushy habit of ornamental plants (Kane 2011; Bhojwani and Dantu 2013a). Micropropagation is especially useful for orchids and plant species that are difficult to be propagated by the traditional methods (Pierik 1991; Nhut et al. 2006; George et al. 2008; Yam and Arditti 2009; Bhojwani and Dantu 2013a). The history of micropropagation is closely related to the development of techniques for in vitro propagation of orchids (Yam and Arditti 2009; Bhojwani and Dantu 2013a). In the past few years, a significant progress has been achieved in the cellular and molecular biology of plants (El-Esawi et al. 2012, 2015, 2016a, b; Sammour et al. 2013; El-Esawi and Sammour 2014; El-Esawi 2015a, b, 2016; Jourdan et al. 2015; Consentino et al. 2015), especially in the in vitro growth and micropropagation of orchids and recalcitrant plants which are very distinct from other plants (Nhut et al. 2005, 2006). Micropropagation technology of orchids, such as *Paphiopedilum delenatii*, has been increasingly optimized using novel practical methods including wounding technique in combination with liquid culture and stem node culture (Nhut et al. 2006). Such novel methods proved to be efficient for the commercial production and conservation of *P. delenatii*. Crop improvement using somaclonal variation found in the in vitro cultured cells has also been accomplished, and many somaclonal variants have been released as commercial varieties worldwide (Gosal and Kang 2012).

23.2 Micropropagation Stages

Successful micropropagation includes five main stages (Bhojwani and Razdan 1996; Bhojwani and Dantu 2013a):

Stage 0: Donor plant selection and preparation
Stage 1: Establishment of aseptic cultures
Stage 2: Multiplication
Stage 3: Rooting of in vitro formed shoots
Stage 4: Transplantation and acclimatization

23.2.1 Stage 0: Donor Plant Selection and Preparation

Quality and responsiveness of explants in vitro are affected by the physiological status, phytosanitary, and genotype of the donor plant (Read 1988; Valero-Aracama et al. 2008; Kane 2011; Bhojwani and Dantu 2013a). Prior to culture establishment, stock plants used as the source of explants should be maintained in clean controlled

conditions that enhance active growth but decrease the possibility of disease infection and contamination (Kane 2011). Maintaining pathogen-tested stock plants under the conditions of the use of basal irrigation, lower humidity, and antibiotic sprays would effectively minimize the contamination problem of candidate explants, which would in turn allow excision of larger and more responsive explants (Kane 2011; Bhojwani and Dantu 2013a).

Several practices can also be used to enhance explant responsiveness by modifying the physiological status of the stock plant (Kane 2011; Bhojwani and Dantu 2013a). Such practices include the following: (1) trimming to induce lateral shoot growth, (2) pretreatment sprays containing gibberellic acid or cytokinins, and (3) use of forcing solutions containing 200 mg/L 8-hydroxyquinoline citrate and 2 % sucrose to stimulate bud break and deliver growth regulators to target explant tissues (Read 1988; Kane 2011).

Stage 0 may also include manipulation of other factors such as temperature and light treatments, under which the mother plants are maintained and the growth regulators are applied, which could affect the explants' responsiveness at the later stages (Kane 2011; Bhojwani and Dantu 2013a).

23.2.2 Stage 1: Establishment of Aseptic Cultures

The goal of this stage is to initiate and establish aseptic cultures of the plant to be propagated using suitable explants. The success at this stage depends on the selection of the best explant, proper sterilization procedure, and prohibition of any hypersensitivity reactions of the explants (Kane 2011; Bhojwani and Dantu 2013a). The selection of the suitable explant depends on the objective of the study as well as the mode of regeneration and multiplication required (Kane 2011). Either apical bud or nodal segments with at least one axillary bud are commonly used explants for micropropagation (Bhojwani and Dantu 2013a). Meristem-tip culture could be used in order to eliminate viruses. Otherwise, meristem-tip culture should be avoided due to its poor survival and complex culture requirements. Propagation from apical and axillary buds has the advantage of true-to-type progeny (Kane 2011; Bhojwani and Dantu 2013a). Direct or indirect adventitious bud formation can be produced from leaf, stem, root, or nucellus explants. Nucellus explants are used for obtaining somatic embryos in *Citrus* and mango. The nucellus is the only tissue from the adult cashew plants that would be used for micropropagation (Cardoza and D'Souza 2000; Kane 2011; Bhojwani and Dantu 2013a). In monocots, the intercalary meristem at the base of bulb scales or young leaves has been used for regenerating adventitious buds (Kane 2011; Bhojwani and Dantu 2013a).

The donor plants in the greenhouse should be established from cuttings to avoid wind-borne contaminants (Kane 2011; Bhojwani and Dantu 2013a). Aseptic shoot tips should be dissected out, inside a sterile hood, by removing the outer leaves or scales from the buds and bulbs, respectively, and the surface should be sterilized by wiping with 70 % ethanol (Bhojwani and Dantu 2013a). The explants from many plants (i.e., tree species and some horticultural crops) release phenols, forming

quinones upon oxidation, and turn the medium black that might be toxic to the tissue (Bhojwani and Dantu 2013a).

23.2.3 Stage 2: Multiplication

The success of micropropagation depends mainly on the efficiency of this stage (Bhojwani and Dantu 2013a). Shoot multiplication can be carried out through (1) regeneration from callus, (2) direct adventitious bud formation from the explant, and (3) forced axillary branching. Each of these methods has its own merits and drawbacks:

1. *Regeneration from callus*: Under suitable culture conditions, cells from almost all plant parts could form callus that could be induced to regenerate plants via organogenesis or somatic embryogenesis (Bhojwani and Dantu 2013a). Both of these methods could produce a large number of plants, but the latter is much more efficient. Somatic embryogenesis is a highly efficient process by which somatic cells can develop into differentiated plants through potential embryological stages without gamete fusion (Bhojwani and Dantu 2013a). This is in contrast to a separate rooting step required for the de novo formed shoots. Furthermore, upon standardization of somatic embryogenesis protocol, the process could be highly controlled and scaled up for mass production in bioreactors (Etienne et al. 2006; Bhojwani and Dantu 2013a). The embryos could be converted into synthetic seeds for possible transplantation on a large scale. However, a reproducible protocol for somatic embryogenesis is not available for many plant species (Bhojwani and Dantu 2013a). Synchronization of embryogenic cultures could not be achieved easily, and the conversion of embryos into plants has been poor due to physiological and morphological abnormalities (Bhojwani and Dantu 2013a). The major drawback of somatic embryogenesis is an intervening callus phase, inducing variability. Therefore, regeneration from callus is not the best mean for mass clonal multiplication (Bhojwani and Dantu 2013a).
2. *Adventitious bud formation*: The adventitious buds are formed directly from any part of the plant other than axillary (apical) bud. Many horticultural plants form adventitious shoots from leaf pieces (*Saintpaulia*, *Begonia*) and root cuttings (raspberry, blackberry) that have been used for clonal propagation of these plants (Bhojwani and Dantu 2013a). The advantages of in vitro propagation by adventitious shoot formation are: (1) enhancing the number of shoots per propagule, (2) small pieces of tissues that do not survive in vivo can form shoot buds in cultures, and (3) many plants that do not form adventitious shoots in nature can do so in vitro. Adventitious bud formation from leaf scales has been used in the Liliaceae and Iridaceae members (Ascough et al. 2009; Bhojwani and Dantu 2013a). Almost 100 bulblets could be regenerated from a single scale of lily (Gupta et al. 1978). This massive regeneration potentiality in lily has raised the technique to mass production on a commercial scale in bioreactors (Takahashi et al. 1992; Bhojwani and Dantu 2013a).

3. *Forced axillary branching*: It is the most widely used method for commercial micropropagation of the desired plants. Removal of the apical meristems stimulates the axillary buds to grow out into shoots (Bhojwani and Dantu 2013a). In the well-established horticultural practice of clonal propagation by stem cuttings, the new plant can be developed from the axillary bud at the node. However, this process of clonal multiplication is slow and limited by the number of cuttings produced from the mother plant. Exogenous application of growth regulators, especially cytokinins, could stimulate the axillary buds to grow even in the presence of terminal bud and increase the number of usable flushes (Bhojwani and Dantu 2013a). However, the effect of exogenous growth regulators does not last long. Excess cytokinins may cause epigenetic changes in the plants regenerated through forced axillary branching (Bhojwani and Dantu 2013a). Bushiness in the micropropagated *Gerbera* plants, accompanied by excessive leaves and short peduncle, could be attributed to the use of excess 6-benzylaminopurine (BAP). Moreover, long exposure to culture conditions affects the size and shape of fruits in strawberry (Bhojwani and Dantu 2013a).

23.2.4 Stage 3: Rooting of In Vitro Formed Shoots

Shoots formed through regeneration from callus, forced axillary branching, or direct adventitious bud formation need an additional step of rooting for a complete plant formation (Bhojwani and Dantu 2013a). In some cases, the shoots formed in vitro by any of the three methods mentioned above, being continually exposed to cytokinins, may remain short and need an elongation step before transfer to rooting medium. The elongation medium may be liquid having the same composition as for shoot multiplication or with a reduced level of cytokinin (Bhojwani and Dantu 2013a).

Cytokinins present in the medium may prohibit the formation of roots and are essential to transfer the shoots to a suitable medium for rooting (Bhojwani and Dantu 2013a). Rooting is generally carried out by transferring individual shoots to a medium provided with a suitable auxin. The rooting stage is labor intensive, accounting for about 70 % of the cost of micropropagated plants. So, the rooting percentage should be high. To cut down the rooting cost of micropropagated shoots, many commercial companies head for in vivo rooting. Therefore, the micropropagated shoots may be treated as microcuttings and planted directly after treating the cut basal end with auxins or a commercial rooting mixture. In vivo rooting not only cuts down the cost but also gets rid of the problems associated with in vitro rooting (Bhojwani and Dantu 2013a).

23.2.5 Stage 4: Transplantation and Acclimatization

The success of micropropagation relies on the establishment of the plants in soil mixture. The plants grown in vitro are exposed to the artificial environment of the

culture vial, characterized by the culture medium rich in organic and inorganic nutrients, growth regulators, sucrose, high humidity, poor gaseous exchange, and low light (Bhojwani and Dantu 2013a). Under these unnatural conditions, plants can grow well but may suffer from many anatomical, morphological, cytological, and physiological abnormalities that necessitate their careful acclimatization to the in vivo conditions (Bhojwani and Dantu 2013a). The two main abnormalities of these plants include poor control of water loss and heterotrophic mode of nutrition. Under high humidity of the culture vials, the leaves exhibit scanty deposition of wax, poor development of cuticle, and abnormally large stomata (Bhojwani and Dantu 2013a). The lack of cuticle and movement of stomata may cause increased water loss on transplantation, resulting in decreasing their survival chances. Hence, in vitro plants should be hardened before transfer to field conditions.

23.2.5.1 Acclimatization

The main aim of acclimatization of the in vitro plants, growing under low light heterotrophic conditions and high humidity, is to help them grow under high light autotrophic conditions and low humidity (Bhojwani and Dantu 2013a). Hardening the in vitro plants may take 4–6 weeks, so that they could survive under normal conditions. The individual micropropagated plants should be taken out of the agar medium; the roots are then washed to remove the agar and individually planted in pots containing a suitable potting mixture. The plants are irrigated with a mild nutritive solution such as Knop's solution (Bhojwani and Dantu 2013a).

To maintain high humidity around the transplanted in vitro plants, they should be covered with plastic bags having small holes for air circulation. The plants are maintained in low light or shade for about 15–20 days and slowly acclimatized to low humidity conditions by removing the polybags for few hours every day in the beginning and slowly increasing the time of exposure till the plants could withstand complete removal of the cover (Bhojwani and Dantu 2013a). During this phase, plants are hardened to survive on inorganic nutrition, and their photosynthetic machinery is reactivated to become autotrophic. The plants can survive under field conditions only when new roots and leaves are formed.

23.2.5.2 In Vitro Formation of Storage Organs

Plant species, producing storage organs (corm, tuber, rhizome, bulb) in nature, can be enhanced to do so in cultures in order to facilitate transplantation with high survival rates (Bhojwani and Dantu 2013a). Moreover, this may remove the rooting step altogether. The storage organs could be easily stored and planted manually or by machines. In vitro formation of storage organs has been carried out in *Crocus* (Plessner et al. 1990), *Gladiolus* (Dantu and Bhojwani 1995), *Dioscorea* spp. (Forsyth and Van Staden 1984), and potato (Donnelly et al. 2003) by increasing sucrose concentration and changing the growth regulator and temperature and light treatments (Bhojwani and Dantu 2013a).

In vitro formation of tubers has been extensively studied in potato. The precise conditions, enhancing in vitro tuberization for various potato cultivars, have been established, and the whole process has been raised to the level of commercial

production (Bhojwani and Dantu 2013a). Shoot multiplication could happen under long-day conditions on a medium supplemented with sucrose (2–3 %), and in vitro microtuberization is enhanced by complete darkness or short-day conditions and increased sucrose level (8–9 %) in the culture medium (Bhojwani and Dantu 2013a). Microtubers are harvested and sown in plastic trays to regenerate minitubers, used as seed tubers for crop production.

The requirement of growth regulators and growth retardants for microtuber formation depends on the propagator (Donnelly et al. 2003). Some varieties could produce better microtubers in the absence of any retardant or growth regulator, while a number of varieties need the presence of either or both (Bhojwani and Dantu 2013a). Dantu and Bhojwani (1987) regenerated corms using micropropagated shoots of *Gladiolus* in liquid cultures with elevated concentration of sucrose (6–10 %). Corm formation could also be achieved in liquid shake cultures treated with biosynthesis inhibitors, such as paclobutrazol (Steinitz et al. 1991). Temperature requirement for corm formation differs for summer- and winter-flowering *Gladiolus* species (De Bruyn and Ferreira 1992). The winter-flowering *G. tristis* formed corms at 15 °C, while the summer-flowering *G. dalenii* formed corms at 24 °C. Light intensity also affects corm formation in some *Gladiolus* varieties (Thun et al. 2008). In *Dierama luteoalbidum*, belonging to the family Iridaceae, corm formation with 6–8 % sucrose has been achieved in 6 months that could be decreased to 3 months by adding aclobutrazol to the culture medium (Madubanya et al. 2006).

23.3 Factors Affecting Micropropagation

23.3.1 Culture Initiation and Shoot Multiplication

MS (Murashige and Skoog 1962) is the most widely used medium for both culture initiation and shoot multiplication. It has been modified for various systems. For a number of plants, the full strength of MS is very toxic and should be decreased to half strength or less (Bhojwani and Dantu 2013a). Bamboos, such as *Dendrocalamus*, revealed a better shoot proliferation on a MS basal medium of one-half strength (Bhojwani and Dantu 2013a). The promoting effect of the reduced salt concentration of MS could be attributed to the reduced strength of nitrogen, particularly the ammonium salt. In *Gladiolus*, decreasing ammonium nitrate to half strength and providing the MS medium with NaH_2PO_4 enhanced the multiplication rate (Dantu and Bhojwani 1992; Bhojwani and Dantu 2013a).

The medium should be provided with suitable growth regulators for culture initiation and later shoot multiplication (Bhojwani and Dantu 2013a). Among various cytokinins available commercially, BAP has been widely used. However, other cytokinins have proved to be more useful for other plants (Bhojwani and Dantu 2013a). For example, 2-isopentenyladenine (2iP) proved to be better than BAP in *Rhododendron* (Anderson 1975), blueberry (Cohen 1980), and garlic (Bhojwani 1980). The expensive cytokinin, zeatin, is not recommended for commercial

micropropagation (Bhojwani and Dantu 2013a). The urea-derived cytokinin, thidiazuron (TDZ), has also proved useful to regenerate species.

Topolins, the latest group of aromatic cytokinins used in tissue culture studies, revealed promising results in different plant species (Bhojwani and Dantu 2013a). mT (meta-topolin) promoted acclimatization and ex vitro survival rate in *Spathiphyllum* species, as well as the multiplication of plantains (Escalona et al. 2003). mTR (meta-topolin riboside) increased the survival rate of potato cultures (Baroja-Fernández et al. 2002), and mT derivatives enhanced anti-senescence effects and histogenic stability in rose and *Petunia* varieties, respectively (Bogaert et al. 2006). mTR proved to be better than BAP in terms of shoot quality and multiplication rate in *Barleria greenii*, an endangered ornamental shrub (Amoo et al. 2011). The most effective concentration of cytokinins varied between 1 and 2 mg L^{-1}. TDZ is effective at concentrations varied between 0.002 and 0.10 mg L^{-1}. Higher concentrations of this cytokinin could stimulate callusing and may cause morphological abnormalities including hyperhydration. To maximize regeneration in plants, the cytokinin should be combined with a suitable auxin (Bhojwani and Dantu 2013a). The most popular used auxins include indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) in a range of 0.1–1 mg L^{-1}. Gibberellic acid (GA3) enhanced the multiplication rate and the shoot quality formed in *Gardenia*.

Media supplemented with agar are used for culture initiation and shoot multiplication (Bhojwani and Dantu 2013a). However, multiplication and survival rate of a large number of plant systems proved to be better in liquid cultures. Cultures could be established only in a liquid medium of a pH of 5.7–5.8 in some orchids, such as *Cattleya* (Bhojwani and Dantu 2013a). However, other plants including *Magnolia* and *Dianthus* prefer a highly acidic medium for enhanced rates of shoot multiplication maintained in light (Bhojwani and Dantu 2013a).

23.3.2 Rooting

Many micropropagated plants could root in vitro on a MS medium of a full strength, provided with a suitable auxin (Bhojwani and Dantu 2013a). However, some plants could not root on a MS medium of a full strength. *Narcissus* and *Rhododendron* enhanced in vitro rooting percentage on a MS medium of a half strength (Bhojwani and Dantu 2013a). Shoots of some apple varieties rooted only when ammonium nitrate was decreased to a half strength or completely removed from the medium (Hyndman et al. 1982; Sriskandarajah et al. 1990; Bhojwani and Dantu 2013a). The period of exposure to auxins differs in plant species. Herbaceous plants require a short exposure, while woody species need a long or continuous exposure to auxins. Long exposure to auxins could also stimulate callusing at the base or cause chlorosis of leaves. Auxins have been used at a concentration of 0.1–1.0 mg L^{-1} for rooting (Bhojwani and Dantu 2013a).

23.4 Micropropagation Systems

Micropropagation systems could be divided into (1) photomixotrophic systems, in which the plant growth depends on photosynthesis and the medium sugar (green shoot cultures), and (2) heterotrophic system, in which the plant growth depends entirely on the culture medium sugar (nonchlorophyllous cell and embryogenic cultures). Furthermore, current efforts are being made to develop photoautotrophic micropropagation systems, in which the plants can grow in sugar-free medium (Bhojwani and Dantu 2013a).

The major drawbacks of the first two systems, in which the plants grow on sugar-containing medium, are that the plants could not perform photosynthesis under low light intensity and low CO_2 concentration in the vessel, resulting in different morphological, phenotypical, and physiological abnormalities in plants (Bhojwani and Dantu 2013a). However, under photoautotrophic conditions, growth and quality of the plants could be improved, and the plant acclimatization ex vitro could also be enhanced (Bhojwani and Dantu 2013a). Photoautotrophic in vitro multiplication has been successfully achieved in many plant species such as *Brassica oleracea*, *Citrus macrophylla*, *Eucalyptus camaldulensis*, *Solanum tuberosum*, *Rubus idaeus*, *Ipomoea batatas*, *Nicotiana tabacum*, and *Lycopersicon esculentum* (Bhojwani and Dantu 2013a).

A comparative study of micropropagation of *Zantedeschia elliottiana* and *Cunninghamia lanceolata* under photomixotrophic micropropagation (PMM) and photoautotrophic micropropagation (PAM) conditions showed that PAM reduced the period of in vitro multiplication and rooting to half, enhanced the shoot quality, improved the rooting quality in the absence of an auxin, lowered the losses due to infection, lowered the production cost, and enhanced higher survival ex vitro (Kozai and Xiao 2006; Bhojwani and Dantu 2013a).

23.5 Advances in Recalcitrant Plant Micropropagation

Paphiopedilum is a terrestrial orchid genus that grows from the Himalayas, Southeast Asia, to Papua New Guinea (Teoh 2005; Nhut et al. 2006). *Paphiopedilum* could be traditionally propagated by seeds or "keikis" (Bahasa Indonesian, referred to plantlets derived from mature orchid plants) (Nhut et al. 2006). *Paphiopedilum* propagation using seeds reveals a low survival rate. In contrast, *Paphiopedilum* propagation using keikis shows a high survival rate, but the mature slipper orchid plant should take 2–3 years to form keikis (Nhut et al. 2006). Therefore, plant cell tissue culture has become an ideal technique for preserving this genus from extinction. Bubeck (1973) was the first to micropropagate *Paphiopedilum*. Many scientists have attempted to micropropagate this unique type of orchids from various plant parts and using different methods (Nhut et al. 2006). Those attempts included plantlet regeneration from meristem culture (Bubeck 1973), lateral bud induction (Stewart and Button 1975), shoot multiplication from seedlings (Huang et al. 2001), and axillary shoot induction and plantlet regeneration from shoot tips (Huang 1988).

Recently, plantlets have been successfully regenerated through protocorm-like bodies (PLBs) from totipotent calli (Lin et al. 2000), and direct shoot bud formation was produced from in vitro leaf explants (Chen et al. 2004).

The endangered species, *Paphiopedilum delenatii*, has been a promising potted plant for centuries due to its distinctive shapes and attractive color. However, the increasing market demand and the low multiplication rate using traditional propagation methods have endangered the survival of this orchid (Nhut et al. 2006). Therefore, two practical methods including wounding technique in combination with liquid culture and stem node culture were established and applied for *P. delenatii* propagation (Nhut et al. 2006).

23.5.1 Wounding Technique and Liquid Culture

Wounding technique included easy propagation procedures for *P. delenatii*, ensures better shoot survival rate, and enhances multiplication rates (Nhut et al. 2005, 2006). Additionally, this technique helps in propagation and preservation of endemic and endangered species, reducing the plantlet cost, and production of a large number of plantlets in a short period (Nhut et al. 2005). In vitro 6-month-old seedlings were exploited as explants for shoot induction using wounding technique (Nhut et al. 2005, 2006). Their roots were carefully removed and the seedling bases were pierced. These wounded seedlings were then placed onto media-containing flask that contains basal MS medium provided with different concentrations of TDZ for investigating the shoot regeneration. A piece of filter paper was also added in the liquid-containing culture vessels. Shoot survival rate and number of shoots per explant have been recorded after 12 weeks of culture at 25 ± 2 °C, 70–80 % relative humidity, and a 12-h photoperiod of 45 μmol m^{-2} s^{-1} photosynthetic photon density flux (Nhut et al. 2005, 2006).

The highest survival rate was measured from wounded seedlings cultured on MS solid medium provided with 0.5 mg L^{-1} TDZ, while no shoot formation was produced in control treatments with non-wounded shoots in all media types (Nhut et al. 2005, 2006). An average of 2.3 green and vigorous shoots were produced on a medium containing 0.25 mg L^{-1} TDZ and 0.5 mg L^{-1} NAA, indicating that the wounding step is a prerequisite for shoot formation in seedlings (Nhut et al. 2005, 2006). Wounded cells at the damaged sites may be responding to stimulating agents such as plant growth regulators present in nutrient media. No shoot formation was produced on non-wounded seedlings in control treatments, indicating that intact cells of these seedlings were not affected by the stimulators (Nhut et al. 2005, 2006). In semisolid auxin-containing media, the number of newly formed shoots decreased due to the elevated TDZ concentration. In contrast, the number of shoots formed in liquid medium was increased by increasing TDZ levels (Nhut et al. 2005, 2006). These results show that TDZ effect on explants depends significantly on the physical properties of the medium and that the medium physical state mainly affected shoot formation in wounded seedlings. Wounded seedlings could easily uptake plant growth regulators and nutrient components due to not being completely

submerged in the culture medium (Nhut et al. 2005, 2006). This induces the differentiation of the affected tissue and results in the formation of a higher number of shoots per explant. Moreover, a significantly lower adverse effect of TDZ on plant tissue was detected in liquid media (Nhut et al. 2005, 2006). The survival rate was relatively high and did not vary considerably among treatments. The survival rate on TDZ-containing medium was lower than that on a medium containing TDZ combined with auxin, indicating that the presence of auxins in liquid media could increase the survival rate (Nhut et al. 2005, 2006). However, auxins of high concentration may play an inhibitory role in liquid media since the shoot number per explant, formed in liquid media without auxin, was two times greater than that in auxin-containing media.

Wounding technique proved to be efficient for inducing shoot regeneration, and highest numbers of shoot were produced in a liquid medium that contained 1.0 mg L^{-1} TDZ (Nhut et al. 2005, 2006). Moreover, rooting plantlets could be produced after 3 months on rooting medium. Because of their efficiency, the wounding method and liquid culture could be used for large-scale micropropagation of this orchid and other endangered species.

23.5.2 Stem Elongation and Stem Node Culture

An innovative protocol for micropropagating *P. delenatii* through shoots from elongated plantlet-derived nodal segments was reported (Nhut et al. 2005, 2006). In vitro plantlets of 1.5 cm in height were placed excessively onto MS medium containing 2.0 mg L^{-1} BA, 1 g L^{-1} AC, 0.5 mg L^{-1} NAA, coconut water (20 %, v/v), 30 g l^{-1} sucrose, and 9 g l^{-1} agar and then incubated in darkness or under different light intensities of fluorescent (FL) tube and red LEDs (Nhut et al. 2005, 2006). The greatest plant height was observed in plantlets incubated in darkness after 4 weeks of culture, followed by the plant height of those exposed to 30 μmol $m^{-2}.s^{-1}$ of red LEDs and 30, 45, and 60 μmol m^{-2} s^{-1} of FL (Nhut et al. 2005, 2006). The plant height of *P. delenatii* shoots was the highest in the dark, but plantlets were yellow and not very vigorous. When *P. delenatii* shoots were incubated under low light intensity of 30 μmol m^{-2} s^{-1} of FL and red LEDs, their shoots elongated, and distances among leaves were widened along the stem. Moreover, it had been reported that red LEDs affect chlorophyll synthesis (Tripathy and Brown 1995; Nhut et al. 2005, 2006), leaf expansion, and stem elongation (Hoenecke et al. 1992). Nhut (2002) also revealed that plantlets of *Eucalyptus*, *Cymbidium*, and *Phalaenopsis* elongated under red LEDs. The elongated plantlets of *P. delenatii* also had a normal growth and thin stems. In this case, 30 μmol m^{-2} $s^{-1}$of FL and red LEDs seemed to be optimal for stem growth and photosynthetic activity of *P. delenatii*. These light intensities together with excessive crowding in culture induced internode elongation of shoots, resulting in their development into rhizome-like structure (Nhut et al. 2005, 2006).

The elongated plantlets developed from this experiment were utilized as explants for the following step in which they were collected; roots and leaves were carefully

removed for exposing nodes (Nhut et al. 2006). These stems were then segmented into nodal segments. The isolated nodal segments were cultured on a nutrient medium containing plant growth regulators including TDZ and zeatin for studying their effects on shoot formation (Nhut et al. 2006). Of the cytokinins (TDZ and zeatin) used for inducing the shoot formation on elongated stem-derived nodal segments, TDZ is the most effective cytokinin for shoot proliferation of this recalcitrant species. The highest shoot formation rate (75 %) was recorded on media provided with 2.0 mg L^{-1} TDZ. However, bigger and more greenish shoots were detected on a modified MS medium containing 1.5 mg L^{-1} TDZ. The shoot size varied among treatments: the biggest on medium comprising 2.0 mg L^{-1} TDZ, whereas the smallest on medium provided with 0.5 mg L^{-1} TDZ. TDZ proved to be more effective for morphogenesis induction such as proliferation and shoot regeneration of several orchids (Chen and Piluek 1995; Nayak et al. 1997; Nhut et al. 2006). However, Huang et al. (2001) stated that TDZ inhibits shoot proliferation of *Paphiopedilum*. Nhut et al. (2005) reported that the combination of TDZ with NAA proved to be efficient for adventitious shoot induction at high concentration. Low concentration of TDZ was not suitable for the shoot formation of *P. delenatii*. Further studies on rooting ability of shoot derived from nodal segment have been carried out. Three-month shoots were subcultured on a rooting media to stimulate root formation. Root primordia were firstly detected after 2 weeks of culture, and rooting plantlets were ready to be transferred to greenhouse after 3 months. Furthermore, it was also revealed that regenerated plantlets derived from stem node were uniform and vigorous (Nhut et al. 2006).

Although this method has been successfully used for micropropagation of many orchids such as *Dendrobium* and *Phalaenopsis*, no reports have been recorded yet on the intensity effect of fluorescent tubes and LEDs on *P. delenatii* elongation and micropropagation using stem node culture (Nhut et al. 2006). Nhut et al. (2005) proved the effectiveness of this method, producing a large number of plantlets with lower cost in a shorter period. Stem node culture proved to be efficient for the commercial production and conservation of *P. delenatii*.

23.6 Somaclonal Variation

Variation among tissues or plants derived from the in vitro somatic cell cultures is known as somaclonal variation (Gosal and Kang 2012). It characterizes genetic mutations that happen during the tissue culture process. It may be genetic or may result from culture-induced epigenetic changes (Larkin and Scowcroft 1981). The epigenetic changes are expressed at cell culture stage, but these changes usually disappear when plants are regenerated or reproduced sexually. Variation derived from anther and pollen culture is known as gametoclonal variation, whereas that occurs through protoplast culture is termed as protoclonal variation (Gosal and Kang 2012). Therefore, it provides a novel way to create a new genetic variation for crop improvement.

23.6.1 Somaclonal Variation Induction

Callus cultures can be established from suitable explants and multiplied through periodic subculturing (Gosal and Kang 2012). Cell suspension cultures could be established by transferring actively growing callus to agitated liquid medium and can be maintained and multiplied through periodic subculturing (Gosal and Kang 2012). Plants are regenerated usually from long-term maintained callus or cell suspension cultures and transferred to soil for screening the variation. In vitro selection at cellular level can be achieved for some traits by growing cells from cell suspensions and calli on a medium provided with elevated levels of various biotic and abiotic stress factors (only the variant cells survive). In vitro selection also decreases the chances of diplontic selection, but needs a high level of correspondence between the traits selected in vitro and expressed in vivo (Gosal and Kang 2012). Somaclonal variants can be determined through screening that involves evaluation of regenerated plant traits such as yield that cannot be assessed at single-cell level or through cell selection.

23.6.2 Somaclonal Variation Causes

Somaclonal variation may be genetic or epigenetic; the genetic variation is heritable, whereas the epigenetic variation is not heritable and has no significance in sexually propagated plants (Gosal and Kang 2012). Genetic variation may result from the following causes:

1. *Chromosomal changes*
   Chromosomal changes in tissue culture-derived plants have been detected with respect to both chromosome number and structure (Gosal and Kang 2012). Besides polyploidy, aneuploidy (monosomics and trisomics) has been detected in ryegrass, oats, wheat, and potato. A number of studies of modified chromosome structure in cultured plant cells have been recorded (Lee and Phillips 1988). Deletions, translocations, inversions, and duplications have been detected in barley, wheat, maize, and potato. Small changes in chromosome structure could change genetic transmission and expression of specific genes. Moreover, recombination or chromosome breakage may occur in preferential regions of particular chromosomes, thereby influencing some regions of the genome (Evans et al. 1984; Gosal and Kang 2012).
2. *Point mutations*
   Genetic changes looking like single-gene mutations have now been observed in numerous crops (Gosal and Kang 2012). The recessive single-gene mutations are doubtful if variant does not express itself in the regenerant (R_0) plant, but the self-fertilized R_1 progeny segregates in an expected 3:1 Mendelian ratio for a morphological trait (Gosal and Kang 2012). This type of analysis has been achieved for several tomato somaclones and used to map somaclones to specific

loci (Evans and Sharp 1986). This process has also been recorded in maize, rice, tobacco, and wheat (Gosal and Kang 2012).

3. *Mitotic crossing over*
   Mitotic crossing over may cause some of the genetic variation that leads to the recovery of homozygous recessive single-gene mutations in some regenerated plants (Gosal and Kang 2012). Dulieu and Barbier (1982) regenerated plants from *Nicotiana tabacum* with specific chlorophyll deficiency markers present in heterozygous condition. A high frequency (9.6%) of variant regenerants at the "a_1" and "y" loci has been attributed to the combination of deletion and mitotic recombination.
4. *Cytoplasmic genetic changes*
   Cytoplasmic genetic changes including mitochondrial DNA (mtDNA) have been characterized in maize by assessing plants for two cytoplasmic traits (Gengenbach et al. 1977; Gosal and Kang 2012). Sensitivity to host-specific toxin of *Drechslera maydis* race T, the causal agent of southern corn leaf blight, is associated with all genotypes containing Texas male sterile (cms-T) cytoplasm (Gosal and Kang 2012). Such traits are tightly linked and controlled by mitochondrial DNA. Gengenbach et al. (1977) used in vitro for resistance to toxin and produced resistant plants with the goal of recovering toxin-resistant cytoplasmic male sterile lines, but among the regenerants, resistance was associated with reversion to male fertility. Restriction endonuclease pattern of mtDNA showed significant changes in mtDNA. This mutation to male fertility and toxin insensitivity has been revealed to be a frameshift mutation in mitochondrial DNA (Gosal and Kang 2012).
5. *Transposable element activation*
   Activation of mutator genes has been detected following plant cell cultures of *Nicotiana* and maize (Gosal and Kang 2012). Chromosome breakage and fusion and genomic stress caused by culture conditions are main causes of transposable element activation. Transposable elements can cause phenotypic changes in plants, and their activation during in vitro culture stimulates somaclonal variation (Kaeppler et al. 2000; Gosal and Kang 2012).
6. *Deamplifications and amplifications*
   Deficiencies in ribosomal DNA (rDNA deamplifications) have been detected at the molecular level in flax, triticale, and potato. On the other hand, gene amplifications have been detected in *Nicotiana* (Santoso and Thornburg 2002; Gosal and Kang 2012).
7. *DNA methylation and demethylation*
   De novo methylation and demethylation events represent a main part of differential genomic changes (Gosal and Kang 2012). Tissue-specific DNA methylation of different sequences has been recorded for several plants. Genome activity and the structural organization of chromatin somehow seem to be related to DNA methylation (Bardini et al. 2003; Bednarek et al. 2007).
8. *Virus elimination*
   Virus infection causes changes in the plant reaction to other diseases. Larkin and Scowcroft (1981) reported that the virus elimination during in vitro passage

could change the plant susceptibility to certain fungal diseases and the somaclones might exhibit resistance.

9. *Modified expression of multigene families*
   Culture conditions may regulate the expression of a multigene family that previously expressed some agronomically important genes, including those for gliadins, glutenins, and zeins (Gosal and Kang 2012).

23.6.3 Origin of Somaclonal Variation

The variation detected in tissue culture-derived plants is a combined effect of the genetic heterogeneity of the initial explant cells and the genetic and epigenetic variations happening during the culture course (Bhojwani and Dantu 2013b).

23.6.3.1 Preexisting Variability

The level of somaclonal variation arising from the explant depends on the age and type of tissue and organ (Castorena Sanchez et al. 1988; Bhojwani and Dantu 2013b), the donor plant genotype (Krikorian et al. 1993), and the cultivation mode of donor plants (Pijnacker et al. 1989). Polyploid plants with a higher chromosome number reveal greater variation in plants regenerated in vitro (Skirvin et al. 1994; Bhojwani and Dantu 2013b).

In most of the angiosperms, normal plant development is accompanied by direct changes in nuclear DNA (D'Amato 1990; Bhojwani and Dantu 2013b). In the apical meristems, the cells are maintained at a uniform diploid level. However, their derivatives could undergo duplication and endoreduplication to various degrees during the differentiation process into specialized tissues, resulting in somatic cells with higher levels of DNA (Bhojwani and Dantu 2013b). Such a phenomenon of polyploidization of body cells is known as polysomaty. *Dendrophthoe falcata*, *Helianthus tuberosus*, *Helianthus annuus*, and *Lilium longiflorum* are some of the nonpolysomatic species (Bhojwani and Dantu 2013b). Torrey (1965) reported that, in root cultures of pea, the first set of tetraploid mitosis was derived from the explant. Aneusomaty is another type of preexisting chromosomal variability, occurring rarely in hybrids and polyploids of recent origin. Every individual of sugarcane clone H50-7209 exhibited chromosome number mosaicism ($2n = 108–128$). In this plant, the apical meristems and the mature tissues contain mosaic of cells with varying proportion of different aneuploid chromosome numbers. The aneusomaty is promoted in callus cultures derived from such tissues.

Genetic chimera breakdown during callusing or direct differentiation of adventitious organs could be another source of somaclonal variation, especially in the vegetatively propagated species (Bhojwani and Dantu 2013b). Highly differentiated tissues such as roots, stems, and leaves produce more variation than explants with the preexisting meristems, such as shoot tips and axillary buds. However, in banana somaclonal variation occurred with higher frequencies in the plants derived from shoot tip cultures than in those derived through somatic embryogenesis

(Shchukin et al. 1998). This could be attributed to the chimeric nature of the shoot tip and dissociation of the chimera in cultures, resulting in the variant recovery.

The level of variability contributed by the explant varies with the explant source. Moreover, meristematic and embryonic tissues may yield more stable cells and regenerated plants than explants involving mature and differentiated cells (Bhojwani and Dantu 2013b). In pineapple, all the plants obtained from syncarp or slip calli were variants, while only 7 % of the plants produced from the crown revealed variations (Wakasa 1979). Most of plants produced from seed callus of *Cymbopogon* species were atypical, but those produced from inflorescence callus closely resembled the parents, with a little variation (Chandra and Sreenath 1982; Bhojwani and Dantu 2013b). The plants produced directly from spadix explants of *Anthurium scherzerianum* were less variable than those regenerated from leaf explants (Geier 1987). In geranium, the plants produced from in vivo stem cuttings were uniform, but those regenerated from in vivo root and petiole cuttings were variable (Skirvin and Janick 1976). In potato, cotyledon-derived protoplasts yielded more tetraploids than leaf-derived protoplasts (Osifo et al. 1989). In cucumber, the plants that were propagated from meristems lacked variation, and low variation occurred in the direct regeneration from leaf explants (Plader et al. 1998). However, a high frequency of changes was detected in plants directly regenerated from protoplasts.

23.6.3.2 In Vitro Induced Variations

Excision of tissue from the stable environment of the plant body and its transfer to culture medium inside the culture vial under artificial conditions could be a potential shock, resulting in a range of abnormalities and mutations, such as structural and numerical changes in the chromosomes and DNA organization (Bhojwani and Dantu 2013b). An obvious evidence of tissue culture-stimulated variation is the occurrence of chromosomal changes in the cultured tissues of nonpolysomatic species, such as *C. capillaris*, and in cultures derived from single cells or protoplasts. Olmos et al. (2002) reported the occurrence of hot spots of instability in the genome. Therefore, somaclonal variation may not be a random process.

In vitro culture conditions and rapid multiplication of tissues influence its genetic stability, causing the occurrence of somaclonal variation (Bhojwani and Dantu 2013b). The frequency of variation increases with the increase in the number of multiplication cycles. This could be attributed to the elevated mutation rate with each cell cycle and accumulation of mutations over a period of time. In shoot tip cultures of banana, somaclonal variation occurred after the fifth subculture, and its frequency was increased thereafter (Rodrigues et al. 1998). Shepherd et al. (1996) recorded similar results in two other banana cultivars.

The culture medium is one of the various factors that induce somaclonal variation. In tissue cultures 2,4-D has proved highly mutagenic (Bhojwani and Dantu 2013b). It either stimulates polyploidy or selectively favors division of polyploid cells. In the presence of 2,4-D, suspension cultures of *Haplopappus* have been converted from wholly diploid state to wholly tetraploid state over a period of 6 months (Sunderland 1977). Substitution of 2,4-D by NAA or IAA decreased the chromosomal aberrations (Chand and Roy 1980). Additionally, in pea, 2,4-D enhanced

polyploid mitosis at a hormonal concentration of 0.25 mg L^{-1}, but favored divisions in diploid cells at herbicidal concentration of 20 mg L^{-1}. High concentrations of BAP and kinetin in cultures induced genetic variation (Trujillo and Garcia 1996). Embryogenic suspension cultures of cucumber in auxin (2,4-D)-containing medium revealed greater genetic variation than those in cytokinin-containing medium (Ładyzynski et al. 2002).

In vitro clonal propagation of oil palm was developed in 1970s, but several forms of somaclonal variation regularly occurred in the micropropagated plants (Bhojwani and Dantu 2013b). Mantled phenotype is a detected floral abnormality that refers to feminization of male parts in flowers of both sexes (Corley et al. 1986), leading to the decrease of productivity or total loss of harvest due to abortion of abnormal fruits (Eeuwens et al. 2002). The abnormality intensity varies according to the nature of the embryogenic calli from which the plants are recovered.

23.6.4 Nature of Somaclonal Variation

Somaclonal variation has been revealed in several crops for both quantitative and qualitative traits, including male sterility in maize; early tasseling in corn; high sucrose content in sugarcane; improved protein content in rice and triticale; changed plant height, tiller number, grain color, and gliadin proteins in wheat; disease resistance in maize, sugarcane, mustard, and potato; and salt tolerance in rice (Jain et al. 1989; Gosal and Kang 2012). However, such somaclonal variations have not been fully exploited because, in many cases, these involve either the existing types or there were desirable changes accompanied by several undesirable changes (Gosal and Kang 2012).

23.6.5 Importance of Somaclonal Variation for Crop Improvement

Various useful traits have been recovered using this methodology in sugarcane (Leal et al. 1994; Kaur et al. 2001; Khan et al. 2004; Jalaja et al. 2006; Gosal and Kang 2012), maize (Zheng et al. 2004), potato (Veitia-Rodriguez et al. 2002; Lara et al. 2003), rice (Cristo et al. 2006; Elanchezhian and Mandal 2007), wheat (Ahmed and Abdelkareem 2005; Sabry et al. 2005), *Prunus persica* (Hammerschlag and Ognjanov 1990), and apple rootstocks (Rosati and Predieri 1990; Donovan et al. 1994). Recovery of novel variants that are rare in the natural gene pool, such as atrazine resistance in maize, improved methionine contents in cereals, jointless pedicels in tomato, increased seedling vigor in lettuce, and *Fusarium* resistance in alfalfa, is of much importance (Sengar et al. 2009; Gosal and Kang 2012). Genetic and molecular evidence for elevated recombination frequency through cell culture has now been provided (Larkin et al. 1993). Tissue culture of wide hybrids also assists in breaking undesirable linkages and achieving introgression from foreign sources (Gosal and Kang 2012). Several new cultivars have been developed through

somaclonal variation in sugarcane, tomato, potato, sorghum, and *Brassica* (Gosal and Kang 2012). This simple and cost-effective technique helps in improving the apomictic and vegetatively propagated species. Somaclonal variant of medicinal plants, such as citronella Java, has been developed and released as commercial varieties that could produce higher yield and oil content than original varieties (Gosal and Kang 2012).

23.7 Conclusions

Micropropagation refers to the in vitro clonal propagation of plants from small plant parts. This technique is preferred over the traditional asexual propagation methods because it ensures true-to-type, rapid, and mass multiplication of genetically identical copies of individual plants, resulting in a quick regeneration of new varieties which are resistant to diseases and abiotic stresses. Novel practical methods including wounding technique in combination with liquid culture and stem node culture have been established and applied for the in vitro growth and micropropagation of recalcitrant plants and orchids, such as *Paphiopedilum delenatii*. These methods proved to be useful for the commercial production and conservation of *Paphiopedilum delenatii*. Many somaclonal variants have been released as commercial varieties worldwide.

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Improvement of Green Leafy Vegetables: The Role of Plant Tissue Culture and Biotechnology

24

Sandopu Sravan Kumar, M.C. Aruna, and Parvatam Giridhar

Abstract

Biotechnological improvement of both ornamental and edible, i.e. food value plants, is one of the important research areas since five decades throughout the world. Especially in vitro propagation technology has come as an aid to accomplish the targets of sustainable propagation of both traditional plants of commercial importance, to rescue the endangered plant species, and also for their qualitative and quantitative improvement. From nutritional and nutraceutical point of view, green leafy vegetables or leafy greens play a pivotal role in view of their potential health benefits. Significant focus has been given for this category of plants by employing in vitro propagation methods to get an efficient mass multiplication, somatic embryogenesis for sustainable cultivation, and also through callus suspension cultures for achieving augmented yield of high-value secondary metabolites. Apart from this, attempts have been made to mobilise the desired traits of nutritionally important biomolecules through genetic engineering into leafy vegetables to alleviate nutrient-deficient diseases in consumers. In addition to this, the well-documented optimised methodologies to leafy vegetables would help as an in vitro model for elucidating the biosynthetic pathways of respective nutritionally important molecules. Under this context, various developments in the above said areas pertaining to green leafy vegetables would be having implications for value addition through food technology. Accordingly, a review on various biotechnological aspects of green leafy vegetables has been contemplated, and the same will be covered in this chapter.

S.S. Kumar • M.C. Aruna • P. Giridhar (✉)
Plant Cell Biotechnology Department, CSIR-Central Food Technological Research Institute, Mysore 570 020, India
e-mail: parvatamg@yahoo.com

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Bioactives • In vitro rooting • Mass multiplication • Nutraceuticals • Secondary metabolites • Somatic embryogenesis • Tissue culture

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
2iP	6-(gamma,gamma-dimethylallylamino) Purine
B5 medium	Gamborg's medium
BAP	6-Benzylaminopurine
GA_3	Gibberellic acid
GLVs	Green leafy vegetables
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Kin	Kinetin
MS	Murashige and Skoog
NAA	1-Naphthaleneacetic acid
TDZ	Thidiazuron

24.1 Introduction

Consumption of green foods especially leafy vegetables benefits human health by improving nutritional status in view of their major phytonutrients and also alleviates risk of diseases like cancer, diabetes and hepatotoxicity due to their nutraceutical potential. Throughout the world these green leafy vegetables (GLVs) are part of daily meals which includes both conventional GLVs that are commercially propagated or wild GLVs often used by people familiar with them and also by ethnic communities residing in forest areas. A wide range of GLVs are in use globally and their usage is limited to a specific geographical location. Since ancient periods GLVs as a food are major source of nutrients and minerals apart from their traditional medicinal use due to the presence of phytochemicals. Being a rich source of carotenoids, anthocyanins, betalains and other pigments and minerals such as iron, they are beneficial in addressing oxidative stress, eye problems, iron deficiency, etc. (Tables 24.1 and 24.2).

During the past few decades, a large number of investigations on phytonutrients and bioactive compounds of various GLVs were reported which substantiate their potential as nutrient source as a fresh form and also as nutraceuticals in either processed or formulations based on the extractives (Saini et al. 2014; Kumar et al. 2015a, b). Nowadays there is a great emphasis to promote GLV-based fortified foods to alleviate hunger, malnutrition-related diseases in developing nations. In this regard, the best example is *Moringa* spp. leaf-based formulations which were in use as a source of major nutrients in African countries (Saini et al. 2014).

Table 24.1 In vitro propagation methods for various green leafy vegetables

Plant	Explant	Response	Hormones	Reference
Bacopa monnieri (water hyssop/ brahmi)	Shoot buds	Shoot regeneration	MS + 0.5–2.5 mg/L BAP or MS + 0.5–2.5 mg/L BAP + 0.5–2 mg/L Kin	Elangovan et al. (1995)
	Leaf and nodal explants	Callus formation	MS + 0.5 mg/L NAA + 0.25 mg/L TDZ	Vijayakumar et al. (2010)
		Shoot formation	MS + 1 mg/L BAP + 0.4 mg/L Kin + 0.4 mg/L NAA	
		Root formation	MS + IAA 0.2 mg/L + TDZ 0.04 mg/L	
	Nodal	Shoot formation	MS + 1.5 mg/L BAP + 1.0 mg/L NAA	Ramesh et al. (2009)
	Internode	Shoot regeneration and root formation	MS + 300 mg/L Bavistin and MS + 0.5 mg/L IBA	Tiwari et al. (2006)
	Shoot tips and nodal explants	Shoot multiplication	MS + 2.0 mg/L BAP + 0.2 mg/L IAA	Haque and Ghosh (2013)
		Root formation	½ MS + 50 % of Aloe vera gel	
		Flowering	MS + 0.2 mg/L BAP	
	Axillary node	Shoot regeneration	MS + 60 % *Aulosira fertilissima* (cyanobacterium)	Banerjee and Modi (2010)
		Shoot multiplication	MS + 60 % *Aulosira fertilissima* (cyanobacterium + Kin (1.0 mg/L)	
		Rooting	MS + 60 % *Aulosira fertilissima* (cyanobacterium)	
	Nodal	Shoot regeneration	MS + 0.2 mg/L BAP	Sharma et al. (2010)
		Root formation	MS + 0.15 mg/L IBA	
	Nodal	Shoot multiplication	MS + 0.5 mg/L BAP + 2.0 mg/L Kin and 0.5 mg/L Kin + 1.0 mg/L BAP	Mehta et al. (2012)
		Root formation	MS + 2.0 mg/L IBA	
	Leaf petiole	Callus induction	MS + 0.25 mg/L 2,4-D + 0.5 mg/L Kin and 0.25 mg/L 2,4-D + 0.1 mg/L BAP	
	Leaf	Callus induction	MS + 2.0 mg/L BAP + 0.5 mg/L NAA	Rout et al. (2011)
		Shoot regeneration	MS + 2.0 mg/L BAP + 0.5 mg /L NAA + 2.0 mg/L BAP + 0.5 mg/L IAA	
		Root formation	½ MS + 2.0 mg/L IAA	
	Shoot tip	Callus formation	MS + 2,4-D-2 mg/L	Talukdar (2014)
	Shoot	Shoot formation	MS + 5 M BAP	Parale et al. (2010)
		Callus formation	MS + 1 M 2,4-D + 5 M NAA	
	Leaf	Callus formation	MS + 1 mg/L NAA + 0.5 mg/L Kin	Rahman et al. (2002)

(continued)

Table 24.1 (continued)

Plant	Explant	Response	Hormones	Reference
Centella asiatica (gotu kola)	Shoot tips	Shoot regeneration	MS + 4.0 mg/L BAP + 0.1 mg/L NAA	Nath and Buragohain (2005)
		Root formation	MS + 2 mg/L BAP	
	Nodal segments	Shoot formation	MS + 22.2 μM BAP + 2.68 μM NAA	Tiwari et al. (2000)
		Shoot elongation	MS + 6.7 μM BAP + 2.88 μM IAA	
		Root formation	MS + 2.46 μM IBA	
	Leaf and internode	Callus formation	MS + 4.52 μM 2,4-D or 5.37 μM NAA + 2.32 μM Kin	Martin (2004)
		Shoot formation	½ MS + 0.054 μM NAA with either 0.044 μM BAP or 0.046 μM Kin	
	Leaf	Callus formation	MS + 9.29 μM Kin + 2.26 μM 2,4-D.	Paramageetham et al. (2004)
		Shoot formation	MS + 2.32 μM Kin + 2.89 μM GA_3	
	Nodal	Shoot multiplication	MS + 2.0 mg/L BAP + 2.5 mg/L Kin	Karthikeyan et al. (2009)
		Root formation	MS + 1.5 mg/L IBA	
	Nodal	Shoot multiplication	MS + 0.5 mg/L BAP or 0.1 mg/L BAP or MS + 1.0 mg/L BAP+ 0.5 mg/L IAA	Raghu et al. (2007)
	Stem and leaf	Callus formation	MS + 2.0 mg/L Kin + 4.0 mg/L NAA	Patra et al. (1998)
		Shoot formation	MS + 4.0 mg/L BAP + 2.0 mg/L Kin + 0.25 mg/L NAA + 20 mg/L adenine sulphate	
		Root formation	½ MS + 0.5 mg/L IAA	
Solanum nigrum L. (black nightshade)	Anther	Callus formation	MS + 1.9 mg/L NAA + 2.2 mg/L 2,4-D + 2.2 mg/L Kin	Harn (1972)
		Shoot formation	MS + 0.0–0.5 mg/L NAA and 0.0–0.5 2,4-D	
	Nodal	Shoot multiplication	MS + 10–15 μM Kin and10–15 μM BAP	Padmapriya et al. (2011)
		Root formation	MS + 10 μM IBA	
	Leaves and internodes	Callus formation	MS + 3 mg/L NAA + 0.5 mg/L BAP	Yogananth et al. (2009)
		Callus formation	MS + 3.0 mg/L NAA + 0.5 mg/L BAP	
		Shoot multiplication	MS + 3.0 mg/L BAP + 0.5 mg/L NAA	
		Root formation	MS + 0.5 mg/L IBA	

	Leaf	Callus formation	MS + 2.0 mg/L IAA + 0.5 mg/L BAP	Kolar et al. (2008)
		Shoot multiplication	MS + 6.0 mg/L BAP + 0.5 mg/L IAA	
		In vitro flowering	MS + 1.5 mg/L 2,4-D + 1.5 mg/L BAP	
		Root formation	MS + 0.5 mg/L IBA	
	Leaf	Callus formation	MS + 1 mg/L 2, 4-D	Xu et al. (2014)
	Shoot	Shoot formation	MS + 1 mg/L IBA	Hassanein and soltan (2000)
		Multiple shoot formation	MS + 0.5 mg/L BAP	
	Shoot tip and nodal	Multiple shoot formation	MS + 1 mg/L BAP + 3 mg/L BAP	Kavitha et al. (2012)
		Root formation	MS + 1 mg/L NAA	
	Leaf	Callus formation	MS + 3 mg/L NAA + 0.5 mg/L BAP	Ewais et al. (2015)
Portulaca spp. (purslane)	Shoot apex	Shoot formation	MS + 2.5 mg/L BAP	Jain and Bashir (2010) and Rajendiran et al. (2015)
		Root formation In vitro cultures	½ MS + 0.75 mg/L NAA UV light tolerance	
Coriandrum sativum L. (coriander)	Shoot	Callus formation	MS + 4.6 µM Kin	Kataeva and Popowich (1993)
		Shoot elongation	MS + 4.6 µM Kin + 1.1 µM IAA	
	Hypocotyls and zygotic embryos	Callus formation	MS + 1 mg/L 2,4-D	Kim et al. (1996)
	Shoot	Shoot formation	MS + 0.1 mg/L IBA + 0.1 mg/L BAP	Barros et al. (2012)
	Hypocotyl	Shoot formation	MS + 1 mg 2,4-D + 1 mg/L Kin	Mujib et al. (1990)
	Cotyledon and hypocotyl	Callus formation	MS + 4.52 mM 2,4-D	Murthy et al. (2008)
Rorippa nasturtium-aquaticum L. (watercress)	Petiole	Callus formation	MS + 1 µM TDZ + 0.2 µM 2,4-D	Arnold et al. (1995)

(continued)

Table 24.1 (continued)

Plant	Explant	Response	Hormones	Reference
Brasssica oleracea L. (kale)	Half and whole stem	Shoot formation	MS + 2 mg/L BA + 1 mg/L NAA or 4 mg/L BAP with 0.5 mg/L NAA	Pua et al. (1999)
	Hypocotyledon and cotyledon	Shoot formation	MS + 2 mg/L BAP, 0.4 mg/L NAA MS + 2 mg/L BAP	Qin et al. (2012)
	Flamingo bill	Shoot multiplication	MS + 2 mg/L BAP	
		Root formation	0.2 mg/L NAA	
	Root segment	Shoot regeneration	$(4.5 \times 10^{-7}$M) BAP	Wong and Loh (1988)
	Hypocotyl	Callus formation	MS + 1 mg/L BAP	Ahmad and spoor (1999)
Lactuca sativa L. (lettuce)	Axillary bud	Shoot formation	MS + 1–2 mg/L Kin and 6.4 mg/L IAA	Pink and Carter (1987)
		Root formation	MS + 6.4 mg/L IAA	
	Cotyledon	Shoot regeneration	SH + 0.44 μM BAP + 0.54 μM NAA	Teng et al. (1992)
Hibiscus sabdariffa L. and *Hibiscus cannabinus* L. (roselle and kenaf)	Cotyledonary petiole	Shoot formation	MS + 0.5 mg/L IAA + 5 mg/L BAP	Zapata et al. (1999)
	Shoot apex	Multiple shoots	MS + 1 μM TDZ	Srivatanakul et al. (2000)
	Shoot	Multiple shoots	MS + 8.8 μM BAP	Herath et al. (2004)
	Cotyledonary leaves	Root formation	MS + 0.5–3.0 mg/L NAA	Kumar et al. (2015e)
		Callus formation	MS + 1.0 mg/L NAA	
	Nodal	Shoot formation	MS+ 0.1 mg/L of Kin	Govinden-Soulange et al. (2009)
		Callus formation	MS + 1.5 mg/L TDZ	
		Root formation	MS + 2.5 mg/L IBA	

R. vesicarius L. (sorrel)	Shoot	Shoot formation	MS + 26.63 µM Kin	Abo El-soud et al. (2012)
		Root formation	2.46 µM IBA or 7.38 µM NAA	
	Nodal	Multiple shoot formation	MS + 8.8 µM BAP + 2.4 µM NAA	Nandini et al. (2013)
		In vitro flowering	MS + 8.8 µM BAP + 2.4 µM NAA + 1.4 µM GA_3	
		Root formation	½ MS + 9.2 µM Kin + 2.4 µM IBA	
	Leaf	Callus formation	MS + 2 mg/L BAP + 0.5 mg/L NAA	Nandini et al. (2014)
		Multiple shoot formation	MS + 4 mg/L BAP + 1 mg/L NAA	
		Root formation	MS + 1 mg/L NAA	
	Nodal	Shoot formation	MS + 2.0 µM BAP + 0.75 µM Kin	Lavanya et al. (2013)
		Root formation	MS + 1.0 µM BAP	
	Leaf	Shoot multiplication	SH + 2 mg/L BAP	Kakarla et al. (2014)
		Callus formation	MS + 0.5–3.0 mg/L 2,4-d + 0.5 mg/L Kin	
		Root formation	1.0 mg/L IBA	
Basella spp. (Indian vine spinach)	Cotyledon and hypocotyl	Callus	MS + 2 mg/L BAP + 0.1–2.0 mg/L NAA	Guo and Xu (2000)
		Shoot regeneration	MS + 2 mg/L BAP + 0.3 mg/L NAA	
		Root formation	MS + 0.3 mg/L NAA	
	Hypocotyls and cotyledons	Callus formation	MS + 0.1–0.3 mg/L, NAA + 1–3 mg/L BAP	Song et al. (1996)
		Shoot formation	MS + 0–0.3 mg/L NAA and 3 mg/L BAP or 3 mg/L Kin	
		Root formation	½ MS + 0.3 mg/L NAA	
	Hypocotyl	Callus formation	MS + 0.1 µM 2,4-D + 5 µM BAP	Pumchausuan and Wongroung (2009)

(continued)

Table 24.1 (continued)

Plant	Explant	Response	Hormones	Reference
Moringa oleifera Lam (drumstick tree)	Nodal	Shoot formation	MS + 4.44 μM BA	Saini et al. (2012)
		Root formation	MS + 2.85 μM IAA + 4.92 μM IBA	
	Nodal	Shoot formation	MS + 1.0 mg/L BAP	Islam et al. (2005)
	Nodal	Shoot formation	MS + 2.5 μM BAP	Marfori (2010)
		Root formation	MS + 0.25 μM NAA	
Amaranthus spp. (amaranth)	Leaf, stem	Callus	MS + 1.5 mg/L IAA + 1.5 mg/L zeatin	Yaacob et al. (2011)
	Hypocotyls, petioles, leaves	Shoot formation	BAP, zeatin, TDZ, and NAA	Flores et al. (1982), Bennici et al. (1992), Bennici and Schiff (1997), and Gajdošová et al. (2013)
Trigonella foenum-graecum L. (fenugreek)	Leaf	Shoot formation	Cytokinins	Aasim et al. (2009, 2010)
	Cotyledons and hypocotyls	Callus	B5 and MS + PGRs	El-Nour et al. (2013) and Elaleem et al. (2014)
	Callus	Shoots	MS + PGRs	Vaezi et al. (2015)

Table 24.2 Some of the important secondary metabolites produced in vitro from various GLVs

Plant	Explants	Metabolites	Reference
Amaranthus spp. (amaranth)	Seedling, leaf, stems	Coloured callus formation (betacyanins)	Bianco-Colomas (1980), Silva-Sánchez et al. (2008), Yaacob et al. (2011), and Biswas et al. (2013)
Bacopa monnieri L. (water hyssop)	Leaf-derived callus culture	Tannins, flavonoids, glycosides, terpenoids, saponins and steroids	Singh (2012)
	Callus culture from shoot tip	Bacoside A	Parale et al. (2010); Sharma et al. (2013), and Talukdar (2014)
Basella spp. (Indian spinach)	Callus	Anthocyanins pigments	Pumchausuan and Wongroung (2009)
Centella asiatica L. (gota kola)	Whole plants derived from nodal explants	Asiaticoside	Kim et al. (2004)
	Leaves	Asiaticoside, madecassoside, madecassic acid, asiatic acid	Randriamampionona et al. (2007) and Satheesan et al. (2012)
Coriandrum sativum L. (coriander)	Callus	C-glycosylated apigenin	Barros et al. (2012)
Solanum nigrum L. (black nightshade)	In vitro shoot cultures and callus cultures	Solasodine	Bhat et al. (2008), Yogananth et al. (2009), and Bhat et al. (2010)
Portulaca grandiflora L. (moss-rose purslane)	Callus	Betalain pigments	Endress (1980)
Trigonella foenum-graecum L. (fenugreek)	Callus, cell suspensions, protoplast cultures, *Agrobacterium tumefaciens*-mediated transformation	Diosgenin, trigonellin, etc.	Trisonthi et al. (1980), Ramesh et al. (2010), Christen (2002), Petropoulos (2002), Rezaeian (2011), Ciura et al. (2015), Reid et al. (2003), and Khawar et al. (2004)

For sustainable production and effective utilisation of GLVs, plant biotechnology interference would help to improve the nutrient profiles and also to develop elite plants for value addition. In vitro propagation methods are helpful to develop elite clones for mass multiplication of plants with vim and vigour. During the past five decades, researchers have made significant investigations to establish in vitro propagation protocols for a quite good number of GLVs (Mahadevan et al. 2009). Similarly, attempts were also made to screen in vitro cultures of GLVs for the presence of various phytochemicals, secondary metabolites, and also for their

improvement (Saini et al. 2012). Although 10–15 GLVs are commonly used, more number of plant genera (herbs) are traditionally in use for edible purposes, and the same needs to be explored for biotechnological improvement. As of now, there are no reviews on the improvement of GLVs through plant tissue cultures and biotechnology. This book chapter information would provide a glance at various developments happened so far in tissue culture of GLVs and also provide the necessity of research activities to be taken up for effective utilisation of GLVs. Some of the methodologies that are already optimised for GLVs could be effectively utilised to elucidate the biosynthetic pathways of respective plant. Some of the herbs such as *Mentha* spp. which finds use as a culinary spice are not covered as they are more familiar for their medicinal applications.

24.1.1 *Allium schoenoprasum* L.

Allium species are well known for their healing properties in curing of numerous problems (Keusgen 2002). *Allium schoenoprasum* (chive) is less known and has curative properties (Timite et al. 2013). It contains sulphur compounds with prominent antibacterial activity (Rattanachaikunsopon and Phumkhachorn 2008). The plants are widely used for culinary purposes, as a food condiment, and milder flavour than onion. They can also be used as an ornamental plant due to their beautiful flowers. The regeneration of plants through both callogenesis and somatic embryogenesis was achieved from different explant types, but the basal plate and young leaves were recognised as the most responsive explants (Kahane et al. 1992; Ayabe and Sumi 1998; Kenel et al. 2010). Lubomski (1990) reported the highest shoot multiplication on 20 g/L sucrose and 1.0 mg/L BAP and rooting was observed in both IAA and IBA with successful cold storage for 6 months. As they are not well studied than other systems such as garlic and onion, there is a limited number of regeneration protocols, namely, leaf explant (Rauber and Grunewaldt 1989), root sections (Zdravkovic-Korac et al. 2010) and the basal plate (Tubic et al. 2011).

In *Allium* spp. auxins were majorly used for induction of regeneration (Mukhopadhyay et al. 2005), and cytokinins (BAP) as a sole PGR has been used seldom (Kahane et al. 1992; Van Staden et al. 2008). Tubic et al. (2011) reported that the three cytokinins (BAP, Kin and TDZ) at 5 μM concentration are best on the regeneration capacity from stalk sections of chives. Tubic et al. (2016) recorded high regeneration efficiency in the presence of 10 μM TDZ for 4 weeks followed by subculture to either 5 μM Kin-containing or PGR-free medium. Similarly, a positive correlation between peroxidase, catalase and superoxide dismutase activity with 10 μM TDZ than 1 μM Kin and the regeneration capacity was observed.

24.1.2 *Amaranthus*

Amaranthus is a flowering plant that belongs to the family Amaranthaceae. *Amaranthus* is having a great interest in the last years as an agronomic crop,

alternative energy source and ornamental plant around the world (Joshi and Rana 1991; Brenner and Makus 1997; Grobelnik Mlakar et al. 2010). It is an annual, broad-leafed plant that is used as a high-protein grain, leafy vegetable or forage crop. There are several varieties of *Amaranthus* available wherein *A. cruentus* (purple amaranth), *A. retroflexus* (common amaranth), *A. spinosus* (prickly amaranth), *A. tricolor* (hybrid amaranth) and *A. viridis* (slender amaranth) which are commonly used as GLVs (Flores and Teutonico 1986). They are drought tolerant and require warm growth conditions for germination (65–75 °F). There are several varieties of *Amaranthus* that are being studied as leafy vegetable. It was found that in general, genus *Amaranthus* has potential to micropropagation. Selected genotypes via direct or indirect regeneration or via somatic embryogenesis were developed, and the importance of the type and concentration of growth regulators used for induction of morphogenic processes was emphasised (Bennici and Schiff 1997). There are reports on shoot formation from calluses derived from hypocotyl segments (Flores et al. 1982; Bennici et al. 1992) from petiole and leaf segments (Bennici et al. 1997). The adventitious shoot induction in *A. cruentus* in vitro in the presence of BAP, zeatin, TDZ and NAA was reported (Gajdosova et al. 2013).

Scoles et al. (2000) reported the pigment separation and characterisation from *A. hypochondriacus* L. Bianco-Colomas (1980) reported accumulation of more betacyanins and amaranthine from seedlings of *A. caudatus* L. cv. Pendula grown in vitro when they are cultivated in the light rather than in the dark. The seed bioactive peptide characterisation and anticancer activity were also investigated (Silva-Sánchez et al. 2008). The coloured callus production from leaf and stem explants of *A. cruentus* on different combination of hormonal medium with most optimum red callus induction on 1.5 mg/L IAA and 1.5 mg/L zeatin with leaf explant. They confirmed those red pigments as flavonoids called betacyanins (Yaacob et al. 2011). However, a recent report investigated the stable callus line with enhanced production of red colour using MS medium containing NAA and BAP, using stem explants of *A. tricolor* (Biswas et al. 2013).

24.1.3 *Apium graveolens* L.

Apium graveolens (celery – Apiaceae) is cultivated as a fresh vegetable (Ryder 1979) because they resemble the leafy vegetable, hence, generally planted in gardens as a cooking herb. Green celery is mostly used in the preparation of salads and cooking as leafy vegetable in the USA. The callogenesis from the petioles followed by embryogenesis in the suspension cultures and finally their development into plantlets was reported (Williams and Collin 1976a). Williams and Collin (1976b) observed little or no phenotypic variation among regenerated plants of celery and concluded that celery is highly stable in tissue culture. Moreover, the subsequent results showed subtle variation in regenerated plants (Browers and Orton 1982; Orton 1983a, b, 1985). Fujii (1982) investigated the field trials of celery cv. Tall Utah 52–70. Orton (1983b) identified the variation at the Pgm2 locus among somatic embryo-derived

regenerants of celery. The callus from leaf explant of celery was good on MS medium containing 2.3 μM 2,4-D + 2.8 μM Kin + 300 mg/L timentin and *Agrobacterium*-mediated transformation using bar gene as selectable marker (Loskutov et al. 2008). Donovan et al. (1994) reported the somaclonal variation from stem explants on callus initiation medium (MS + 0.5 mg/L 2,4-D + 0.6 mg/L Kin) followed by regeneration on MS medium with 30 g/L sucrose. The cell suspension immobilisation in Ca-alginate reveals that the immobilised cells showed reduced dry weight and uniform respiratory rate with 2,4-D. However, there is no increase in dry weight of the immobilised cells but small increase in respiratory rate with 2,4-D.

24.1.4 *Bacopa monnieri* L.

Being an edible, medicinal and multipurpose herb, *Bacopa monnieri* has been extensively investigated for its conservation, sustainable production by employing in vitro propagation methods. It is considered as a memory vitaliser like *Centella asiatica* in herbal formulations. In many parts of India, this plant is considered as edible and finds use in making various recipes mainly due to various health benefits attributed to this plant (Basu et al. 1967). Enumerated tissue culture protocols were optimised for its mass multiplication by using various explants such as nodal segments, axillary buds, shoot tips, leaf explants, etc. (Basu et al. 1967; Elangovan et al. 1995). Micropropagation methods for conservation of *B. monnieri* by using adventitious shoot buds on MS medium supplemented with cytokinins such as 0.5–2.5 mg/L BAP alone or combined with 0.5–2 mg/L Kin were developed (Elangovan et al. 1995). In the same study, the significance of synergistic blend of BAP or NAA with that of 2,4-D was reported for obtaining callus. Similarly, Praveen (2009) achieved the highest rate of shoot regeneration on semi-solid MS medium fortified with BAP or Kin or TDZ wherein 2 mg/L Kin showed best response. Highly proliferative callus could be obtained under synergetic activities of 1 mg/L NAA, 0.5 mg/L Kin and 1 mg/L casein hydrolysate (Rahman et al. 2002). Tiwari et al. (2006) and Ramesh et al. (2009) demonstrated the efficiency of Bavistin interference on rapid proliferation of shoot buds (45 shoots) from encapsulated single-nodal micro-cuttings of micropropagated plants. Both leaf and nodal explants were also good choice (Vijayakumar et al. 2010) for obtaining multiple shoots preferably in the presence of 2 mg/L BAP or Kin or TDZ or even in the presence of fungicide Bavistin which exhibits somewhat cytokinin-like activity. Haque and Ghosh (2013) optimised a micropropagation protocol by using shoot tips and nodal segment as explants, wherein 15–19 shoots were obtained from shoot tips and also achieved in vitro flowering of these shoots up on subculturing onto MS medium containing 0.2 mg/L BAP. The efficiency of synthetic cytokinin TDZ at 6.8 μM on high-frequency shoot bud initiation (93 shoot buds) from leaf explants was reported. Interestingly subculture of these leaf explants onto medium containing 2.2 μM BAP produced up to 130 adventitious shoot buds by the end of three subcultures. In addition to these, a novel tissue culture medium formulation was explored by blending MS medium with a cyanobacterium extract (*Aulosira fertilissima*) which could generate up to 400 shoots per nodal explant upon three

subcultures (Banerjee and Modi 2010). In most of the cases, the explant source, viz. ex vitro or in vitro, is the key factor to show varied responses for shoot multiplication. Especially, the concentration of cytokinin such as BAP or Kin (Kin-0.01–0.3 mg/L) found to be good enough to get 40 multiple shoots (Sharma et al. 2010) from nodal explants. A glance at various reports reveals that subculturing onto the respective shoot bud induction media or quite low cytokinin-containing media supports further shoot elongation. In vitro rooting of microshoots is not a difficult task in *B. monnieri* which could be achieved on MS or half MS media devoid of any growth regulators or very low concentration of IBA or IAA or NAA or combination of auxins (Banerjee and Modi 2010; Mehta et al. 2012).

Efficient callus induction and multiplication from leaf petiole explants in the presence of 0.25 mg/L 2,4-D and 0.5 mg/L Kin or 0.25 mg/L 2,4-D and 0.1 mg/L BAP were reported (Mehta et al. 2012). Rout et al. (2011) achieved callus-mediated organogenesis from *B. monnieri* by using leaf explants on MS medium with 2 mg/L BAP and 0.5 mg/L NAA. Subsequently shoot regeneration from callus was obtained in combination of 2 mg/L BAP and 0.5 mg/L NAA or 2 mg/L BAP and 0.5 mg/L IAA.

24.1.4.1 In Vitro Production of Metabolites

Compared to in vitro culture such as shoot cultures, leaf-based callus suspension cultures were proved to be highly effective in producing bacoside A, wherein up to five- to six fold increased in bacoside A could be obtained in 40 days on MS liquid medium comprising 1 mg/L NAA, 0.5 mg/L Kin, 1 g/L casein hydrolysate and 30 g/L sucrose (Rahman et al. 2002). Singh (2012) investigated the presence of various photochemicals in leaf-derived callus culture wherein the presence of glycosides, terpenoids, tannins, flavanoids, saponins and steroids was formed. Similarly, Talukdar (2014) established callus culture from shoot tip explants in the presence of 2,4-D and screened these cultures for the biosynthesis of bacoside A tetracyclic triterpenoid saponin which showed 1.53 % of total bacoside content. In a recent study, Sharma et al. (2013) are able to augment bacoside production in in vitro shoot cultures by challenging with abiotic elicitors such as jasmonic acid, copper sulphate and salicylic acid, wherein 1.24-fold higher bacoside was produced compared to control shoots. Similarly by feeding various organic supplements to culture medium, enhanced production of bacoside A in both shoot and callus cultures with up to 1.2–4 times higher was shown (Parale et al. 2010). In a recent study, 1.8-fold increase in bacoside content was achieved in in vitro shoot cultures on medium containing 50 μM methyl jasmonate (Sharma et al. 2013).

24.1.5 *Basella* spp.

Basella spp. (family – Basellaceae) are commonly known as Ceylon spinach, Indian spinach and Malabar nightshade, and the plant is highly exploited for its medicinal properties in Chinese traditional medicine and in ancient Indian medical system to treat constipation and also used as a diuretic, a toxicide and an anti-inflammatory (Toshiyuki et al. 2001). There are two common species such as *Basella rubra* L.

(red stem and leaves) and *Basella alba* L. (green stem and leaves), which are twining herbaceous vines, perennial, succulent, branched, smooth and annual and found in tropical and subtropical areas (Khare 2004). Recently, the nutritional facts of the leaves of *Basella* spp. were explored (Kumar et al. 2015a), a method for red-violet pigment extraction, characterisation and food application Kumar et al. (2015b, c), and functional attributes of *B. rubra* fruit extracts against human cervical carcinoma cells (Kumar et al. 2015d) were also been reported.

There are limited data on the in vitro micropropagation of *Basella* spp. *B. alba* hypocotyl explants induced callus in MS medium containing BAP, NAA and 2,4-D individually or in combinations wherein cotyledon explants failed. However, there is bud regeneration from callus of hypocotyl supplemented with BAP and NAA. Rooting was best in medium containing half MS medium and 0.3 mg/L NAA (Guo and Xu 2000). Similarly, Song et al. (1996) reported the use of different explants (hypocotyls and cotyledons) on MS medium containing different combinations of NAA, BAP and Kin in which 0.1–0.3 mg/L NAA and 1–3 mg/L BAP with hypocotyls explants showed callus growth. The same callus was transferred to 0–0.3 mg/L NAA and 3 mg/L BAP or 3 mg/L Kin-induced buds which in turn induced rooting on half MS medium supplemented with 0.3 mg/L NAA (Song et al. 1996). In vitro production of anthocyanin-pigmented callus from hypocotyl explants on MS medium containing 0.1 μM 2,4-D and 5 μM BAP was achieved (Pumchausuan and Wongroung 2009). The coloured callus culture developed on MS medium contains 0.1 μM 2,4-D and 5 μM BAP. The cell suspension cultures with different concentrations of sucrose (3 %) and UV illumination for 20 min on the production of anthocyanins pigments have also been reported (Pumchausuan and Wongroung 2009).

24.1.6 *Brassica oleracea* L.

Kale is an important GLV in both oriental countries and Europe and also other parts of the world. In vitro propagation methods for these GLVs were initiated for decades ago. Different plant parts such as cotyledons (Hu et al. 2006), hypocotyls (Zee and Hui 1977; Huang et al. 2004), stems (Pua et al. 1999), root segments (Wong and Loh 1988) and axillary bud explants (Huang et al. 1999) were used for in vitro propagation. The nature of explants is reported to be important for optimising growth media and also to get maximum response for shoot bud proliferation. Ahmad and Spoor (1999) investigated the role of NAA and BAP combinations on induction of callus, its growth and regeneration of shoots. Pua et al. (1999) opined that half- and whole-stem explants were more responsive for regeneration compared to cotyledons and hypocotyls. Growth medium comprising MS salts with 2 mg/L BAP in synergy with 1 mg/L NAA or 4 mg/L BAP with 0.5 mg/L NAA is able to induce 100 % response. Recently, Qin et al. (2012) established in vitro regeneration protocol for Chinese kale wherein silver nitrate incorporation into culture medium

comprising 2 mg/L BAP and 0.4 mg/L NAA induced 86.7 % and 94 % response for hypocotyl and cotyledon explants, respectively. Explants such as flamingo bill (the portion of a seed upon removal of one cotyledon and primary and secondary meristems from seedlings but retaining the radical hypocotyledon and one cotyledon) were used. Up to four to five multiple shoots were induced in the presence of 2 mg/L BAP. Effective rooting of adventitious shoots could be obtained on MS medium comprising 0.2 mg/L NAA (Qin et al. 2012).

24.1.7 *Centella asiatica* L.

Centella asiatica is an important medicinal plant in traditional medicine (Warrier et al. 1994; Sivarajan and Balachandran 1994), and also its leaves are consumed as leafy vegetables in view of its role in improving the memory and strength. The important metabolites of this plant comprise a bitter compound, vellarin; an alkaloid, hydrocotylin; and the glycosides, centalloside and asiaticoside (Anonymous 1950).

Extensive in vitro propagation methods have been studied for this plant to obtain organogenesis through callus cultures (Patra et al. 1998; Josekutty 1998), shoot tips (Nath and Buragohain 2003), axillary buds (Tiwari et al. 2000), leaf explants (Banerjee et al. 1999), stolons (Sampath et al. 2001) and somatic embryos (Martin 2004; Paramageetham et al. 2004). Up to 15 shoots were obtained in the presence of 2 mg/L BAP in MS medium (Karthikeyan et al. 2009). Callus-mediated organogenesis showed rapid in vitro shoot bud regeneration from stem-derived callus (42.8 shoots/culture) and leaf-derived callus (54.3 shoots/culture), respectively, upon culturing on medium supplemented with 4 mg/L BAP, 2 mg/L Kin, 0.25 mg/L NAA and 20 mg/L adenine sulphate (Patra et al. 1998). In an interesting study, low-cost alternatives were explored for efficient in vitro shoot multiplication wherein laboratory-grade sucrose and tap water were used (Raghu et al. 2007). As per many reports, in vitro rooting of shoots is not a difficult task as it could be achieved in the presence of low concentration of auxins such as IAA or IBA or even on medium devoid of any auxins (Patra et al. 1998; Karthikeyan et al. 2009). High-frequency somatic embryogenesis and plant regeneration were reported by Martin (2004), wherein up to 203 somatic embryos/100 mg callus were obtained from embryogenic callus on half-strength MS medium provided with 0.54 μM 2,4-D and 1.16 μM Kin followed by 88 % of them conversion to plantlets on half-strength semi-solid MS medium with 0.054 μM NAA and 0.044 μM BAP. Callus cultures were successfully established along with suspension culture development by using leaf explants on MS medium comprising 1 mg/L BAP and 1 mg/L NAA or 0.5 mg/L BAP and 1 mg 2,4-D (Nath and Buragohain 2005). The established callus cultures are able to produce asiaticoside 494.62 mg/g dry mass.

24.1.7.1 In Vitro Production of Metabolites

Stimulation of different secondary metabolites of importance from *Centella asiatica* plant parts was investigated especially asiaticoside derivatives in view of their possible role in treating Alzheimer disease (Mook-jung et al. 1999). Preliminary studies in this report showed 50 % increase in asiaticoside content in micropropagated shoot. Subsequent studies (Kim and Botella 2004) proved that whole plants derived from nodal explants are able to produce significantly high content of asiaticoside compared to ex vitro plant material. Elicitor-mediated stimulation of asiaticoside accumulation in plants grown in bioreactor condition was investigated (Kim et al. 2004), wherein, to achieve 1.53–1.41-fold increase in asiaticoside production, the plants were challenged with methyl jasmonate and yeast extract. The contents of various triterpenoid metabolites such as asiaticoside, madecassoside, madecassic acid and asiatic acid were detected in the leaves of in vitro-propagated plants (Randriamampionona et al. 2007) to get large quantity of asiaticoside generation 7.12 mg/g dry weight. Satheesan et al. (2012) attempted to enhance asiaticoside production in *Centella asiatica* by employing root colonisation method wherein the tissue-cultured plants were infected with an endophytic fungus *Piriformospora indica* which not only enhances efficient rooting biomass but also metabolite production.

24.1.8 *Coriandrum sativum* L.

This edible herb is having great economic importance and most commonly used as a flavouring spice in Indian, Chinese and Oriental cuisine. The seeds contain an unusual fatty acid petrocelinic acid which comprises 85 % (w/w) of the total seed fatty acid. Establishment of in vitro culture of this plant would pave way for in vitro production of various bioactives from this plant. Initial attempts were made by Kataeva and Popowich (1993) to establish *C. sativum* shoot through micropropagation. Similarly attempts were also made for in vitro shoot cultures and callus cultures for this plant (Liu et al. 2002; Barros et al. 2012).

Somatic embryogenesis is reported to be very successful and reproducible for regeneration of large number of shoots (Zee 1981). Initially, hypocotyls callus-mediated somatic embryogenesis in the presence of 1 mg/L 2,4-D and 1 mg/L Kin in MS medium was reported (Mujib et al. 1990). Subsequently, similar studies were carried out by Stephen and Jayabalan (2000). Kim et al. (1996) reported high-frequency plant regeneration via somatic embryogenesis by using callus suspension culture. In vitro flowering in shoots derived from shoot tip explants was also reported (Stephen and Jayabalan 1998). Embryogenic callus could be obtained in the presence of 1 mg/L 2, 4-D and 0.2 mg/L Kin. Repeated subculturing on the same medium lead to globular embryogenic callus development. Subculturing of the same embrogenic callus in differentiated media comprising 1 mg/L NAA or 0.5 mg/L IAA along with 2 g/L casein hydrolysate induced embryogenesis. Further refinement to the protocol for somatic embryogenesis of *C. sativum* was attempted by Murthy et al. (2008) by inducing embryogenic callus from cotyledon and

hypocotyl segments. Barros et al. (2012) attempted to find variation in phenolic profiles of in vitro and in vivo grown plants of *C. sativum*, wherein the richness of phenolic acids and derivatives in fruits of in vivo grown plants and C-glycosylated apigenin as a main compound in in vitro samples were shown.

24.1.9 *Hibiscus sabdariffa* L. and *Hibiscus cannabinus* L.

Hibiscus sabdariffa and *H. cannabinus* belong to the family Malvaceae that are familiar as roselle and kenaf, respectively, in many parts of the world. They are an annual erect, herbaceous subshrub, grown in tropical and subtropical climates. The plants are bushy, with nearly smooth, cylindrical, typically red stems with red petioles and a red or pale yellow colour calyx and green stem and green leaves that is considered edible (Brouk 1975; Purseglove 1986; Morton 1987). They are one of the preferred GLVs in southern parts of India especially in Andhra Pradesh and Telangana. The plants are reported to have medicinal properties such as antihypertensive, antidiabetic, antimicrobial and chemopreventive and other folklore medicinal applications as a diuretic and mild laxative (Mayol-Soto and Aragón-Vargas 2002; Kuriyan et al. 2010; Lin et al. 2011). Micropropagation of roselle provides a powerful tool to eliminate virus, leading to the production of healthy and vigorously growing planting material (Narayanaswamy 1997). The vegetative propagation methods offer many benefits including the ability to regenerate clones, convenience and ease of propagation, combination of genotypes and reduction of length of juvenile period (Hartman et al. 1994). Most of in vitro propagation studies were on kenaf (Zapata et al. 1999; Srivatanakul et al. 2000; Khatun and Naher 2002; Herath et al. 2004), except a sporadic report on plant regeneration from hypocotyl-based callus in *H. siryacus* (Jenderek and Olney 2001) and somatic embryogenesis in *H. sabdariffa* (Raoul et al. 2010). A very recent study on the in vitro production of ascorbic acid from normal root cultures initiated from leaf explants of kenaf was reported (Kumar et al. 2015e). Recent investigations have further added the value to this plant, as evidenced by the ascorbic acid content of *H. sabdariffa* (Sarkiyayi and Ikioda 2010) and optimisation of leaf-drying methods for effective utilisation of foliage for food formulations (Kumar et al. 2015f).

The callus induction and somatic embryogenesis from *H. sabdariffa* by using different sugars on three types of explants (root, hypocotyl, cotyledon) and diverse combinations of plant growth regulators in MS and Drivers and Kuniyuki walnut (DKW) medium were studied (Raoul et al. 2010). Ayadi et al. (2011) reported the formation of multiple buds from young buds and cotyledons. In another study, the high-frequency multiple shoot induction and proliferation to complete propagation were reported in kenaf through apical and nodal shoot explant on hormone-free MS medium and hardened in greenhouse conditions with 70 % survival rate (Ayadi et al. 2011). Govinden-Soulange et al. (2009) reported that the 8 weeks of culturing of *H. sabdariffa* microshoots in the presence of 2.0 mg/L IBA on MS medium was required for induction of rooting.

According to earlier reports for *H. cannabinus* (kenaf), BA and Kin at different concentrations alone (Herath et al. 2004) or in combination with auxins like NAA and other substances such as M-topolene (Gomez-Leyva et al. 2008), IAA were used, wherein rosette-like shoot bud mass along with microshoots was prevalent, followed by subsequent subculturing to get 12–13 shoots per explant (Chen et al. 2010). Chen et al. (2010) reported that in kenaf 12–14 shoot production in MS medium supplemented with 5 mg/L BA, 0.3 mg/L IAA and 0.2 % (m/v) F-68 with 100 % response. The rooting was achieved with IAA and NAA combination. Srivatanakul et al. (2000) reported multiple shoot induction in MS medium supplemented with 1 μM TDZ wherein the highest shoot number in different cultivars of kenaf was noticed and upon subculturing in hormone-free medium-induced shoot elongation and rooting. In fact, in kenaf (*H. cannabinus*) too, in vitro rooting could be achieved efficiently on both hormone-free medium (Ayadi et al. 2011) and in the presence of respective auxins (Herath et al. 2004) which varies with the cultivar. Ayadi et al. (2011) reported a micropropagation protocol for kenaf (*H. cannabinus*) wherein 3.66 multiplication rates could be achieved upon five subcultures with 4–6 weeks of culturing at each level.

24.1.10 *Lactuca sativa* L.

In vitro propagation of lettuce to develop breeding material by employing tissue culture was reported (Pink and Carter 1987). Effective shoot growth from maxillary bud explants was induced in the presence of 1–2 mg/L Kin and 6.4 mg/L IAA. In vitro rooting of these shoots was obtained in the presence of 6.4 mg/L of IAA followed by 90–95 % of survival upon field transfer. The main constraint in micropropagation of lettuce is its long culturing period. To overcome this, efforts were made (Teng et al. 1992) for rapid regeneration from suspension cultures of cotyledon-derived callus that was used to establish suspension cultures followed by optimisation of various parameters such as quality of callus, light intensity, the type of carbohydrate and its concentration and various auxins and cytokinins influence on growth and differentiation in suspension cultures. Hundreds of shoot production from 50 to 55 mg of cell aggregates within 2 weeks were reported when suspension cultures were grown in Schenk and Hildebrandt (SH) (1972) basal medium comprising 1 g/L inositol, 1.5 % glucose, 0.4 μM BAP and 0.54 μM NAA (Teng et al. 1992). The main advantage in this protocol was that there is no necessity of cell aggregates from suspension culture transferring onto solid media as reported earlier for lettuce by Alconero (1983) and Sasaki (1975) which requires 5 weeks to 3 months.

In order to address salinity-related problems in cultivation of lettuce crop, salt tolerance was induced in lettuce via plastid genetic engineering wherein betaine-aldehyde dehydrogenase (BADH) was transferred to the plant (Suleiman et al. 2012). Similarly in a study, a genotype-independent transformation of lettuce was investigated by Curtis et al. (1994) wherein 13 lettuce cultivars were assessed for the suitability to *Agrobacterium*-mediated gene transfer. Genetic manipulation

technology for improvement of lettuce was initiated by Kim and Botella (2004). Recently, somaclonal variation and selection approach were used (Cheng et al. 2014) to develop polyphenol-rich Rutgers Scarlet Lettuce (RSL), i.e. *L. sativa*. Lettuce being an important vegetative crop having economic importance, tissue culture studies (Koevary et al. 1978; Pink and Carter 1987; Bloksberg and Salveit 1986; Ampomah-Dwamena et al. 1997) were attempted. Apart from these, to determine the response of apical and axillary shoot tip explants of lettuce to the storage time and head maturity was demonstrated by Jenni et al. (2006) wherein core length as a source of variation between maternal explants was also studied.

24.1.11 *Moringa oleifera* L.

Moringa oleifera is commonly known as drumstick or ben oil tree. It is a widely cultivated species of Moringaceae and native to the sub-Himalayan tracts of Northwestern India. The plant is a fast growing tropical perennial soft-wooded tree with a long history of traditional medicine and culinary uses. There are some other species of genus *Moringa* like *M. stenopetala* which is an important crop in Kenya and Ethiopia (Verdcourt 1985). Similarly, *M. peregrine* was known to the ancient Egyptians who utilised its seed oil. However, some of the species are in danger of extinction, especially *M. hildebrandtii* which is now extinct in the wild (Olson and Razafimandimbison 2000). *M. oleifera* leaves are a very good source of food rich in nutrients and minerals, and the tree has maximum leaves at the end of the dry season when other foods are typically scarce (Fuglie 1999). There are few reports on the tissue culture of *M. oleifera* especially through clonal propagation using nodal explants taken from non-aseptic source, either from young seedlings or matured plants (Stephenson and Fahey 2004; Islam et al. 2005; Marfori 2010). Hence, the preservation of the *Moringa* spp. is thus of great concern from biodiversity, ethnobotanical, dietary and pharmacological perspectives. In a recent report, Saini et al. (2012) reported that the young nodal sections grown aseptically on MS medium supplemented with 4.44 μM BAP producing an average of 9–10 shoots per explant after 15 days of inoculation. Similarly, the rooting of individual shoots was obtained on 2.85 μM IAA along with 4.92 μM IBA. The micropropagated plants were more superior over control plants to contain 13.4% and 14.7% higher amount of α-tocopherol and total carotenoids, respectively.

24.1.12 *Portulaca spp.*

Portulaca species are succulent herbs with profuse branching. They are pharmaceutically important plants (Chavalittumrong et al. 2007). In some parts of India, *P. grandiflora* H. and *P. oleracea* L. are popularly known as edible purslane are used as leafy vegetable in view of nutrients and nutraceuticals. The arial part of this plant contains metabolites, viz. portulal, portulenone, portulenol, portulene, etc. In vitro propagation of this plant has been reported by using nodal segments (Jain and

Bashir 2010). Shoot proliferation up to 57 shoots per explant was observed in MS medium containing 4 mg/L BAP followed by efficient rooting in the presence of 0.75 mg/L NAA. Shoot explants are also able to induce rapid direct shoot multiplication on MS medium containing 2 mg/L BAP followed by rooting on half MS medium with 0.75 mg/L NAA (Jain and Bashir 2010). Similar studies for *P. olaracea* was also reported (Rajendiran et al. 2015).

P. grandiflora callus cultures were able to produce betalain pigments. Especially, accumulation of betacyanins can be regulated by inhibiting the accumulation of DOPA. Tyrosine being a precursor of DOPA investigations was carried out to produce tyrosine from biochemical conversions by using *Portulaca* callus (Endress 1980) wherein phenylalanine was used as a substrate.

24.1.13 *Rorippa nasturtium-aquaticum* L.

Watercress is one of the popular high-value salad crop grown in the UK and other parts of Europe. This plant commonly grows in large gravel-based beds of shallow flowing calcarious spring water in southern parts of UK (Ryder 1979). As the breeding programmes are very limited and also the absence of any cultivars (Ryder 1979), in vitro propagation methods for its propagation were attempted. Moreover, crook root is a fungal disease caused by *Spongospora subterranea* f. sp. that causes a huge loss to this crop. To address this, the potential use of somaclonal variations in watercress was investigated (Arnold et al. 1994, 1995; Claxton et al. 1998). Callogenesis could be obtained from petiole explants on MS medium comprising 1 μM TDZ and 0.2 μM 2,4-D which subsequently produces up to 4–5 shoots per gram of callus in 4 weeks. The regenerated adventitious roots were screened to find the resistant clones against fungal diseases (Arnold et al. 1994). Similarly various explants such as hypocotyl, cotyledons, petiole, true leaf explants, stem and shoot tip cultures were also tested for callus induction and organogenesis. Especially, the shoot tip culture obtained from 14-day seedlings was grown on MS medium supplemented with 0.44 μM BAP that leads to shoot proliferation.

24.1.14 *Rumex vesicarius* L.

Rumex vesicarius (Polygonaceae), commonly called as dock sorrel or bladder dock, is an annual, pale green herb. This plant finds use as a leafy vegetable in some part of India and also as a medicinal plant. The seed germination, growth, development and effect of exogenous gibberellic acid and the effect of different temperatures on the germination rate, germination percentage and some chemical contents of sorrel for 16 days was reported (Asrar 2000, 2011). The percentage of seed germination was recorded with 200 ppm GA_3 (62 %) followed by 100 ppm GA_3 (53 %) after 48 h of soaking at 20 °C in growth chamber, whereas the percentage seed germination decreases by increasing the incubation temperature (Asrar 2011).

Micropropagation of *R. vesicarius* shoots with MS + 8.88 μM BAP with an average of 11 shoots, MS + 26.63 μM Kin produced 4.6 shoots and rooting was best

achieved in medium containing 2.46 μM IBA or 7.38 μM NAA (Abo El-soud et al. 2012). Similarly, multiple shoots were induced on medium containing 8.8 μM BAP and 2.4 μM NAA with a maximum of 4–6 shoots, and rooting was induced in half-strength MS medium containing Kin, IBA supplemented with 1.5 % activated charcoal (Nandini et al. 2013). In vitro flowering was also successful on MS medium containing BAP, NAA and GA_3 (Nandini et al. 2013). The somatic embryogenesis, profuse callusing and regeneration on MS medium with 2 mg/L BAP and 0.5 mg/L NAA were reported (Nandini et al. 2014). The shoot organogenesis from leaf-derived callus on MS medium supplemented with 4 mg/L BAP, 1 mg/L NAA and rooting of the microshoots were done in half-strength MS with 1 mg/L NAA (Nandini et al. 2014). High concentration of auxin in combination with less concentration of cytokinin induced the somatic embryogenesis and its maturation (Nandini et al. 2014). Lavanya et al. (2013) too reported the in vitro clonal propagation methods for *R. vesicarius*. The leaf-derived callus-mediated shoot multiplication in Schenk and Hildebrandt (SH) medium with 2 mg/L BA was studied, wherein 90 % of callusing and 85 % multiple shoots were induced (Kakarla et al. 2014). The in vitro bioactive compound production was also demonstrated (Alam 2012).

24.1.15 *Solanum nigrum* L.

Solanum nigrum is commonly known as black nightshade. It is well familiar for its medicinal properties in view of two important alkaloids solamargine and solasoline in its plant parts. This plant is considered as a model plant in view of high multiplication rate and easy in vitro propagation. Accordingly reports on direct regeneration of shoots from leaf explants (Sreedhar et al. 2008; Saritha and Naidu 2008; Mingozzi and Morini 2009), protoplast cultures (Hassanein and Soltan 2000), production of plants from anthers (Harn 1972), shoot tips (Verma et al. 2010) and nodal explants (Padmapriya et al. 2011) were available. In fact, the tissue culture attempts were initially attempted in the 1970s (Harn 1972) to produce haploid plants by anther culture through callus-mediated organogenesis on MS medium supplemented with 1.9 mg/L NAA, 2.2 mg/L 2,4-D and 2.2 mg/L Kin followed by their transfer onto 0.5 mg/L NAA and 2,4-D. In vitro shoot cultures are easily established from shoot cuttings from in vitro-germinated seedlings on MS medium or B5 medium with suitable plant growth regulators. B5 medium supplemented with 0.5 mg/L BAP was found to be good for multiple shoot production followed by its in vitro shoot production with 1 mg/L IBA (Hassanein and Soltan 2000). By using shoot tip explants, up to 20 multiple shoots could be obtained in the presence of 1 mg/L BAP and 3 mg/L BAP from shoot tips and nodal explants, respectively (Kavitha et al. 2012). Supplementation of media with NAA (1 mg/L) supports efficient in vitro rooting within 2–3 weeks. Both Kin and BAP found to be more responsive for shoot induction from nodal explants, wherein up to 40–49 multiple shoots were produced from nodal explants (Padmapriya et al. 2011). The obtained shoots were able to respond effectively for in vitro rooting in the presence of 10–15 μM of IBA or 2,4-D. Efficient methods for induction of callus culture from leaf explants and highly proliferative

cultures that able to produce multiple shoots (indirect organogenesis) were also developed (Yogananth et al. 2009; Sridhar and Naidu 2011; Mahadev et al. 2014).

In vitro flowering induction in established microshoots of *S. nigrum* was reported on MS medium containing 2,4-D and BAP (Kolar et al. 2008). An efficient somatic embryogenesis protocol by using root, stamen and leaf explants was reported by Xu et al. (2014), wherein 93–100 % response for producing embryogenic calli on MS medium comprising 1 mg/L 2,4-D under dark condition was achieved. Subsequently the development of frog egg-like bodies was achieved followed by plantlet formation in the presence of 0.5 mg/L BAP and 0.1 mg/L GA_3. Ewais et al. (2015) evaluated the influence of biologically synthesised silver nanoparticles on *S. nigrum* callus response from leaf explants. Attempts were also made for *Agrobacterium rhizogenes* mediated transformation of *S. nigrum*. Even for bioremediation studies, the in vitro cultures of *S. nigrum* are reported to be a good choice. Being a cadmium hyperaccumulator, cadmium toxicity was evaluated by studying protective effects of proline in callus and in vitro-regenerated shoots on *S. nigrum* (Xu et al. 2009). Verma et al. (2010) explored the possibility of preservation of germplasm through encapsulation of shoot tips by using alginates.

24.1.15.1 In Vitro Production of Metabolites

Significant contributions towards secondary metabolites production in in vitro cultures of *S. nigrum* were made by researchers wherein metabolites such as solasodine were analysed, quantified and also improved in established in vitro shoot cultures and callus cultures (Bhat et al. 2008, 2010; Yogananth et al. 2009). Similarly analysis of various phytochemical constituents that exhibit antimicrobial activities from in vitro callus cultures and crude plant extracts from *S. nigrum* was reported. The maximum solasodine content of 2.34 mg/g dry mass was observed in in vitro-regenerated shoots derived from leaves and 0.76 mg/g dry mass in non-regenerative callus which work better than 0.5 mg/g dry mass field-grown shoots (Bhat et al. 2010). Prior to this, similar observations for higher content of solasodine in in vitro callus cultures of *S. nigrum* were achieved by Yogananth et al. (2009) wherein up to 0.142 mg/g dry mass and 0.116 mg/g dry mass of solasodine was documented in callus culture grown in terms of 2.5 mg/L IAA +0.5 mg /L BAP and 2 mg/L NAA + 0.5 mg/L BAP, respectively.

24.1.16 *Spinacia oleracea* L.

Spinach (*S. oleracea*) is a dioecious plant native to West Asia and probably Iran and at present is widely cultivated in the world as one of the most popular vegetables and known as a rich source of iron, vitamins and minerals (Bao et al. 2009). It is an important vegetable crop of which dioecy in nature has made cultivar improvement difficult using traditional breeding methods (Neskovic and Radojevic 1973) attempted micropropagation of spinach using seeds. The callus induction from leaf

explants of spinach could be achieved with MS medium containing 2 mg/L Kin and 0.5 mg/L 2,4-D, and shoot regeneration occurred upon transferring to medium containing 2 mg/L Kin, 0.01 mg/L 2,4-D and 1 mg/L GA_3. The established shoots were rooted on 1 mg/L IBA medium and transferred to field condition with 60 % survival rate (Al-Khayri et al. 1991). Al-Khayri et al. (1992) studied the stimulator action of coconut water in two cultivars of spinach, High Pack and Baker, and showed that the addition of 15 % (v/v) coconut water to the culture medium improved callus growth, shoot regenerative capacity and shoot growth in leaf disc cultures of spinach. The callus weight obtained after 5 weeks showed direct relationship to the varied concentration of coconut water added to the media. The shoot regeneration was noticed to be faster in coconut water containing media which took 4–5 weeks compared to 8–12 weeks on a coconut water-free media. Shojaei et al. (2010) identified the best explants and media for spinach tissue culture. Though the effects of explants were not significant except on regeneration phase, the medium for callus induction was found to be MS media comprising 1.5 mg/L IAA and 2.5 mg/L GA_3. Shoot regeneration was best in MS media containing 0.5 mg/L NAA and 2 mg/L GA_3. The highest shoot regeneration frequency of 84 % was obtained in this media. The best rooting medium reported was MS medium containing 0.5 mg/L IBA.

24.1.17 *Talinum portulacifolium* L.

T. portulacifolium is an erect shrub belonging to the family Portulacaceae. It is a very well-known important medicinal plant in the local system of medicine and commonly wild grown in Tamil Nadu, Karnataka and other parts of India (Nair and Henry 1983). It is commonly used as GLVs due to its rich vitamin A and mineral content and to strengthen the body. The low survival rate by stem cuttings restricts its mass propagation via conventional methods. The tissue culture plants have been reported to possess superior field performance to those derived from stem cutting in terms of survival rate, fruit yield, rhizome production and plant weight (Gustavsson and Stanys 2000).

There is a very limited data on the in vitro micropropagation of *T. portulacifolium* compared to other species of *Talinum*. The shoot proliferation of *T. portulacifolium* was efficient in MS medium with 6 μM BAP and 2 μM IAA (Thangavel et al. 2008). It could be possible to get eight new shoots from single explant after three subcultures with 15 days of interval. The rooting was facilitated on MS medium containing with 4 μM IBA and 1 μM NAA (Thangavel et al. 2008).

Rao et al. (2009) reported the anti-hyperglycemic activity of *T. portulacifolium* leaf methanol extracts against alloxan-induced diabetes. Similarly, the antidiabetic activity and antioxidant effects in the liver and kidney like malondialdehyde (MDA), reduced glutathione (GSH) and catalase levels of methanol extracts showed a significant effect in rat model (Thalapaneni et al. 2011).

24.1.18 *Trigonella foenum-graecum* L.

Trigonella foenum-graecum is a medicinal plant extensively distributed in most regions of the world belonging to the family Leguminaceae. The seeds and leaves have been extensively studied not only for medicinal aspects (Shani et al. 1974) but also for nutritional value (Rajagopalan 1998). Elaleem et al. (2014) reported the in vitro callus induction on both B5 and MS medium from cotyledons and hypocotyl explants with different combinations of plant hormones where B5 medium showed good response. The seeds have been extracted for polysaccharides, galactomannan and different saponins such as diosgenin, yamogenin, mucilage, volatile oil and alkaloids such as choline and trigonelline (Seasotiya et al. 2014). El-Nour et al. (2013) reported that the two auxins on callus induction using cotyledons and hypocotyls explants. There are many bioactive, i.e. trigonelline, coumarin and nicotinic acid, compounds isolated from fenugreek seeds having medicinal activity especially diabetes (Moorthy et al. 2010). The in vitro shoot regeneration using different cytokinins from fenugreek has been explored (Aasim et al. 2009, 2010).

24.1.18.1 In Vitro Production of Shoots and Metabolites

There are several studies on *Trigonella* covering fields from secondary metabolite production from cell suspension culture (Cerdon et al. 1945; Khanna et al. 1975; Trisonthi et al. 1980; Ramesh et al. 2010), callus culture (Joshi and Handler 1960; Khanna and Jain 1973; Radwan and Kokate 1980), protoplast culture (Shekhawat and Galston 1983; Christen 2002; Petropoulos 2002) and organogenesis (Khawar et al. 2002; Prabakaran and Ravimycin 2012), and *Agrobacterium tumefaciens*-mediated genetic transformation (De La Riva et al. 1998; Merkli et al. 1997; Reid et al. 2003; Khawar et al. 2004) has been reported. Prabakaran and Ravimycin (2012) investigated the chlorophyll pigment content in the callus with different morphology and the in vitro-regenerated plants. An optimised method for in vitro culture conditions for diosgenin accumulation has been reported (Rezaeian 2011; Ciura et al. 2015). Vaezi et al. (2015) reported the indirect plant regeneration using different plant growth regulators in vitro established shoot from callus cultures.

24.2 Future Prospects

GLVs being a rich source of nutrients and nutraceuticals are easily accessible to wide range of consumers at affordable price. Modern agricultural practices, including polyhouse/greenhouse growing techniques that adopted of late in many developing countries to combat the adverse effects of environment contributed to a greater extent to their sustainable production and availability throughout the year to consumers. However, some of these GLVs are specific to geographical conditions. As explained above in this chapter, successful efforts were made by researchers to improve the quality of some commonly used GLVs with the intervention of plant tissue culture and other biotechnological methods. During this course, some

important biomolecules are also identified which are otherwise known for their antioxidant potential. Many of such secondary metabolites identified are known for their potential applications in medicine hence could be potential nutraceuticals. But due to their low concentrations in intact plant parts, alternate methods for their production through callus cultures or root cultures are having paramount significance as it will lead to scale-up studies and also to pursue downstream processing for economic prospects. In in vitro cultures of plants such as *Basella rubra* and *Amaranthus* species, betalain pigments were streamlined. Whether it is for a pigment or other metabolites, this alternate mode of production could act as a model for elucidating respective metabolite biosynthetic pathway. So, sustainable efforts are required to further fine-tune the existing methods for improvement of the quality of GLVs and also to embrace emerging technologies for large-scale production of GLVs and their effective utilisation upon postharvest (Fig. 24.1). As explained above, recent research has identified many of GLVs as a valuable resource of nutrients and metabolites with potential for multipurpose uses and also as a source for preparing raw materials of pharmaceutical industry. Now it is a well-established

Fig. 24.1 Scope for biotechnological improvement of green leafy vegetables

fact that GLVs are the source of bioactives and other constituents such as protein, mucilage and fixed oils, as well as culinary uses for both traditional and modern flavouring. The demand for such plant-based metabolites, mainly with a higher content, prompted more directed tissue culturing efforts. Successful efforts by researchers assert that there are possibilities for enhancing the chemical constituents by the use of tissue and static or suspension cell cultures (Khanna and Jain 1973; Trisonthi et al. 1980) and by biological manipulation of yield (Petropoulos 2002).

Some of these GLVs contain antinutritional compounds such as oxalates, phytates, hydrocyanides, etc. which pose a threat to consumer's health (Agbaire and Emoyan 2012). Through genetic engineering it could be possible to either reduce such antinutritional compound content or to arrest their production (Fig. 24.1). Apart from this, the rampant use of fertilisers, pesticides and insecticides in the field leads to environmental pollution and also leads to accumulation of poisonous chemicals and heavy metals in GLVs especially when they are grown in areas of industry-polluted soils and waters. Under this context, organised cultivation of elite varieties of GLVs in polyhouse is a solution to get healthy plants for better health of consumers. Many parts of the world are endowed with various types of indigenous leafy vegetables which provide not only food, income and employment but also as herbal medicine to the population. Some GLVs are unexplored and have uncharacterised germplasm, susceptible to pests and diseases, and contain antinutritional factors, recalcitrant seed and seed dormancy. Due to their perishable nature upon harvesting, influence against the realisation of potentials of the GLVs. In many third world countries, traditional indigenous leafy vegetables are important in the agricultural development of respective state and country as the case may be. For example, in African countries like Nigeria, some indigenous leafy vegetables, viz. *Amaranthus cruentus*, *Corchorus olitorius* L., *Celosia argentea* L., *Gongronema latifolium*, *Vernonia amygdalina* Del, *Talinum triangulare*, *Telfairia occidentalis* Hook.f., *Talinum triangulare* Willd., *Solanecio biafrae* (Olive and Hiern) C. Jeffrey and *Launea taraxicifolia* (Willd.) Amin ex *C. Jeffrey*, are considered as a good source of nutrients (Opabode and Adebooye 2005).

These leafy vegetables are a key source of income for those who engage in its farming either at small scale or commercial way. Majority of these vegetables have proven to possess economic potentials and contribute to food security in rural areas. The research institutes in India focus mainly on the regularly cultivated species, and most of the unexplored or underutilised leafy vegetables often do not receive attention. Therefore, the research priorities have to be oriented to GLVs so that it will contribute substantially to promote the underutilised GLVs. It is necessary to promote GLVs propagation in large scale with the help of tissue culture methods such as clonal propagation, organogenesis, somatic embryogenesis and anther culture as they can solve improvement and production problems and also help to pick up elite lines with quality traits for mass multiplication and commercial propagation. Advocating such less-known GLV cultivation through ethnic group participation under respective local NGO monitoring or government-funded schemes or through homestead concept would be helpful to alleviate nutrient deficiency diseases in poor and ethnic communities. If sustainable production of highly nutrient underutilised

leafy vegetables is promoted, the same would become a base for the concept of 'biofortification through biotechnology to combat malnutrition'.

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Nonzygotic Embryogenesis for Plant Development

25

Mohamed A. El-Esawi

Abstract

Nonzygotic or somatic embryogenesis is a specialized developmental mode by which somatic cells, under appropriate induction conditions, undergo restructuring pathway to form embryogenic cells. These cells then undergo a sequence of morphological and biochemical alterations that lead to the formation of a nonzygotic embryo and the production of new plants. Nonzygotic embryogenesis is a model system for a large-scale plant production. In vitro nonzygotic embryogenesis has wide practical and commercial applications in basic and applied aspects of agriculture and plant sciences. This chapter discusses the factors affecting nonzygotic embryogenesis and provides valuable information on induction, development, origin, and maturation of nonzygotic embryos, being useful for biotechnological applications. It also highlights the physiological, biochemical, and molecular aspects of nonzygotic embryogenesis. Moreover, this chapter surveys the characteristics of zygotic and nonzygotic embryos, as well as the synthetic seed technology and the practical applications of nonzygotic embryogenesis for crops improvement.

25.1 Introduction

The life cycle of higher plants comprises the haploid gametophyte and the diploid sporophyte generations. The diploid sporophyte generation starts with a fertilization process which produces a zygote embryo and an endosperm nucleus (Yang and Zhang 2011). The developmental pathway of the zygotic embryogenesis includes

M.A. El-Esawi (✉)
Botany Department, Faculty of Science, Tanta University, 31527 Tanta, Egypt
e-mail: mohamed.elesawi@science.tanta.edu.eg

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globular, scutellar, and coleoptilar stages in monocots; globular-shaped, heart-shaped, torpedo-shaped, and cotyledonal stages in dicots; and globular and early and late cotyledonary embryos in conifers (Yang and Zhang 2011). Accomplishment of these developmental stages leads to the production of a new plant. Alternatively, the new plant could be originated from a single somatic cell or a group of somatic cells. This propagation technology, differing from the zygote embryogenesis pathway, is called somatic or nonzygotic embryogenesis, and the embryos derived from somatic cells are known as somatic or nonzygotic embryos (Yang and Zhang 2011), being the main focus of this chapter.

Nonzygotic embryogenesis is a specialized developmental mode by which somatic cells, under appropriate induction conditions, undergo restructuring pathway to produce embryogenic cells. These cells then undergo a sequence of morphological and biochemical alterations that lead to the formation of a somatic embryo and the production of new plants (Yang and Zhang 2011). Nonzygotic embryogenesis, occurring among widely tissue types, is a model system for the study of morphological, physiological, biochemical, and molecular events happening during the embryos development in higher plants. In vitro nonzygotic embryogenesis has wide practical applications in basic and applied aspects of agriculture and plant sciences (Bhojwani and Dantu 2013).

The formation of nonzygotic embryos in vitro was first demonstrated in carrot by Steward et al. (1958). Since then, carrot has become a model system to investigate the different aspects of nonzygotic embryogenesis in several plant species. The generation of nonzygotic embryos has been recorded in more than 500 species of monocots and dicots including carrot, citrus, alfalfa, coffee, maize, cotton, mustard, sunflower, rice, wheat, and *Ranunculus sceleratus* (Bhojwani and Dantu 2013). In *Ranunculus sceleratus*, the floral and somatic tissues produce callus on a coconut milk-containing media, and several nonzygotic embryos are formed on this callus within a short period on the original medium (Bhojwani and Dantu 2013). These nonzygotic embryos grow in situ or upon transfer to a fresh medium. In citrus, the nonzygotic embryos originate from potential nucellar cells or from a callus in subcultures (Bhojwani and Dantu 2013). Nonzygotic embryogenesis has been also reported from single somatic cells of *Macleaya cordata*. Moreover, nonzygotic embryogenesis of *Arabidopsis thaliana* is being developed as a model system to investigate the molecular biology and genetic aspects of embryogenesis (Raghavan 2006; Bhojwani and Dantu 2013).

25.2 Factors Affecting Nonzygotic Embryogenesis

Nonzygotic (somatic) embryogenesis procedures include induction of embryogenesis, embryo development, embryo maturation, and their growth to give complete plants. The widely used method to induce somatic embryogenesis is to culture an appropriate plant tissue in a suitable nutrient medium supplemented with auxins (Bhojwani and Dantu 2013). The requirement for embryogenesis induction differs based on the plant species. Successful somatic embryogenesis requires the right

selection and manipulation of plant tissues and genotype, basal media, growth regulators, and physical culture environment.

25.2.1 Plant Tissue and Genotype

The selection of explant (plant tissue) for generating somatic embryos is limited to the less differentiated tissues such as hypocotyl segments, embryonic shoot tip, young leaves and floral parts, and immature zygotic embryos (Bhojwani and Dantu 2013). The choice of immature zygotic embryos as explants plays the most important role in the successful induction of nonzygotic embryogenesis in monocots and many dicots. The more differentiated explants contain cells which are converted to be embryogenic and are known as induced embryogenic determined cells (IEDCs), while the zygotic embryos comprise cells that have embryogenic competence and are known as pre-embryogenic determined cells (PEDCs) (Bhojwani and Dantu 2013). Immature zygotic embryo is used as the best explant for somatic embryogenesis induction in *Arabidopsis thaliana*. Immature embryos of this plant species cultured on a liquid medium may exhibit indirect somatic embryogenesis through a callus phase (Raghavan 2004). In contrast, the older embryos cultured on semisolid medium reveal direct somatic embryogenesis from the protoderm cells of the cotyledons (Gaj 2001; Kurczyńska et al. 2007).

Remarkable intervarietal variations for somatic embryogenic potential have been recorded in many crops such as maize, groundnut, rice, and soybean (Bhojwani and Dantu 2013). A mutant line (2HA) of *Medicago truncatula* cv. Jemalong revealed 500-fold greater capacity to produce somatic embryos than the parent genotype (Nolan et al. 2003). Genotypic variations could be due to varying levels of growth regulators. The endogenous levels of the cytokinin and auxin in the ovules of highly embryogenic genotypes of maize were lower than that in the poorly embryogenic and nonembryogenic genotypes (Bhojwani and Dantu 2013).

25.2.2 Basal Media

Basal medium of Murashige and Skoog (1962) or its modifications have been mainly used for the successful somatic embryogenesis. However, White's or SH basal medium has been rarely utilized with appropriate supplements. Sucrose is the most widely used carbon source, but glucose and galactose/lactose were preferred in scarlet runner bean and citrus nucellus cultures, respectively (Bhojwani and Dantu 2013). Moreover, the nitrogen form in the culture medium also influences in vitro somatic embryogenesis. For example, carrot cultures initiated in White's medium that comprises KNO_3 as the only source of inorganic nitrogen could not form somatic embryos upon transfer to auxin-free medium. However, the embryos were successfully developed upon addition of a reduced nitrogen source such as NH_4Cl mixed with KNO_3 (Halperin and Wetherell 1965).

Other nitrogen sources such as casein hydrolysate (CH) and amino acids could also enhance somatic embryo development (Bhojwani and Dantu 2013). Casein hydrolysate (CH; 3 g L^{-1}) stimulated somatic embryos development in orchard grass cultures. Proline, alanine, arginine, and glutamine also induced the development of somatic embryos in alfalfa. Therefore, nitrogen sources play an important role in the continued synthesis of protein and nucleic acids, as well as maintaining an appropriate pH during embryogenesis.

25.2.3 Growth Regulators

Growth regulators can exert large effects in plants in terms of gene expression, growth, and development (Beyl 2011). Endogenous plant growth regulators (PGRs) that the plant biosynthesizes for itself are known as hormones which control different processes such as root and bud initiation, dormancy, cell division and enlargement, flowering, and ripening (Beyl 2011). When a plant is developing from a germinating seed, such hormones direct the growth and development of shoots and roots through cell division and enlargement. The following five categories of hormones have been traditionally identified and classified as auxins, cytokinins, gibberellins, abscisic acid, and ethylene. Auxins and cytokinins are the most important hormones used in plant tissue culture media. Other new PGRs have been discovered such as jasmonates, polyamines, salicylic acid, brassinosteroids, triazoles, and oligosaccharins (Beyl 2011).

25.2.3.1 Auxin

Auxins enhance root initiation and cell enlargement (Beyl 2011). Additionally, auxins play an essential role in apical dominance, phototropism, geotropism, root induction, and wounding responses (Beyl 2011). Auxins are commercially exploited as herbicides or to stimulate parthenocarpy to prohibit fruit abscission (Beyl 2011; Bhojwani and Dantu 2013). Indoleacetic acid (IAA) is the most common auxin found naturally and of a limited use in tissue culture media due to its sensitivity to light and tendency to be oxidized, metabolized, or broken-down by microorganisms (Beyl 2011). The concentration of endogenous IAA in plants is regulated by the rate of biosynthesis and oxidation by the enzyme IAA oxidase and the synthesis of conjugates with amino acids and sugars. Other auxins found naturally include phenylacetic acid (PAA) and indole-3-butyric acid (IBA). Auxins including naphthalene acetic acid (NAA), 2,4- dichlorophenoxyacetic acid (2,4-D), dicamba (DIC), and picloram (PIC) can induce callus growth in tissue culture and are horticulturally used as herbicides against dicotyledonous weeds (Beyl 2011; Bhojwani and Dantu 2013). PAA and IAA are relatively weak auxins, while 2,4-D, NAA, DIC, and PIC are strong (Beyl 2011; Bhojwani and Dantu 2013). The carrot embryogenic cultures could be initiated and propagated in a medium containing 2,4-D of a concentration varied between 0.5 and 1.0 mg L^{-1}. On this medium, callus could differentiate localized groups of meristematic cells, known as proembryogenic masses (PEMs)

(Bhojwani and Dantu 2013). These PEMs could be developed into dicot embryos only upon transfer to auxin-free media or in the presence of low levels of auxin.

25.2.3.2 Cytokinins

Cytokinins proved to have impressive effects on the induction of somatic embryogenesis. Cytokinins play an essential role in controlling different processes in plants such as cell division and enlargement, development of chloroplasts and shoots, vascular development, resource uptake, as well as nodulation in leguminous species (Beyl 2011). They could also mitigate apical dormancy and induce shoot proliferation and cell division in tissue culture techniques. Cytokinins include two general groups known as adenine and phenylurea cytokinins (Beyl 2011). Adenine cytokinins may be aromatic such as benzyladenine (BA) and meta-topolin (mT) or isoprenoid such as zeatin (ZEA) and isopentenyladenine (IPA). Phenylurea cytokinins comprise thidiazuron (TDZ) and diphenylurea.

Synthetic cytokinins including BA and 6-furfurylaminopurine (KIN) have proved to have an important role in inducing shoot proliferation and callus formation in different woody and herbaceous species (Beyl 2011). IPA and ZEA found naturally were isolated from *Zea mays* kernels (Letham 1963). In carrot tissue culture, ZEA promoted the process of somatic embryogenesis at a concentration of 0.1 μM. Meta-topolin (mT; Strnad et al. 1997; Beyl 2011), isolated from poplar leaves, could be more active than ZEA or BA in inducing shoot formation of sugar beet (Kubalakova and Strnad 1992) and *Spathiphyllum floribundum* (Werbrouck et al. 1996). TDZ, used as a defoliant for cotton, is a potent cytokinin showing activity at low concentrations (10 pM) (Preece et al. 1991). TDZ plays an essential role in the micropropagation of recalcitrant species and may act through endogenous hormones modulation (Murthy et al. 1998; Beyl 2011). Many plant species including tobacco, peanut, geranium, and chickpea showed responsiveness to TDZ in terms of embryogenesis (Murthy et al. 1998). A mixture of TDZ and IPA stimulated the growth of floral structures from stamen explants in *Rhododendron* (Shevade and Preece 1993; Beyl 2011). Furthermore, Skoog and Miller (1957) studied the relationship between auxins and cytokinins in regulating the formation of shoots and roots from callus. If the ratio of cytokinin to auxin is high, the shoot formation occurs, but, if the ratio of auxin to cytokinin is high, the root formation happens. High concentrations of both auxins and cytokinins can induce callus formation.

25.2.3.3 Abscisic Acid

Abscisic acid (ABA) has an important role in stomatal control, bud and seed dormancy, leaf abscission, and senescence (Beyl 2011). In tissue culture, ABA is positively effective at low concentrations but prohibits both callus growth and production of buds and embryos at high concentrations (Gaspar et al. 1996). Brown et al. (1989) reported that the low concentrations of ABA could promote embryogenesis in cereals. ABA proved to be effective in pre-treating somatic embryos of carrot to enhance their survival rate (Kitto and Janick 1985; Beyl 2011). ABA could also promote the development of conifer somatic embryos (Becwar et al. 1987; Hakman and von Arnold 1988; Boulay et al. 1988; Misra and Green 1990). ABA can prohibit shoots

proliferation when germplasm preservation is required (Singha and Powell 1978; Beyl 2011). ABA may also inhibit growth of grape somatic embryos as reported earlier by Rajasekaran et al. (1982).

25.2.3.4 Gibberellins

Gibberellins have strong effects on stem elongation and sex expression in plants (Beyl 2011). They also play an essential role in dormancy and germination but do not have a large role in regulation of in vitro development (Gaba 2005). Gibberellins may reduce root formation and interfere with bud initiation and embryogenesis (Beyl 2011). Rosati et al. (1980) proved that GA3 has been efficient for rooting of Japanese plum in vitro. However, Jiminez (2001) reported that gibberellins could prohibit embryogenesis or somatic embryo maturation in different plant species. Exogenous GA3 decreased the number of somatic embryos produced in carrot (Fujimura and Komamine 1975). A combination of gibberellic acid, zeatin, and ABA was needed in caraway (Ammirato 1977), while GA3 and 2iP were required in grapevine (Bhojwani and Dantu 2013).

25.2.3.5 Ethylene

Ethylene plays a role in plant morphogenesis, ripening, senescence, abscission, and stress (Beyl 2011). High concentrations of ethylene can prohibit the in vitro growth and maturation of plant tissues. Ethylene used exogenously prohibited somatic embryogenesis in *Daucus carota*. 2,4-D induced the inhibition of embryo development that might be due to the production of endogenous ethylene. Ethylene biosynthesis (nickel, cobalt, and salicylic acid) and action inhibitors could promote somatic embryogenesis in plant species such as rubber and carrot (Beyl 2011).

25.2.4 Selection of Subculture and Electric Current-Dependent Stimulation

Few multicellular explants could express their cellular totipotency under specific culture conditions (Bhojwani and Dantu 2013). The suspension cultures and calli that came from these explants are heterogeneous in terms of the embryogenic potential of its component cells. Sometimes, the embryogenic parts of calli are distinct from the nonembryogenic portions based on their morphological characteristics, and it is essential to prepare appropriate subcultures to initiate regenerating tissue cultures (Bhojwani and Dantu 2013). Nabors et al. (1983) reported that the embryogenic calli of various Poaceae species were granular and with a smooth surface, but the nonembryogenic calli were rough and translucent. Similarly, the embryogenic calli of *Coffea arabica* appeared brown and hard while the nonembryogenic calli were friable and pale (Quiroz-Figueroa et al. 2006).

Dijak et al. (1986) reported that a moderate electric current could stimulate the differentiation of embryos and shoots. For example, in alfalfa, the exposure of the protoplasts to a moderate electric current promoted the direct embryogenesis from 40 to 100 % and the number of embryos per plate from 76 to 116 (Bhojwani and

Dantu 2013). Such electrical stimulation of a plant development could be attributed to changes in microtubules organization that influence the cell polarity (Dijak and Simmonds 1988; de Jong et al. 1993; Bhojwani and Dantu 2013).

Other factors including initial cell density and light and oxygen concentration in cultures play an important role in the induction of plant somatic embryogenesis (Bhojwani and Dantu 2013).

25.3 Induction, Development, and Origin of Nonzygotic Embryos

Depending on the culture conditions and explant nature, somatic embryos may originate either directly from the explant cells or indirectly via a phase of callus formation after many cycles of cell divisions (Bhojwani and Dantu 2013).

25.3.1 Induction

Plant growth regulators, especially auxin, are widely exploited to induce dedifferentiation required to get embryogenic competence (Bhojwani and Dantu 2013). Auxin, such as 2,4-D, is the most important hormone used to activate cell division in the differentiated plant cells both in vivo and in vitro. An important process correlated with the somatic embryogenesis stimulation is the change of cellular polarity. Plant growth regulators applied for the induction of somatic embryogenesis may change the cell polarity and enhance asymmetric division (Bhojwani and Dantu 2013). In carrot cell suspensions, the smaller daughter cells were developed into nonzygotic embryos (Backs-Hüsemann and Reinert 1970). The polarity of the entire somatic embryogenesis is estimated before the first division of the embryogenic cells. Dijak et al. (1986) reported that the exposure of freshly isolated mesophyll protoplasts to an electric current enhanced the embryogenic response in alfalfa. The electric current would stimulate the differentiation of embryos and shoots through affecting cell polarity and changing the microtubules organization.

25.3.2 Development

In carrot, embryogenic cells are formed on the medium after reinitiation of cell division and cell proliferation in the presence of auxin (Bhojwani and Dantu 2013). The suspension cultures comprise proembryogenic masses (PEMs) and two types of single cells, known as cytoplasmically rich small cells and vacuolated long cells. The cytoplasmically rich small cells may form PEMs or globules (Komamine et al. 1990). However, the majority of the PEMs originate from the pre-existing PEMs (de Vries et al. 1988; Emons et al. 1992). PEMs contain embryogenic cells held together by nonembryogenic cells. The continued presence of auxin induces cell elongation and adhering cells disruption (Bhojwani and Dantu 2013). The embryogenic cells

excrete specific proteins into the culture media that support maintaining the embryogenic potential of cultures and stimulate the appearance of small embryogenic cells in nonembryogenic cultures (Kreuger and Van Holst 1993). Upon transfer of carrot embryogenic cultures to auxin-free medium, the disruption of cells from each other's ends and the globules could be developed into globular embryos (Bhojwani and Dantu 2013). In this case, the first differentiation step is the production of a protoderm outside the globule. The globular embryos could be developed further into typical embryos. The developing embryos could then produce their own auxin, and further morphogenesis could occur.

25.3.3 Origin

Nonzygotic embryos originate from single cells (Gray 2011; Bhojwani and Dantu 2013). Many studies reported the somatic embryogenesis in callus and plant suspension cultures leading to the production of somatic embryos resembling the zygotic embryos (Bhojwani and Dantu 2013). Backs-Hüssemann and Reinert (1970) reported the formation of bipolar somatic embryos resembling zygotic embryos from a single cell separated from carrot tissue cultures. The cell was divided by unequal division and the smaller derivative was further divided to form an embryo.

25.4 Maturation of Nonzygotic Embryos

Nonzygotic (somatic) embryos generally skip the final stage of zygotic embryo maturation and grow to form weak seedlings (Bhojwani and Dantu 2013). To promote maturation, the morphologically fully developed nonzygotic embryos need special treatments such as abscisic acid treatment, exposure to a high concentration of sucrose, and gradual drainage. The maturation of nonzygotic embryos of soybean was significantly increased in the presence of sucrose as reported earlier by Buccheim et al. (1989). Maize somatic embryos could also undergo a maturation phase to form a typical storage organ in the presence of a high sucrose concentration (Emons and Kieft 1993). Senaratna et al. (1989) also reported that nonzygotic embryos of alfalfa could resist desiccation by treating them with abscisic acid at the torpedo to cotyledonary stages. These abscisic acid-treated embryos were converted into plantlets upon a direct sowing in sterile soil. The vigor of these plantlets was greater than that of the plantlets originated from non-desiccated embryos. Sometimes the somatic embryos show structural abnormalities which should be minimized to enhance the maturation and development of nonzygotic embryos (Bhojwani and Dantu 2013). Moreover, the maturation and conversion of somatic embryos could be enhanced under a reduced humidity or in the presence of auxin inhibitors.

25.5 Physiological and Biochemical Traits of Nonzygotic Embryogenesis

Physiological and biochemical changes could happen within the cell which gets embryogenic competence before the appearance of any morphological differentiation of nonzygotic embryo (Bhojwani and Dantu 2013). The most important change detected is in the endogenous levels of hormones and their associated effects. Noma et al. (1982) reported that, in nonembryogenic callus, the levels of polar gibberellic acids were higher than the less polar ones. However, when the callus became embryogenic, the levels of less polar gibberellic acids were higher than the polar ones. Abscisic acid also exhibits changes in endogenous levels relying on the embryo stage (Kamada and Harada 1981; Bhojwani and Dantu 2013).

The levels of arginine and aliphatic amines were maximum during globular and torpedo developmental stages but were reduced in geminating embryos (Bhojwani and Dantu 2013). The increase in the endogenous level of polyamines is associated with the somatic embryogenesis induction in carrot (Altman et al. 1990). Further studies on mango and *Solanum melongena* supported the causative role of polyamines in nonzygotic embryogenesis (Litz et al. 1993; Yadav and Rajam 1998). During the development of globular embryo, an increase in the turnover rate of RNA and protein followed by active DNA synthesis was observed (Fujimura et al. 1980).

25.6 Molecular Aspects of Nonzygotic Embryogenesis

Changes in gene expression may occur during somatic embryogenesis. Several molecular markers and embryo-specific genes have been recognized and cloned from somatic embryos (Zimmerman 1993; Schmidt et al. 1997; Montero-Córtes et al. 2010; Bhojwani and Dantu 2013). Calcium plays a key role in regulation of several cellular and physiological processes in plants (Yang and Zhang 2011). In *Daucus carota* system, Ca2+ proved to promote somatic embryogenesis. The vacuolar Ca^{2+} is the first signal that helps in the recognition of embryogenic cells (Bhojwani and Dantu 2013). Cellular calcium signals are observed and inherited by sensor molecules. Three major classes of Ca^{2+} sensors have been identified in plants (Yang and Zhang 2011): (1) Calmodulin (CaM) is found in the meristematic regions of developing embryos and embryogenic cell cultures. In cultures of *Saccharum officinarum*, CaM expression was specific to the embryogenic stage. (2) Calcium-dependent protein kinase (CDPK) has a C-terminal CaM-like domain which can directly bind Ca2+. CDPKs play regulatory roles in several developmental and metabolic processes. Anil and Rao (2000) reported that blocking the CDPK-involved signaling pathway prohibits nonzygotic embryogenesis in *Santalum album*. (iii) Calcineurin B-like proteins (CBLs) play an essential role in decoding calcium transients and regulating a family of protein kinases (CIPKs). Various CBL proteins and CIPKs were identified and implicated as important components of

abiotic stress responses and ion transport processes (Yang and Zhang 2011). *CIPK9* is essential in the early stage of somatic embryogenesis (Zhu et al. 2008).

Cell wall-associated proteins have a signaling role in plant development (Bhojwani and Dantu 2013). Oligosaccharin group of these proteins can participate in embryogenesis regulation. The wall of the embryogenic cells of maize has arabinogalactan proteins (AGPs) (Samaj et al. 1999; Bhojwani and Dantu 2013). Chapman et al. (2000) reported the key role of AGPs in the induction of somatic embryogenesis in *Cichorium*. Arabinogalactan proteins of tomato could also induce the somatic embryogenesis in carrot (Kreuger and van Holst 1996). Moreover, two callus-specific proteins (Cl and C2) are formed in proliferating carrot cell cultures (Bhojwani and Dantu 2013). Upon transfer to an embryogenic-specific medium, the protein profile of callus changes resulting in the appearance of two new embryo-specific proteins (E1 and E2). The callus-specific and embryo-specific proteins could be regulated (Sung and Okimoto 1981, 1983).

Germin-like proteins (GLPs), embryogenic cell proteins (ECPs), and Trx H proteins (Karami et al. 2009; Bhojwani and Dantu 2013) have been identified at different stages during somatic embryogenesis. Moreover, Schmidt et al. (1997) isolated many genes from carrot suspension cultures, such as Somatic Embryogenesis Receptor Kinase (DcSERK) that act as a marker of single competent cells. AtSERK1 gene has been separated from *Arabidopsis thaliana*. Overexpression of AtSERK1 gene in *Arabidopsis thaliana* seedlings showed three- to fourfold increase in the initiation efficiency of somatic embryogenesis (Bhojwani and Dantu 2013). LEAFY COTYLEDON (LEC) 1 and 2 are two genes identified through the use of loss-of-function mutations in *Arabidopsis* (Bhojwani and Dantu 2013). LEC1 and LEC2 affect embryo maturation and partially convert cotyledons into leaves. Expression of both LEC1 and LEC2 stimulate the embryos formation on vegetative tissues (Lotan et al. 1998; Stone et al. 2008). LEC2 stimulates somatic embryogenesis in vegetative tissues and can act through AGAMOUS-Like 15 (AGL15), stimulating the enzyme that inactivates GA and thus promote the formation of somatic embryo (Stone et al. 2001, 2008; Wang et al. 2004).

Analysis of the conditioned cell-free medium, used to induce somatic embryogenesis in fresh cultures, revealed the presence of three types of extracellular proteins (EPl, EP2, and EP3) which have been released by embryogenic cells. Only nonembryogenic cells can release EPl (van Engelen et al. 1991; Bhojwani and Dantu 2013). EP2 is only produced by the somatic embryos and embryogenic cells and could be expressed in the PEMs peripheral cells and the somatic embryos protoderm (Sterk et al. 1991). EP2 may also be involved in the transport of cutin monomers to the specific sites of cutin synthesis. EP3 could induce the normal protoderm formation (de Jong et al. 1992). Furthermore, PLANT GROWTH ACTIVATOR 6 (PGA6) gene has been identified in *Arabidopsis* (Bhojwani and Dantu 2013). Overexpression of PGA6 gene enhanced the formation of somatic embryo from different vegetative tissues and zygotic embryo. PGA6 is similar to WUSCHEL (WUS) gene that regulates the stem cells fate in shoot and floral meristem and helps in maintaining the embryonic stem cells identity (Zuo et al. 2002). MYB118 and MYB115 genes have also been identified and could play an important regulatory

role in inducing vegetative-to-embryonic transition as well as enhancing somatic embryos formation in *Arabidopsis* from vegetative parts (Wang et al. 2009; Bhojwani and Dantu 2013).

25.7 Characteristics of Zygotic and Nonzygotic Embryos

Unlike the zygotic embryos, nonzygotic embryos may exhibit secondary embryogenesis and their development is asynchronous (Bhojwani and Dantu 2013). Zygotic and nonzygotic embryos have the same developmental patterns, passing through globular, scutellar, and coleoptilar stages in monocots, or globular, heart, torpedo, and cotyledonary stages in dicots and conifers (Gray 2011). During early developmental stage, the embryo gains a globular shape and remains undifferentiated but with a well-defined epidermis. However, the final stages of development are distinguished by coleoptilar enlargement in monocots and increase in cotyledon size in dicots (Gray 2011). At the same time, the embryonic axis becomes increasingly developed. In dicots, the root apical meristem becomes well-established. In monocots, the embryo axis grows parallel to the scutellum. The root apical meristem is embedded, whereas the shoot apical meristem grows externally and is protected by the coleoptile (Gray 2011).

The major difference in the gross morphology between zygotic embryos in seeds and nonzygotic embryos growing in vitro is due to the physical constraint on zygotic embryos. Zygotic embryos exhibit a compressed shape due to their flattened shape during development (Gray 2011). However, somatic embryos become larger and have wider hypocotyls and cotyledons (Gray and Purohit 1991). Additionally, more developmental abnormalities occur during somatic embryogenesis when compared to zygotic embryogenesis. Somatic embryos may also exhibit structural abnormalities including poorly developed apical meristems and extra cotyledons (Gray 2011).

Unlike the zygotic embryos, nonzygotic embryos lack a suspensor which is the pathway for all nutrients required (Gray 2011; Raghavan 1976). Moreover, nonzygotic embryos often lack a quiescent resting phase. In contrast, during seed maturation, zygotic embryos of several crops have a resting period, being the main factor allowing seeds to be conserved and used in agricultural practices (Gray 1986; Gray and Purohit 1991).

25.8 Synthetic Seed Technology and Applications of Nonzygotic Embryogenesis

In the whole plant, the biochemical and molecular markers have been used for improving crops through breeding practices (El-Esawi et al. 2012, 2015, 2016a, b; Sammour et al. 2013; El-Esawi and Sammour 2014; El-Esawi 2015a, b, 2016; Jourdan et al. 2015; Consentino et al. 2015). However, in tissue culture, the artificial or synthetic seed technology has emerged with the aim of developing somatic embryogenesis into a commercially beneficial method of plant multiplication and

improvement (Gray 2011). A synthetic seed is a nonzygotic embryo engineered to be of practical uses in the commercial plant regeneration (Gray 2011). Due to these useful practical reasons, somatic embryos should be converted into synthetic seeds (synseeds) by encapsulating in a protective covering for field planting (Pinto et al. 2008). The synseeds coating must be mild and protective to the somatic embryos and should incorporate growth regulators, nutrients, and other constituents necessary for germination (Bhojwani and Dantu 2013). The synthetic seeds should also be compliant to the existing farm machinery. The successful synthetic seed technology also relies on the quality and development of nonzygotic embryos which should have high rates of maturation and conversion on planting (Pinto et al. 2008). The most common widely used method for encapsulation of single somatic embryos is the coating with calcium-alginate (Bhojwani and Dantu 2013). The important advantages of synthetic seeds include their easy handling and transportation, higher scale-up capacity, potential long-term storage, uniformity in production, and potential for automation of the whole production process (Bhojwani and Dantu 2013). Seedless watermelon is an attractive candidate for synthetic seed technology since the per-plant cost could be reduced (Gray 2011). Similarly, conifers, which are difficult to be improved using breeding practices, would benefit from the artificial seed technology applications (Farnum et al. 1983; Gray 2011).

Since the nonzygotic embryogenesis has been induced in the majority of crop plants, very large numbers of somatic embryos have been obtained. In carrot, nonzygotic embryos at the same development stage could be produced in gram quantities (Bhojwani and Dantu 2013). These characteristics have made somatic embryogenesis, a model system for a large-scale plant propagation in automated bioreactors (Quiroz-Figueroa et al. 2006).

25.9 Conclusions

The formation of nonzygotic embryos in vitro was first demonstrated in carrot which has become a model system to investigate the different aspects of nonzygotic embryogenesis in several plant species. The generation of somatic embryos has been recorded in more than 500 species of monocots and dicots. Successful somatic embryogenesis requires the right selection and manipulation of plant tissues and genotype, basal media, growth regulators, and physical culture environment. Nonzygotic embryogenesis, occurring among widely tissue types, is a model system for a large-scale plant production and for understanding the morphological, physiological, biochemical, and molecular aspects happening during the embryos development in higher plants. In vitro nonzygotic embryogenesis has wide practical and commercial applications in basic and applied aspects of agriculture and plant sciences.

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Somatic Hybridization and Microspore Culture in *Brassica* Improvement

26

Mohamed A. El-Esawi

Abstract

Brassica species have an economic and medicinal importance. Somatic hybridization is a widely used technology for the transfer of nuclear and cytoplasmic traits in *Brassica* species. Many wild *Brassica* species have important agronomic traits, especially those for disease resistance, that could be transferred into the cultivated brassicas. Somatic hybridization enhanced the development of interspecific and intergeneric hybrids in the sexually incompatible *Brassica* species. Additionally, the microspore culture is one of the most effective technologies used for developing microspore-derived embryos and double haploid plants. The formation of haploids and doubled haploids using microspores enhanced the generation of homozygous genotypes in *Brassica* species. This technology played an important role in breeding self-incompatible and out-crossing genotypes. This chapter discusses the advanced applications of somatic hybridization and microspore culture in *Brassica* improvement over the past years.

26.1 Introduction

Somatic hybridization is a widely used technology for the transfer of nuclear and cytoplasmic traits in *Brassica* species. Protoplast fusion allows DNA to be introgressed from sexually incompatible *Brassica* species (Sigareva and Earle 1997; Christey 2004). Many wild plant species have important agronomic traits, especially those for pathogen resistance and altered fatty acid composition, which could be transferred into the cultivated crops (El-Esawi et al. 2012, 2015, 2016a, b;

M.A. El-Esawi (✉)
Botany Department, Faculty of Science, Tanta University, 31527 Tanta, Egypt
e-mail: mohamed.elesawi@science.tanta.edu.eg

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El-Esawi and Sammour 2014; El-Esawi 2015a, b, 2016; Consentino et al. 2015; Jourdan et al. 2015). Protoplast fusion enhanced the development of interspecific and intergeneric hybrids in the sexually incompatible *Brassica* species (Christey 2004). Protoplast fusion could be used in the production of new nuclear cytoplasmic organelle combinations, resulting in an increase in cytoplasmic diversity (Christey 2004). In *Brassica napus*, various cytoplasmic male sterility systems have been characterized, including cytoplasmic male sterility Ogura, Tour, and Polima and cytoplasmic male sterility from Kosena radish. Somatic hybridization has been utilized for embedding them into new genotypes (Sigareva and Earle 1997; Christey 2004).

The microspore culture is one of the most effective technologies used for developing microspore-derived embryos and double haploid plants. This technology played an important role in breeding self-incompatible and out-crossing genotypes (Ferrie and Keller 2004). Since the first promising production of microspore-derived embryos from *Datura* anthers (Guha and Maheshwari 1964, 1966), various species including *Brassica* formed microspore-derived embryos from cultured anthers (Keller and Armstrong 1978, 1979). Microspore culture of *Brassica* species is a highly effective system for producing several embryos and double haploid lines (Ferrie and Keller 2004). This haploidy system has been used in various applications, including genetics, breeding, mutagenesis, transformation, genomics, and physiological and biochemical analyses (Ferrie and Keller 2004).

26.2 Somatic Hybridization in *Brassica*

26.2.1 Protoplast Culture and Shoot Regeneration

Creating an effective protoplast culture and shoot regeneration system for the required somatic hybrids is essential for their successful regeneration and application. Generally, rates of protoplast regeneration could be reduced after fusion (Christey 2004). However, some cabbage genotypes exhibited improved regeneration after fusion (Sigareva and Earle 1997). Many *Brassica* species exhibited shoot regeneration from protoplasts with varying levels of success. *Brassica oleracea* and *Brassica napus* revealed the highest rates of shoot regeneration (Christey 2004). However, *Brassica campestris* showed low rates of shoot regeneration. Plant regeneration has been achieved using protoplasts isolated from different explants such as cotyledons, leaves, and hypocotyls from in vitro seedlings (Christey 2004). Other explants used for the subsequent shoots regeneration involve roots (Xu et al. 1982), inflorescence (Yang et al. 1994), cell suspensions (Simmonds et al. 1991), peduncle stem peels (Chuong et al. 1987a), and microspore-derived embryos (Swanson et al. 1988). Other factors including choice of media and culture system should also be considered during protoplast fusion process (Christey 2004).

Pelletier et al. (1983) founded a unique protocol which is the basis of most other protoplasm regeneration protocols. This protocol utilizes the liquid medium during initial culture which is then transferred to a series of solid media for callus

development and shoot regeneration (Christey 2004). Various culture methods have been utilized, including agarose beads (Jaiswal et al. 1990), liquid culture in 24-well plates (Jourdan and Earle 1989), and the alginate bead technology (Yamagishi et al. 2002). Additionally, *Brassica napus* feeder layer system, described by Walters and Earle (1990), is currently used for protoplast fusion. Hu et al. (1999) studied 28 cultivars of *Brassica napus*, *Brassica rapa*, and *Brassica juncea. Brassica rapa* cultivars did not regenerate shoots, whereas *Brassica juncea* genotypes showed high regeneration rates ranging between 56 and 60 %. Eleven of the 17 *Brassica napus* lines exhibited regeneration rates ranging between 1 and 24 % (Christey 2004).

26.2.2 Protoplast Fusion and Agronomic Traits Transfer

Sigareva and Earle (1997) established protocols for *Brassica* protoplast fusion which involved high concentrations of high molecular weight polyethylene glycol in the presence of calcium, followed by washing at high pH and high calcium. Additionally, more fusion protocols have been established, such as electrofusion (Hagimori et al. 1992; Gaikwad et al. 1996; Christey 2004) and dextran application (Kameya et al. 1989). Protoplast fusion could result in desired or undesired outcomes. However, various strategies could be used to get rid of the undesired outcomes and enhance the chances of developing desired traits (Christey 2004). For example, irradiation combined with chemicals could be used to prohibit the division of both fusion partners, resulting in developing only somatic hybrids (Christey 2004). Sometimes, protoplast fusion does not require pretreatment due to the lack of regeneration ability of one fusion partner, such as fusions including *Brassica rapa* (Christey et al. 1991; Ren et al. 2000), radish (Kameya et al. 1989), *Moricandia arvensis* (Toriyama et al. 1987a), and *Camelina sativa* (Sigareva and Earle 1999a).

Molecular markers have been utilized to select the desired cybrid or hybrid fusion products surviving on antibiotic- or herbicide-containing medium (Christey 2004). In *Brassica napus*, hygromycin-resistant *Brassica nigra* (Gerdemann-Knörck et al. 1995) and Basta-resistant *Arabidopsis thaliana* (Forsberg et al. 1998) have been utilized to help selecting fusion products and prohibit the growth of unfused *Brassica napus* protoplasts. Furthermore, selectable marker genes have been used to select the fusion products of hygromycin-resistant *Brassica juncea* and phosphinothricin-resistant *Brassica oleracea* (Arumugam et al. 1996; Christey 2004). After protoplast fusion, the putative hybrids produced must be identified and characterized by a range of methods, including analyses of morphological and cytological traits, isozymes, and molecular markers (AFLP, RFLP, mtDNA, and cpDNA) (Dixelius 1999; Fahleson et al. 1997; Christey 2004).

Protoplast fusion has also been utilized to resynthesize *Brassica* amphidiploid species, including *Brassica carinata*, *Brassica napus*, and *Brassica juncea*, through fusing the appropriate diploid species, resulting in generating new forms as well as increasing the genetic variation in the amphidiploid species and exploiting cytoplasmic traits from both parents (Christey 2004). Since the formation of the first intertribal somatic hybrids between *Brassica campestris* and *Arabidopsis thaliana*

(Gleba and Hoffmann 1979, 1980), various intertribal somatic hybrids have been accomplished between *Brassica* species and four other Brassicaceae tribes, including genera from the Arabideae, Lepidieae, Sisymbrieae, and Drabeae tribes. The agronomic traits of interest that have been successfully transferred involved disease resistance, altered oil quality, herbicide resistance, and drought tolerance and cold tolerance (Christey 2004).

Bacterial and fungal diseases affect *Brassica*, causing a high economic damage. Protoplast fusion has been utilized to overcome interspecific, intergeneric, and intertribal crossing barriers in the transfer of genes conferring bacterial and fungal disease resistance (Christey 2004). Furthermore, the genus *Moricandia* of Brassicaceae contains C_3–C_4 intermediates that exhibit reduced photorespiration. To enhance the production of C_3 *Brassica* species, modifying their photosynthetic characteristics should be achieved through the transfer of this trait (Christey 2004). Several intergeneric somatic hybrids have been produced by fusion of *Brassica oleracea* and *Moricandia nitens* (Yan et al. 1999). Six hybrids exhibited a gas-exchange trait which was intermediate between the C_3 and C_4 *Moricandia nitens* and C_3 *Brassica oleracea*. Other agronomic traits, including pest resistance, altered oil quality, and metal hyperaccumulation, have been transferred through *Brassica* protoplast fusion (Christey 2004). Asymmetric somatic hybrids have been produced via the fusion of *Brassica napus* and *Crambe abyssinica* having greater amounts of erucic acid using UV irradiation (Wang et al. 2003; Christey 2004). Furthermore, several somatic hybrids have been produced via the fusion of the metal hyperaccumulator *Thlaspi caerulescens* and *Brassica napus* (Brewer et al. 1999). Several hybrids survived on high zinc-containing medium. AFLP markers have been utilized to identify and characterize these somatic hybrids (Christey 2004).

26.2.3 Cytoplasmic Traits and Fertility

Protoplast fusion could be used in the production of new nuclear cytoplasmic organelle combinations, causing increasing of cytoplasmic diversity (Christey 2004). Additionally, protoplast fusion exhibited reassortment processes in the cytoplasmic organelles produced to repair any defects that emerged because of nuclear-organellar incompatibility. Recombination of organellar DNA could happen, resulting in creating new cytoplasmic traits (Christey 2004). Several methods have been used to produce cybrids which comprise a nucleus from one source and a cytoplasm from another one. These methods included treatment with gamma rays or UV irradiation (Christey 2004). The most widely used method for cybrid production is the asymmetric hybridization which includes iodoacetate treatment of the recipient source and irradiation of the donor one. Sigareva and Earle (1997) reported that the gamma-irradiation method was the most efficient in cybrid production. UV irradiation proved to be uneffective method. However, irradiation of cytoplasts was also needed because some of the cytoplasts kept nuclear DNA.

Cytoplasmic male sterility (CMS) plays an important role in hybrid seed production since the self-incompatibility technologies have some disadvantages, involving

breakdown of incompatibility, genetic complexity of the system, and labor intensiveness (Christey 2004). In *Brassica napus*, various cytoplasmic male sterility systems have been characterized, including cytoplasmic male sterility Ogura, Tour, and Polima and cytoplasmic male sterility from Kosena radish. Somatic hybridization has been utilized for embedding them into new genotypes. The CMS Ogura system from *Raphanus sativus* is commonly used. However, *Raphanus* chloroplasts are not completely functional at low temperature, causing chlorosis and limited growth (Christey 2004). Organelle segregation after protoplast fusion prevented this issue. Moreover, cabbages have been produced via fusion of fertile cabbage and cold-tolerant CMS broccoli (Sigareva and Earle 1997). Only diploid fusion products containing the Ogura-specific mtDNA sequence should be selected. Fusion of *Brassica napus* and *Brassica tournefortii* produced male sterile cybrid *Brassica napus* genotypes (Liu et al. 1996). MtDNA analyses of male sterile genotypes showed that the *atp6* locus of *Brassica tournefortii* could play a role in CMS expression. CMS was also introduced into *Brassica juncea* after protoplast fusion of *Trachystoma ballii* and *Brassica juncea*, followed by repeated backcrossing (Kirti et al. 1995b). These new lines had a recombinant plastome (Baldev et al. 1998) and a large mitochondrial recombination (Kirti et al. 1995b). In male sterile systems of alloplasmic origin, the lack of appropriate fertility restorer genes could reduce the use of such genotypes in generating hybrid seeds (Christey 2004). Protoplast fusion between *Raphanus sativus* and *Brassica napus* along with backcrossing process could be used to introduce a restorer gene (Sakai et al. 1996). Furthermore, new CMS types proved to be efficient for F1 hybrid seed production in *Brassica oleracea*. Yarrow et al. (1990) and Christey et al. (1991) used protoplast fusion to transfer Polima CMS and *nigra* CMS to broccoli, respectively. The combination of the two cytoplasmic traits of CMS and atrazine resistance (ATR) through protoplast fusion would be much better to improve growth on atrazine-containing soils (Christey 2004). The combination of these two cytoplasmic traits has been mainly used in *Brassica napus* and *Brassica oleracea*. Moreover, CMS Ogura and Polima have been combined with ATR in *Brassica napus* (Christey 2004).

Intertribal somatic hybrids form few fertile plants. In *Brassica carinata* hybrids produced by fusion of *Brassica oleracea* and *Brassica nigra*, somatic hybrids comprised 34 chromosomes, and the majority had meiosis of 17 bivalents (Narasimhulu et al. 1992). The low level of fertility indicates the presence of other chromosomal alterations which could be due to the effect of fusion or cell culture (Christey 2004). The formation of asymmetric hybrids results in more fertile hybrids. Intertribal fertile somatic hybrids between *Brassica napus* and *Lesquerella fendleri* have also been produced (Skarzhinskaya et al. 1996).

26.3 Microspore and Haploidy System in *Brassica*

Since the first promising production of microspore-derived embryos from *Datura* anthers (Guha and Maheshwari 1964, 1966), several species including *Brassica* have produced microspore-derived embryos from cultured anthers (Keller and

Armstrong 1978, 1979; Ferrie and Keller 2004). Embryogenesis frequency from *Brassica* anther culture was low compared to the microspores number available per anther (Keller and Armstrong 1978; Swanson 1990; Ferrie and Keller 2004). Therefore, other methodologies have been developed for culturing the isolated microspores instead of the whole anther. The isolated microspores of *Brassica napus* produced haploid plants as demonstrated earlier by Lichter (1982). Microspore culture of *Brassica* species is a highly effective system for producing several embryos and double haploid lines (Ferrie and Keller 2004). This haploidy system has been used in various applications, including genetics, breeding, mutagenesis, transformation, genomics, and physiological and biochemical analyses (Ferrie and Keller 2004).

Haploidy systems have been used in genetics and breeding programs to develop new genotypes. Double haploidy could fasten breeding program, develop homozygous genotypes in one generation, and use smaller population sizes (Ferrie and Keller 2004). Most of the *Brassica* genotypes have been developed through microspore culture or spontaneous doubling (Ferrie and Keller 2004). Schuler et al. (1992) reported that heterosis could affect the seed yield in *Brassica rapa*. Developing hybrid lines might use this heterosis. In *Brassica oleracea*, genotypes developed from anther culture had different ploidy levels ranging from haploid to polyploid (Farnham 1998; Wang et al. 1999). This could be attributed to the irregular polyploidization and spontaneous doubling occurring during the microspore culture process (Ferrie and Keller 2004).

Mutation methods have been utilized to develop several genotypes of different crop species through treating seeds with physical or chemical mutagens (Ferrie and Keller 2004). Seed mutagenesis proved to be efficient for enhancing genetic variation and developing genotypes. Single-celled haploid microspores would be the most appropriate material to be used for mutagenesis studies (Ferrie and Keller 2004). Chemical or physical mutagens have been successfully used to stimulate mutations in *Brassica* microspores as well as to induce embryogenesis (Ferrie and Keller 2004). Macdonald et al. (1988) reported that treating *Brassica napus* buds with gamma rays may result in increasing the embryo and double haploid plants. Microspore mutagenesis has been carried out in *Brassica rapa* and *Brassica napus* to develop disease- or herbicide-resistant genotypes (Beversdorf and Kott 1987; Swanson and Erickson 1989; Ahmad et al. 1991; Zhang and Takahata 1999). Examination and choice during the haploid stage could assist in producing genotypes carrying disease resistance trait (Ferrie and Keller 2004). Bansal et al. (1998) inoculated haploid *Brassica napus* plantlets with *Leptosphaeria maculans*. These plants were then treated with colchicine to double the chromosomes and rescreened for blackleg symptoms. Many resistant double haploid plants were generated from the resistant haploid plantlets (Bansal et al. 1998; Ferrie and Keller 2004). Double haploidy has also been used in improving canola meal (McClellan et al. 1993; Ferrie and Keller 2004).

Microspores are ideal tool for transformation studies in *Brassica*. Successful microspore transformation studies have been successfully achieved using *Agrobacterium*-mediated transformation, particle bombardment, and electroporation

(Ferrie and Keller 2004). The first commercial transgenic canola genotype was developed using *Agrobacterium*-mediated transformation method (Oelck et al. 1991). *Brassica* microspore culture has been utilized to investigate the early events and regulation process of embryogenesis (Ferrie and Keller 2004). Several methods have been used to identify the early stages of embryogenic microspores, including morphological and cytological analyses (Hause and Hahn 1998; Simmonds and Keller 1999; Ferrie and Keller 2004). Flow cytometry has been used to separate and distinguish between induced and non-induced cells (Pechan and Keller 1988; Schulze and Pauls 2002). Fluorescence microscopy has also been used to identify embryogenic microspores (Telmer et al. 1992). Gene mapping and investigating their linkage relationships to other genes are essential in breeding programs (Ferrie and Keller 2004). Double haploids proved to be invaluable in this case. Double haploids could be exploited to assess traits within a population based on bulked segregant analysis. Double haploids could also be used for studying quality trait loci. Application of genome-wide expression profiling in such systems could also be possible in *Brassica* (Ferrie and Keller 2004).

26.4 Conclusions

Protoplast fusion plays an important role in the transfer of nuclear and cytoplasmic important traits between different *Brassica* species, genera, and tribes. Protoplast fusion could be used in the production of new nuclear cytoplasmic organelle combinations. In *Brassica napus*, various cytoplasmic male sterility systems have been characterized, including cytoplasmic male sterility Ogura, Tour, and Polima and cytoplasmic male sterility from Kosena radish. The microspore culture is an ideal technology for developing microspore-derived embryos and double haploid plants. Since the first promising production of microspore-derived embryos from *Datura* anthers, several plants including *Brassica* produced microspore-derived embryos from cultured anthers. This haploidy system has been used in various applications, including genetics, breeding, mutagenesis, transformation, genomics, and physiological and biochemical analyses.

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