Iram Siddique *Editor*

Propagation and Genetic Manipulation of Plants

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Editor
Iram Siddique
Department of Botany
Aligarh Muslim University
Aligarh, Uttar Pradesh, India

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About the Editor

Iram Siddique is an Assistant Professor in the Department of Botany, Aligarh Muslim University, Aligarh, India. She has also worked as an Assistant Professor in the Department of Botany and Microbiology, King Saud University, Riyadh, Saudi Arabia, where she was also a Co-Principal Investigator in a research group project funded by DSR-KSU, Riyadh. She has also worked as a Senior Research Fellow and Research Associate of CSIR, New Delhi. She has more than ten years of teaching and research experience in Plant Tissue Culture and Molecular Biology. She has published about 35 research papers in the journals of International repute. The same have been cited 446 times with 11 h-index. She has also participated in several national and international symposiums and seminars held at various universities and institutes of India and abroad. She was conferred by a number of prestigious awards like Young Scientist award of the Year 2010 of UPCST, Lucknow, Junior Scientist of the Year 2011 of NESA, New Delhi, and Excellence in Research (Scientist) award 2018. She visited Faculty of Agriculture, University of Mauritius, Mauritius, Singapore, Addis Ababa University, Ethiopia, and UAE under various research exchange programs. She holds membership of national and International scientific bodies and editorial boards of various journals.

Somaclonal Variation and Methods Used for Its Detection

1

V. M. Ranghoo-Sanmukhiya

Abstract

During plant tissue culture, the genetic stability of in vitro plantlets is often compromised and the underlying factors include the plant species or varieties involved, mode of regeneration used, genotype and ploidy level, composition of the growth medium, duration of the callus phase, and total time in culture. During tissue culture, conversion of the explant into in vitro tissue in fact imposes stress to the plant cells, which undergo a genomic shock and require restructuring of their genomes. An array of morphological, biochemical, and molecular methods has been used to assess somaclonal variation in different plant species and variable efficiency has been observed. The array of methods including molecular markers used for ensuring genetic stability of regenerated cultures in different plants is reviewed in this chapter.

Keywords

Somaclonal variation · Tissue culture · Molecular markers

1.1 Introduction

Tissue culture experiments involve growing plant tissues under a set of specific laboratory conditions different from their normal growth conditions in nature in an aim to eventually propagate and conserve them or for ultimate genetic transformation. When the explant is placed in vitro, a specific tissue culture protocol has to be optimized or designed for each species used. Moreover, adjustments in physiology, anatomy, and metabolism have to be made for them to be able to grow and

V. M. Ranghoo-Sanmukhiya (✉)
Faculty of Agriculture, University of Mauritius, Réduit, Moka, Mauritius
e-mail: m.sanmukhiya@uom.ac.mu

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multiply in culture. During tissue culture, any plant cell type or organ including embryos, spores, roots, leaves, and protoplast are used as explants. There is normally a cycle of dedifferentiation of tissues from the explant referred to as (callus production) under conditions set by the researcher followed by proliferation for a number of generations (callus proliferation) culminating in the regeneration of the plants (Larkin and Scowcroft 1981). Regeneration only involves repeated mitotic divisions, which ideally should yield genetically uniform plants (Larkin 1998). The "somaclone" is the regenerated plant obtained following tissue culture, while "calliclones" and "protoclones" refer to regenerated plants obtained from stems and protoplast (Chawla 2000).

During tissue culture, conservation of the genetic integrity of the mother plant is a desirable component, but regeneration by tissue culture has been reported to yield plants which exhibit unexpected and undesired phenotypic anomalies and variation (Karp 1994). Conditions inherent in vitro include regimented media components, low light levels, and more importantly high humidity which eventually cause several physiological and developmental aberrations of the regenerated plantlets. In fact, somaclonal variation refers to culture-induced anomalies or variation, which eventually becomes genetic and is inherited by the clonal progenies (Chawla 2000; Us-Camas et al. 2014). There are other variations induced in culture which are only temporary and reverse back to the normal state when faced with other culture conditions. The temporary changes which are nonheritable and not reversible are in fact caused by epigenetic and physiological factors, otherwise heritable and permanent changes represent "de novo" genomic variation (Kaeppler et al. 2000). Initially, somaclonal variants were classified as artifacts caused by overexposure to phytohormones and epigenetic events (Chawla 2000; Isah 2015). In fact, genetic variation observed following regeneration is a result of aberration of genome expression which is due to the formation of chromosome mosaics and spontaneous mutation. Moreover, somaclonal variation can be induced by a directed approach through in vitro selection and can be minimized as well without any in vitro selection (Brar and Jain 1998; Chawla 2000). The genetic basis of somaclonal variation, its uses in agriculture, associated disadvantages and methods of detection are discussed.

1.2 Uses of Somaclonal Variation in Agriculture

In the current era, we need to secure food availability faced with the impending crisis of global warming and declining arable soil. Somatic embryogenesis can be exploited to produce plants with certain variable characteristics as compared to the starting material and without using transgenics. Under tissue culture conditions, the plant will do what it is programmed to do but variation will result by using epigenetic regulation and hence inducing variation. In addition, the number of embryos produced can be increased by making use of specific chemicals, e.g., 5-Aza for plants which are recalcitrant for SE and hence increase production. Somaclonal variation can be considered as an advantage as it allows creation of new genotypes which have been exploited in agriculture for the following:

1.2.1 Generation of New Agronomic Variants with Favorable Traits

Somaclonal variation has given rise to new breeding lines with variants of important agronomic traits. Many variants in different crops have been generated using somaclonal variation which can further be integrated in breeding programs for introgression of important agronomic traits in different lines. Somaclonal variants were selected for: high oil yield in *Cymbopogon winterianus* (Dey et al. 2015), higher flowering and fruiting ability in strawberry (Biswas et al. 2009), high sucrose yielding sugarcane varieties (Rastogi et al. 2015), finger millet of higher seed and biomass yield (Baer et al. 2007), non-browning potato varieties (Arihara et al. 1995; Thieme and Griess 2005).

1.2.2 Generation of Disease Resistance Varieties

Disease resistance for different crop varieties has been obtained through induced somaclonal variation. Somaclonal variants from different crops with increase disease resistance include sugarcane which were resistant to eyespot and red rot disease (Rastogi et al. 2015), wheat resistant to white blotch disease (Arun et al. 2003), and *Fusarium* wilt-resistant banana (Asif and Othman 2005).

1.2.3 Generation of Biochemical Variants with Abiotic Resistance

Biochemical variants can be generated and these can be used in plant metabolic pathway studies. Somaclonal variants in wheat which exhibit frost and freezing tolerance have been obtained and these have been directly related to the production of proline (Dorffling and Melz 1997). Somaclonal variation has also successfully yielded plants which exhibit salt and herbicide resistance in various crop species, e.g., drought and salt resistance in sugarcane (Rastogi et al. 2015) as well as drought tolerance rice (Adkins et al. 1995).

1.3 Disadvantages of Somaclonal Variation

1. Somaclonal variation generates uncontrollable and unpredictable variation which are of no agricultural use. Most changes are cultivar independent.
2. Genetic changes during regeneration occur at variable frequencies and are not stable and nonheritable.
3. Somaclonal variants are not novel genotypes and have frequently to be checked for novelty.
4. Generation of somaclonal variants is limited only to those plants which have the power to multiply in culture and regenerate into whole plants.
5. Somaclones have reduced growth rate, reduced fertility as well as low-performance rate. They sometimes lose the power of regenerating.

1.4 Causes and Genetic Basis of Somaclonal Variation

Conditions in tissue culture which induce mutations include wounding, removal of the cell wall, sterilization which includes exposure to high concentration of chemicals including sterilants, sugars, and plant growth regulators in the nutritive media. Genetic alteration of the regenerants in culture is inevitable and increases with prolongations (Duncan 1996; Us-camas et al. 2014). The in vitro environment is stressful and hampers proper morphogenesis of tissues and this is due to erratic gene expression which results in disrupted hormonal synthesis and signaling. In addition, oxidative stress has been reported in plant tissue culture which normally results in elevated reactive oxygen species production (Isah 2015). During regeneration of plants using tissue culture, there is normally differentiation and redifferentiation of cells leading to qualitative and quantitative genomic changes. Somaclonal variation can be traced back to changes which have occurred at the genome level and include:

1. Single gene mutation: Somaclonal variants in tomato have been linked to single gene mutation inside the genome of crop species including wheat, maize, rice (Edallo et al. 1981), and tomato (Evans and Sharp 1983).
2. Activation of transposable elements: Transposable elements have been linked with somaclonal variation and were first reported to cause colored spots in *Nicotiana tabacum* species (Lorz and Scowcroft 1983). Activation of the transposable elements has been observed in in vitro maize and tobacco tissues (Peschke and Phillips 1991). In vitro genetic rearrangements (Hirochika et al. 1996; Sato et al. 2011) as well as insertion and excision of transposable elements have also been observed (Gupta 1998; Sato et al. 2011).
3. Karyotype changes: Variant plants with altered chromosome numbers inducing ploidy level changes namely polyploidy which includes chromosomal abnormality have been observed (Hang and Bregitzer 1993; Mujib et al. 2007; Leva et al. 2012).
4. Changes in chromosome structure: Changes in chromosome structure which include chromosome breakage and rearrangement, centric and acentric fragments, abnormal arrangement of chromosomes, ring chromosomes, and formation of micronuclei (Kaeppler et al. 1998; Alvarez et al. 2010).
5. Plastid genetic change: Plastid genetic changes including deletion, addition, and intramolecular recombinations have occurred in somaclones (Cassells and Curry 2001; Bartoszewski et al. 2007).
6. Methylation: Methylation in single-copy sequences, methylation of the genome as well as histone modifications have been reported in tissue culture (Kaeppler et al. 2000; Linacero et al. 2011; Miguel and Marum 2011).

1.5 Methods Used for Assessing Somaclonal Variation

1.5.1 Morphological Assessment of Somaclonal Variation

Morphological assessment of somaclonal variation has been traditionally used to detect "variants" been referred to as "off types" regenerated plantlets. Characters most often used for the assessment include plant stature, leaf morphology, and pigmentation. Examples of somaclonal variants include banana dwarf off-types (Rodrigues et al. 1998) or excessive vegetative growth in palms (Zaid and Al Kaabi 2003). Morphological assessment for somaclonal variation may not be reliable since the number of characters used may be limited and more importantly be artifacts and caused by the impeding stress inherent of the tissue culture conditions (Cloutier and Landry 1994). Moreover, genomic changes caused during tissue culture but these may not be reflected in the phenotype (Bairu et al. 2011).

1.5.2 Biochemicals Used to Assess Somaclonal Variation

Responses of explants to certain chemicals such as gibberellic acid have been used for the assessment of somaclonal variation (Phinney 1985; Sandoval et al. 1995; Graebe 2003). Gibberellic acid is well known for its regulation of growth and development and somaclonal variants differ from normal plantlets in having disturbed gibberellic acid metabolism and endogenous level allowing somaclone identification (Bairu et al. 2011). Normal banana plants have been found to be gibberellic acid responsive and hence developed significantly greater leaf sheaths as compared to dwarf somaclonal variants which were nonresponsive (Damasco et al. 1996). Synthesis of chlorophyll and carotenoids has been used to differentiate somaclones with normal plantlets (Mujib et al. 2007; Wang et al. 2007).

1.5.3 Protein Markers Used to Assess Somaclonal Variation

Isozymes are different molecular forms of proteins which are used to actively control biochemical processes inside cells (Kunert et al. 2003). Isozymes fingerprints are normally generated and can be used as a basis for finding differences between genomes of parental plants and regenerated individuals. Enzymes commonly measured include esterase (EST), endopeptidase, alcohol dehydrogenase, peroxidase, polyphenol oxidase amongst others (Kunert et al. 2003). Isozyme analysis has advantages such as facilities of usage and low cost. Isozymes assays, however, have limited polymorphisms which are highly influenced by environmental conditions (Kunert et al. 2003). Somaclonal variation has been assayed using isozymes in palms (Salman et al. 1988; Saker et al. 2000), soybean (Amberger et al. 1992), citrus (Carini and De Pasquale 2003), and potato (Afrasiab and Iqbal 2012).

1.5.4 Cytogenetic Analysis for the Determination of Somaclonal Variation

Chromosomal abnormalities can occur in tissue culture plantlets and could be the causal factors of somaclonal variations (D'Amato 1985; Kaeppler et al. 2000). Presence of an abnormal number of chromosomes, aneuploidy, has been reported during callus induction and cell suspension phases of citrus and oil palm (Hao and Deng 2002; Giorgetti et al. 2011). Chromosomal alterations due to non-separation of chromatids during mitosis and meiosis can lead to the production of chromosomal abnormalities and segmental or complete aneuploidy (D'Amato 1985; Holland and Cleveland 2009; Kaeppler and Phillips 1993; Kaeppler et al. 2000). Variations in chromosome number among cells in cultures can be detected using fluorescence in situ hybridization, flow cytometry, and microscopic examination. Flow cytometry is a technique which allows the calculation of the DNA content analysis through the use of a fluorochrome specific to the DNA found in whole cells, protoplast, nuclei, or chromosomes. Using flow cytometry, diploids and tetraploids were recovered from calli cells of *Arapidopsis* which had cytogenetic abnormalities such as chromosomal translocations, deletions, and duplications (Orzechowska et al. 2013). Flow cytometry has allowed detection of chromosomal abnormalities during somaclonal variation in many plants including eucalyptus (Pinto et al. 2004), cotton (Jin et al. 2008), papaya (Abreu et al. 2014), and *Viola uglinosa* (Slazak et al. 2015).

1.5.5 Detection of Somaclonal Variation Using RFLP

The restriction fragment length polymorphism (RFLP) is one technique which is used for genome analysis and it involves digestion of genomic DNA using enzymes restriction endonucleases, followed by separation of the digested products on agarose gel for the generation of DNA profiles. Variations in the number and size of the bands will occur in the DNA profiles of the true to type regenerated plantlet as compared to the somaclonal variant. Variations in the band number and sizes observed inside the genome can be explained by insertion, deletion, or single nucleotide polymorphisms (Agarwal et al. 2008; Bairu et al. 2011). RFLP markers have been used to screen for somaclonal variation in rice (Müller et al. 1990), maize (Brown et al. 1991) sugar beet (Levall et al. 1994), *Hypericum perforatum* (Halušková and Čellárová 1997), and oil palm (Jaligot et al. 2002).

1.5.6 Detection of Somaclonal Variation Using Random Amplified Polymorphic DNA (RAPD)

Random amplified polymorphic DNA (RAPD) is a PCR-based technique which relies on the selective amplification of fragments by using primers of a random nature. These primers are around ten nucleotides in length which have the ability to bind to complementary sites within the genome followed by amplification of the

sites delimited. RAPD represents one among the most popular markers used for the assessment of somaclonal variation due to low input cost and low technology required. Presence of certain bands in the parental strains and absence in regenerated plantlets indicates genomic rearrangement during tissue culture and account for somaclonal variation. Common bands which are found in the somaclones originate from mutation inherited from the mother embryogenic callus tissues. Variations in the genome level caused by somaclonal variation include nucleotide substitution events such as transition and transversion (Ngezahayo et al. 2007). RAPDs have been however associated with low reproducibility, reliability, and transferability among different labs. RAPDs have actually been used in the assessment of somaclonal variation in sugarcane (Taylor et al. 1995; Tawar et al. 2008), banana (Trujillo and Garcia 1996; Tang 2005; Abdellatif et al. 2012), orchids (Chen et al. 1998), rice (Yang et al. 1999), maize (Osipova et al. 2001), tomato (Soniya et al. 2001), family of Zingiberaceae (Islam et al. 2004; Wondyifraw and Wannakrairoj 2004; Mohanty et al. 2011), and *Cymbopogon* (Dey et al. 2015).

1.5.7 Detection of Somaclonal Variation Using Simple Sequence Repeat (SSR)

Simple sequence repeat (SSR) also referred to as microsatellite markers consist of short DNA sequence motifs of few base pairs which are repeated tandemly inside the eukaryotic genome. Each genome varies in having a characteristic number of repeats and these can be amplified by using the polymerase chain reaction (PCR) using special primers. Different somaclones will have different numbers of these repeats units which are exhibited with high levels of polymorphism following amplification (Levinson and Gutman 1987; Coggins and O'Prey 1989). SSRs are highly reproducible and codominant which make them ideal, rapid, simple genotyping assays. SSRs are very versatile in asserting genetic variation and are highly used in marker assisted breeding. Moreover, SSR markers have also been used to screen for somaclonal variants in cotton populous (Rajora and Rahman 2003), bananas (Hautea et al. 2004; Ray et al. 2006), rice (Khai and Lang 2005), pine (Marum et al. 2009), sorghum (Zhang et al. 2010), and grapevine (Nookaraju and Agrawal 2012).

1.5.8 Detection of Somaclonal Variation Using the Inter-Simple Sequence Repeat (ISSR) Markers

Inter-simple sequence repeat markers represent the DNA fragments which are found between simple sequence repeats which are oppositely oriented. The ISSRs can be amplified by using primers which are complementary to the microsatellite regions. These inter-simple sequence repeats can be amplified by using the PCR technique which makes use of primers specially designed to amplify part of the microsatellite DNA core sequences as well as selectively amplifying the adjacent regions. The targeted region inside the genomes is found between two closely spaced SSRs which

may also be oppositely oriented. Following amplification around 20–50 fragments will be amplified from multiple loci containing the SSR motifs but they will be polymorphic in their sizes. The ISSR technique has been successfully used on several occasions for the genome screening of somaclones of mulberry (Vijayan and Chatterjee 2003), tea (Thomas et al. 2006), and gerbera (Bhatia et al. 2009).

1.5.9 Detection of Somaclonal Variation Using the AFLP Technique

Molecular markers mentioned above targeted highly repetitive sequences inside the genome and genotyping relies on the variation in the number of repeat counts. However, since multiple targets are being exponentially amplified at the same time, DNA profiles obtained may not be reproducible and be variable due to the quality of DNA and other reaction conditions.

The AFLP method involves both restriction enzymes digestion with PCR amplification of fragments obtained and allows generation of fragment length polymorphisms. The AFLP technique relies on the use of primers which are complementary to known adapter sequences to selectively amplify restriction-digested fragments and DNA profiles obtained are more reproducible. The AFLP technique is a more suitable and sensitive marker system with higher reproducibility (Meudt and Clarke 2007). The AFLP technique has been recognized as the ideal marker system for assessing somaclonal variation in *Arabidopsis thaliana* (Polanco and Ruiz 2002), *Coffeea arabica* (Sanchez-Teyer et al. 2003), bean (Rosales-Serna et al. 2005), *Asparagus officinalis* (Pontaroli and Camadro 2005), pea (Smýkal et al. 2007), grapevine (Schellenbaum et al. 2008), and *Feesia hybrida* (Gao et al. 2010).

1.5.10 Detection of DNA Methylation Events Using the Methylation-Sensitive Amplification Polymorphism (MSAP)

DNA methylation of the genome is normally associated with several inheritance processes such as transcriptional silencing, genetic imprinting, and genomic stability. Methyl groups are added to DNA cytosine to form 5-methyl cytosine (He et al. 2011) during the methylation process without any change occurring in the DNA sequence but results in an increase in the compression level of chromatin affecting gene expression eventually as access to transcription by RNA Polymerase II is debarred. The tissue culture environment induces abiotic stress during regeneration and is responsible for global genome-wide methylation. The long callus generation phase allows for the methylation event and induces variation during plant regeneration. In cassava, genome-wide methylation changes have been observed during meristem micropropagation using genome sequencing (Kitimu et al. 2015). DNA methylation events appear to be exclusive to repetitive DNA more specifically to transposons (Lippman et al. 2004). Decrease in the level of methylation may be due to gene activation during cell reprogramming to facilitate plant regenerations.

Changes in methylation have been reported to be the cause of somaclonal variation in many crops (Gonzalez et al. 2013). Epigenetic changes to DNA of plant tissues in culture appear to allow the reactivation of multiple transposon classes. Insertions of the retrotransposon family Tos and that of the class II TE family (nDiaZ) have been reported to increase with tissue culture period (Huang et al. 2009a). Transcriptional activity of the MERE1 retrotransposon family (associated with a decrease in cytosine methylation) has also been found to increase in two accessions of *Medicago truncatula* and has led to 40% of novel insertions mostly within coding sequences (Rakocevic et al. 2009). In grapevines, an increase in TE insertions has also been noted (Lizamore 2013).

The methylation-sensitive amplification polymorphism (MSAP) technique is used for the evaluation of the total methylation level of a genome. The MSAP is actually a modification of the AFLP (Vos et al. 1995) technique in which methylation-sensitive restriction endonucleases such as isoschizomers HpaII and Msp I to digest the DNA. These enzymes have different sensitivity to methylated cytosines and generate digestion patterns which allow identification of the methylated DNA (Yaish et al. 2014). The MSAP technique has efficiently been used to detect somaclonal variants in different plants including coffee and oil palm (Matthes et al. 2001; Bobadilla Landey et al. 2013; Francischini et al. 2017).

1.5.11 Detection of Somaclonal Variation Using Transposon-Based Marker Systems

Transposable elements represent DNA fragments which have the ability to make copies of themselves and can insert into new loci of the genome during transposition. When transposable elements insert themselves within genes there will be a hindrance to normal gene function (Makarevitch et al. 2015) and gene expression will be affected (Hollister and Gaut 2009; Lisch 2009). Induction of transposable elements has been observed in rice (Huang et al. 2009b) and maize (Makarevitch et al. 2015). Retrotransposons were also identified in tobacco and rice and have been reported to be involved with induced mutations in tissue culture (Hirochika 1993; Hirochika et al. 1996).

With the advent of genome sequencing projects, transposable elements have been recognized as being dynamic and abundant for the development of markers namely sequence-specific amplified polymorphism (SSAP), inter-retrotransposon-amplified polymorphism (IRAP), and retrotransposon-based insertion polymorphisms (RBIP) (Fig. 1.1). The sequence-specific amplified polymorphism (SSAP) marker which made use of transposon-specific sequence within an AFLP reaction was developed by Waugh et al. (1997). The SSAP technique has been found to be more specific than SSR-based markers in different agricultural crops namely oats, grapevine, tomato, sweet potato, and pea (Berenyi et al. 2002; Labra et al. 2004; Tam et al. 2005; Lizamore 2013). IRAP markers make use of the long terminal repeat primers (singly or in pairs) to amplify different retrotransposons distributed inside the genome of the somaclones, hence, amplifying regions of the genome which are found between

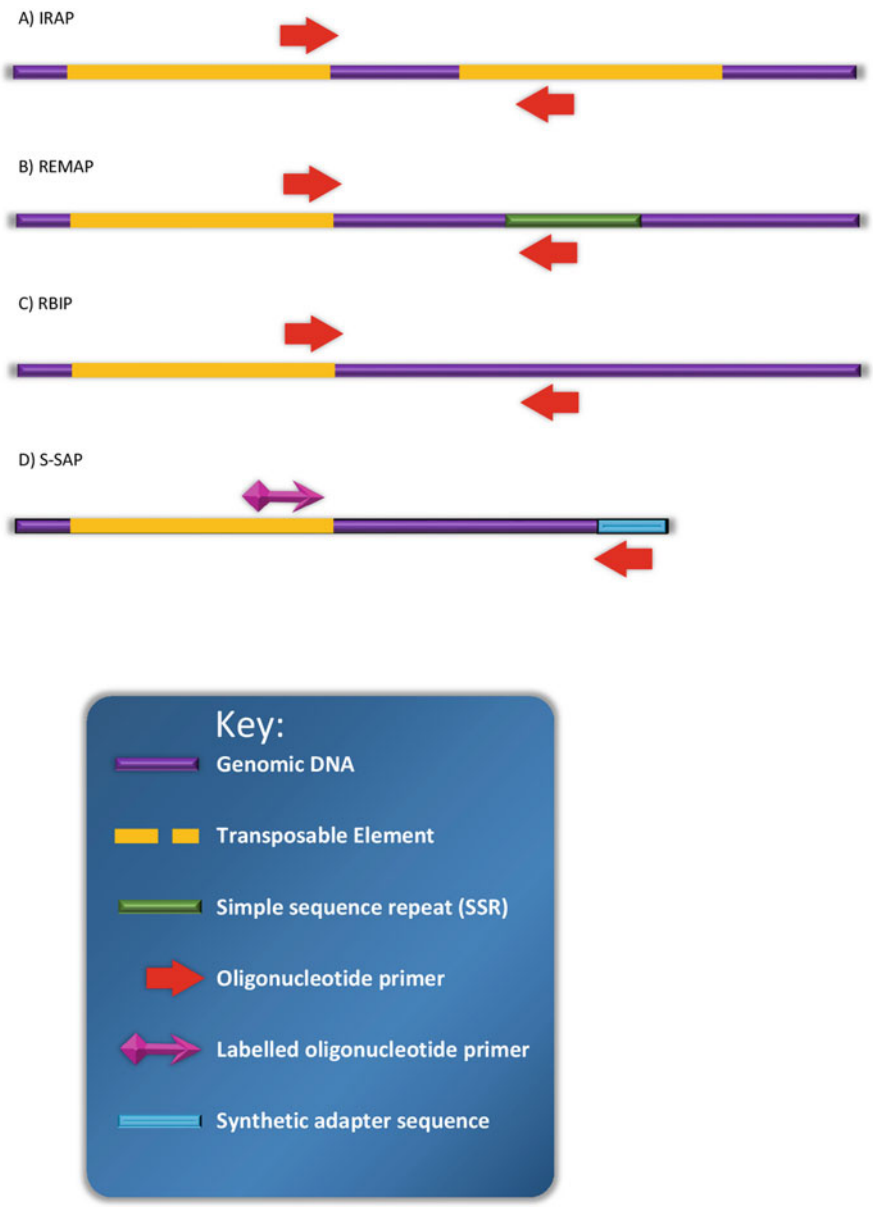

Fig. 1.1 Shows the different types of markers involving transposons discussed above

retrotransposons insertion sites (Kalendar et al. 1999; Kalendar and Schulman 2006). IRAP markers have been used to ensure genetic fidelity in banana, barley, wheat, and *Diospyros* (Muhammad and Othman 2005; Guo et al. 2006; Li et al. 2007; Carvalho et al. 2010; Campbell et al. 2011). Retrotransposon-based insertion

polymorphisms (REMAP) use primers specifically targeting transposon insertion sites and aim at testing presence or absence of insertions hence amplifying regions between microsatellites and adjacent retrotransposons (Carvalho et al. 2010; Lizamore 2013). REMAP aims at amplifying regions between microsatellites and adjacent retrotransposons (Lizamore 2013). All markers are represented in Fig. 1.1.

1.5.12 Next-Generation Sequencing (NGS) for the Determination of Somaclonal Variation

Regeneration of plants by tissue culture is stressful and can lead to both genetic and epigenetic changes which lead to phenotypic polymorphism. NGS-based analyses have been carried out in *Arabidopsis* (Jiang et al. 2011) and rice (Sabot et al. 2011; Miyao et al. 2012) to confirm the extent of genomic change which occurs during tissue culture for induction of somaclonal variation. An elevated genome-wide DNA sequence mutation rate was observed in *Arabidopsis* regenerated plantlets with base substitution as major genomic change but with no detectable change in transposable element reactivation (Jiang et al. 2011). In rice, single nucleotide polymorphism (SNPs), nucleotide changes (including insertions and deletions) as well as transpositions were observed (Sabot et al. 2011; Miyao et al. 2012; Zhang et al. 2014). In wheat, however, transposable elements were found to be the most likely factors causing somaclonal difference at the genome level (Bara Anek et al. 2016).

1.5.13 MicroRNA Involvement in Tissue Culture

MicroRNA or miRNA, first discovered in *Caenorhabditis elegans*, has strong gene regulatory activity. These miRNAs are noncoding, approximately 19–24 nucleotides in length which have been recognized to play crucial regulatory roles in many biological processes and pathways (Carrington and Ambros 2003). MiRNAs have been reported to be highly involved in plant defense especially in stress responses, plant development, hormonal signaling, and seed germination. Recently miRNAs have been reported to be involved during in vitro culture of plants (Miguel and Marum 2011; Rodriguez-Eneiquez et al. 2011). Somaclonal variation is thought to be caused by both genetic and epigenetic factors which relate to transposable element transposition and small RNA-directed methylation (Smulders and De Klerk 2011; Neelakandan and Wang 2012).

Differential microRNA production (namely Mir169a and miR390) has been recorded in strawberry plantlets regenerated by tissue culture and these have been correlated with existing differences with the phenotype (Li et al. 2012). Regulatory roles of miRNAs have been highlighted during somatic embryogenesis of many plants (Nodine and Bartel 2010; Su et al. 2015; Zhang et al. 2015a, b) as well as their involvement in gene regulation of epigenetic processes. However, to date, no direct relationship has been found between somaclonal variation and microRNA. Further studies are required to characterize the miRNA expressed during somaclonal

variation from different plants so that diagnostic miRNA associated with this phenomenon can be compiled and their possible use as biomarkers facilitated.

1.6 Conclusion

Regenerating plants by tissue culture may be aimed at maintaining the genetic integrity and the production of true to types but at times somaclonal variation may be desirable to increase the gene pool of a population with a narrow genetic base. Various methods for the detection of somaclonal variation have been reviewed and methods for assessing variant genomes at the molecular level allow for early and easy selection during a breeding experiment. These methods may be used in isolation but preferably in concertation to ascertain genetic fidelity.

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Synthetic Seed Technology in Some Ornamental and Medicinal Plants: An Overview

2

Mehpara Maqsood, Mir Khusrau, A. Mujib, and Zahoor A. Kaloo

Abstract

Synthetic seed technology has been successfully employed in various medicinal and ornamental plants. It has immense potential in preserving callus, somatic embryo (SE), and other important tissues of valuable germplasm. The method can also be exploited as a tool of multiplication in diverse plant groups. Synthetic seed production in plants facilitates season-independent propagation of elite germplasm, accelerates seedless, ornamental, and ploidy plants multiplication with unique traits. Synthetic seed/encapsulation approach has a high beneficiary role in improved and fast transportation of propagules. The obligatory requirement for application of artificial seed technology en masse involves high-scale production of quality micro-propagules, which nowadays is a serious limiting factor. There are numerous factors controlling the success of development and germination of synthetic seeds. These include matured quality embryos, temperature, storage time, sodium alginate percentage, calcium chloride concentration, hardening time, and plant growth regulators (PGRs) concentrations. Encapsulation technology is an emerging area to open new vista in conserving, propagating, and shipment of valuable plant material for research affecting floriculture and pharmaceutical industry. In this chapter, the artificial seed technique status is reviewed in some plants of ornamental and medicinal importance.

M. Maqsood
Govt. College for Women, Srinagar, Jammu & Kashmir, India

M. Khusrau
Govt. Degree College (Boys), Anantnag, Jammu & Kashmir, India

A. Mujib (✉)
Department of Botany, Jamia Hamdard, New Delhi, India

Z. A. Kaloo
University of Kashmir, Hazratbal, Srinagar, Jammu & Kashmir, India

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2.1 Introduction

The production of seed is an outcome of usual sexual process; in cross-pollinating plants, however, the produced seeds are genetically different from parental stock (Senaratna 1992). An important seed characteristic is its ability to undergo dormancy by self-drying, enabling seeds to be preserved for a long time. This is primarily due to plants' vegetative nature in which the distribution and exchange of seeds become inconvenient and a threat of disease dissemination is quite high (Chaudhury and Malik 2003). Recently, an increasing interest is noted on preparation of artificial seed through encapsulation of seeds/other plant parts—an important way of preservation and exchange of germplasm (Gantait et al. 2015). This synthetic seed technology is of immense importance in mass propagation of elite plant species where sexual seed propagation is plagued with incompatibilities. Frequently, the somatic embryos (SE) are used in encapsulation study; in recent times, enough efforts have been given to encapsulate non-embryogenic and other vegetative parts like axillary buds, shoot tips, and nodal segments (Sundararaj et al. 2010; Verma et al. 2010). Synthetic/artificial seeds are analogous to botanical seeds in which SEs are covered with one or more artificial layers, looking like a capsule (Cangahuala-Inocente et al. 2007). These encapsulated embryos can be used in conservation purposes of important germplasm including endangered plants; it also helps facilitating the exchange of materials across the world research centers (Rai et al. 2009).

The concept of artificial seed is floated since the discovery of SE induction in various investigated plant species. Artificial seed, also known as synseed, is first described by Murashige (1977) as an encapsulated single somatic embryo. It was later defined as a system engineered for practical use in commercial plant production (Gray et al. 1991). Unfortunately, very little attention has been paid to encapsulate vegetative plant parts like calli, nodal segments, buds, and shoot apices (Zych et al. 2005). The successful production of artificial seeds containing embryos or other nonsexual plant parts can open up new vistas in medicinal and floriculture sectors. But the application of artificial seeds depends primarily on the quality of somatic embryos similar to zygotic embryos analogous in morphology, physiology, and biochemistry (Redenbaugh et al. 1988).

There are two types of synthetic seeds, i.e., desiccated and hydrated (Bhojwani and Razdan 2006). The desiccated types were first introduced as naked or encapsulated SEs in Polyox, followed by desiccation (Kitto and Janick 1982). Desiccation is applied slowly over 1 or 2 weeks using decreasing relative humidity or is petri dishes placed overnight on laminar airflow chamber bench rapidly (Ara et al. 2000).

The hydrated artificial seed was first developed by encasing embryos of *Medicago sativa* and the technique is used in plant species where SEs are recalcitrant

and sensitive to temperature (Redenbaugh et al. 1984). These seeds are prepared by encapsulating SEs or other vegetative parts in a hydrogel. Various methods have been attempted in producing artificial seeds in which the use of calcium alginate is found most successful (Redenbaugh 1983). Both the hydrated and desiccated synthetic seeds are plagued with technical limitations. The gaseous exchange/embryo respiration is often an issue with hydrated synthetic, which also dried out rapidly unless provided with humid conditions. At the same time, desiccation methodology damages the rudimentary embryo within the capsule very fast reducing the application of synthetic seed concept (Redenbaugh 1983).

Since the first report of Steward et al. (1958) and Reinert (1958), the incidence of somatic embryogenesis is observed in a number of tropical plants. These include cereals, beverages, spices, fruits, legumes, fibers, etc. In most of the situations, only a few plants were produced and conversion frequencies, i.e., percentage of embryo forming plants was not reported at all (Redenbaugh 1983). For commercial viewpoint and for artificial seed technology to be successful, large-scale, high-quality embryo production and faster germination comparable to true seeds are absolutely essential. In ornamentals, in particular, tissue culture technique is applied successfully for plant propagation but in vitro embryogenesis research has not been taken off for production system. In other groups, however, this technology has been attempted to a wide number of plants of different families. The propagation and simultaneous conservation of pharmaceutically important herbs using axillary buds was earlier reported (Mandal et al. 2000). A method of producing encapsulable units of synthetic seeds was reported in *Asparagus officinalis* (Kanji and Yuji 2001). The vasaka (*Adhatoda vasica*) shoot buds were similarly encapsulated in Na alginate solution in forming artificial seeds (Anand and Bansal 2002).

The current effort aimed to give a brief description of artificial seed production in some select medicinal and ornamental plants, in which the technology is developed. The protocol of encapsulation is presented in the below scheme:

2.2 Preparation of Synthetic Seeds

Selection of explants for encapsulation.
↓
Preparation of MS medium.
↓
Surface sterilization of explants for encapsulation.
↓
Preparation of alginate (%) and calcium chloride (mM) solution.
↓
Encapsulation of explants in sodium alginate solution using a wide bored pipette tip.

↓

Dropped sodium alginate along with explants into calcium chloride.

↓

Kept on rotary shaker for some time.

↓

Washed with autoclaved double distilled water.

↓

Dried synthetic seeds with autoclaved paper.

↓

Placed seeds on suitable MS medium.

↓

Hardened the plant in greenhouse.

The sodium alginate and calcium chloride level varies with plant species. The duration of time to harden the seeds also varies with the different plant species. The mechanism involves the replacement of Na^+ of sodium alginate (water soluble) with Ca^+ of calcium chloride to form calcium chloride (water insoluble). In an important medicinal plant, *Catharanthus roseus*, sucrose was added to the alginate which functions as endosperm provided nourishment to the growing embryo (Mehpara et al. 2012).

2.3 Synthetic Seeds of Medicinal Plants

Several medicinal plants occurring naturally come under rare, endangered, and endemic category. This decline in number is due to habitat destruction by a variety of anthropogenic activities like overgrazing, unregulated harvesting, global warming, and pollution and also by diverse natural phenomena (fewer seed setting, dormancy, poor rate of germination owing to incompatibility, etc.). It is therefore important to propagate these plants fast and conserve medicinal plants in vitro along with other preservation methods. In medicinal plants, the duration of incubation temperature had direct influence on artificial seed conversion, i.e., longer storage decreased conversion frequency irrespective of temperature in *Tylophora indica* (Gantait et al. 2017). Similar promising outcome was reported in oil palm in which cold storage influences metabolic rate in artificial seeds, embryos remain in a quiescent state and aiding preservation (Palanyandy et al. 2015). *Rumex vesicarius* L. belonging to the family Polygonaceae is a wild edible medicinal herb, propagated through seeds. The seeds are called dormant/recalcitrant with low seed germination rate (20%) which lessens its survivability (Asrar 2011). In such situation, the induction of mass-scale embryo formation is essential and encapsulation of SE by forming synthetic seed has huge applications. This methodology is noted to be highly successful in many plants like *Adhatoda vesica* (Anand and Bansal 2002), *Allium sativa* (Bekheet 2006), and *Daucus carota* (Latif et al. 2007). In *C. roseus,* an encapsulation process was developed for making synthetic seeds by using sodium

Table 2.1 Some important medicinal plants and various explants used for making synthetic seeds (Courtesy and source: Reddy et al. 2012; Gantait et al. 2015)

Medicinal plants	Explants used	Citations
Catharanthus roseus *Adhatoda vasica*	*SE* *Shoot buds*	Mehpara et al. (2012) Anand and Bansal (2002)
Allium satium L.	*Bulblets*	Bekheet (2006)
Bacopa monnieri L.	*Nodal microcutting*	Ramesh et al. (2009)
Cannabis sativa L.	*Nodal segments*	Lata et al. (2011)
Coriandrum sativum L.	*SE*	Stephen and Jayabalan (2000)
Cucumis sativus L.	*SE*	Tabassum et al. (2010)
Daucus carota L.	*SE*	Latif et al. (2007)
Helianthus annuus L.	*Shoot tip*	Katouzi et al. (2011)
Hyoscyamus muticus L.	*SE*	Pandey and Chand (2005)
Morus indica L.	*Axillary bud*	Kavyashree et al. (2006)
Rauvolfia serpentina (L.) Benth. Ex Kurz.	*Micro shoot*	Faisal et al. (2012)
Ocimum sanctum L.	*Axillary bud*	Mandal et al. (2000)
Simmondsia chinensis L.	*Shoot apex and axillary bud*	Hassan (2003)
Solanum nigrum L.	*Shoot tip*	Verma et al. (2010)
Valeriana wallichii	*Apical and axial shoot bud*	Mathur et al. (1989)
Ocimum basilicum	Axillary bud	Mandal et al. (2000)
Paulownia elongata	*SE*	Ipekci and Gozukirmizi (2003)
Chlorophytum borivilianum	*Shoot bud*	Dave et al. (2004)
Rhodiola kirilowii	Axillary bud and callus	Zych et al. (2005)
Withania somnifera	*Shoot tip*	Singh et al. (2006)

alginate (2.5%) + calcium chloride (100 mM) added solution. The encapsulated embryos germinated well and produced seedlings in NAA- and BAP-supplemented MS medium (Mehpara et al. 2012). The artificial synthetic seeds were preserved at low temperature (4 °C) for about 10 weeks, which later produced plantlets without losing germination ability.

Beside embryos, the use of other explants is reported for the production of artificial seeds in several studied medical plants like *Fragaria ananasa* and *Rubus idaeus* (Lisek and Orlikowska 2004), *Valeriana wallichii* (Mathur et al. 1989), *Rauvolfia serpentina* (Ray and Bhattacharya 2008), *Cineraria maritime* (Srivastava et al. 2009), *Cannabis sativa* (Lata et al. 2009), etc. The development of synthetic seeds in various medicinal plants is presented in Table 2.1:

In several studied plants like *R. vesicarius* L., the torpedo-shaped SEs were chosen for encapsulation (Nandini et al. 2014). The 2.0% alginate concentration in 100 mM $CaCl_2$ was optimum for encapsulation when kept for 45 min for complexation.

2.4 Synthetic Seeds and Ornamental Plants

A number of ornamentals/plants have desiccation-sensitive seeds showing storage time very limited (a few weeks to months). High pathogenic incidences make things more difficult. Synthetic seed procedure is an alternative way of in vitro propagation and preservation of elite germplasm including ornamentals. Ornamental plants constitute an important component in floriculture industry (Ali et al. 2007) and these groups composed of bulbous; tuberous; and foliage used in pot, landscaping, and house/office decorative purposes. Several protocols have been developed for the encapsulation of in vitro-regenerated SEs, protocorms, and other important explants (Lambardi et al. 2006). Some important ornamentals and the explants used for encapsulation are presented in Table 2.2.

The synthetic seed technology and conservation of important germplasm like medicinal, spices, and ornamental have been attempted in author's laboratory (Mujib et al. 2016; Ali et al. 2018). *Caladium,* an important monocotyledonous genus, belongs to the family Araceae. There are 12 species with about 2000 different cultivars. The foliage of *Caladium* is colored and very attractive; the leaves have green; white; red pigments, some are spotted, with red/green veinations, and are used as indoor decorative pot plants (Deng et al. 2007). In *Caladium,* the induction of SEs was indirect, i.e., callus was induced first from cultivated explants on which variable numbers of embryos were produced (Mujib et al. 2016). Compact callus cultured on

Table 2.2 Some ornamental plants and various explants used for the development of synthetic seeds (Courtesy and source: Reddy et al. 2012; Gantait et al. 2015)

Ornamental plants	Explants used	Citations
Caladium bicolor	SE	Mehpara et al. (2015)
Hyoscyamus muticus L.	SE	Pandey and Chand (2005)
Eustoma grandiflorum	SE	Ruffoni et al. (1994)
Genista monosperma	SE	Ruffoni et al. (1994)
Betula pendula	Soot tips	Piccioni and Standardi (1995)
Lilium longiflorum.	Bulbs	Standardi et al. (1995)
Syringa vulgaris	Axillary buds	Refouvelet et al. (1998)
Anthurium andreanum	Embryogenic calli	Nhut et al. (2004)
Gypsophila paniculata	Shoot tips	Rady and Hanafy (2004)
Saintpaulia ionantha	*Micro shoots*	Daud et al. (2008)
Nerium oleander	Axillary nodes	Ozden et al. (2008)
Photinia fraseri	Axillary nodes	Ozden et al. (2008)
Syringa vulgaris	Axillary nodes	Ozden et al. (2008)
Spathoglottis plicata	Protocorms	Khor et al. (1998)
Geodorum densiflorum	"Protocorm-like" body	Datta et al. (1999)
Cremastra appendiculata	"Protocorm-like" body	Zhang et al. (2009)
Coelogyne breviscapa	"Protocorm-like" body	Mohanraj et al. (2009)
Flickingeria nodosa.	"Protocorm-like" body	Nagananda et al. (2011)
Vanda coerulea	"Protocorm-like" body	Sarmah et al. (2010)

BAP- and NAA-added medium started to differentiate embryos in numbers. The embryo-forming ability (i.e., the embryo numbers/callus mass and embryogenesis frequency) however, varies with plant growth regulators used**.** On an average, three to five SEs were differentiated per callus mass. The embryo number gradually improved with time and in NAA + BAP-amended medium, good numbers of embryos were observed. The embryos progressed into matured embryo (swollen base and pointed tip) and these somatic embryos were selected for artificial seed production study. Mehpara et al. (2015) developed synthetic seeds by mixing various sodium alginate + calcium chloride levels in *Caladium bicolor*. The synthetic seeds/beads were developed by using embryos in sucrose (3.0%), sodium alginate (3.0%), and calcium chloride (100 mM) amended gel and successful plantlet recovery was obtained (Fig. 2.1d–f). The prepared artificial beads were kept at low temperatures at 4 °C, which showed good germination rate following 12 weeks of low-temperature storage. The conversion percentage (synthetic seed to plantlet regeneration) was, however, reduced with passage time.

2.5 Factors Affecting Development of Synthetic Seeds

The various factors that have noticeable effect on the formation of encapsulated seeds are explants used, percentage of sodium alginate, concentration of calcium chloride, duration for hardening, regulation of rotary rpm for hardening (Fig. 2.1a, b), plant growth regulators in culture medium, incubation time, etc. Various types of explants are used in the formation of synthetic seeds that govern their germination/viability:

2.6 Factors Affecting Synthetic Seeds' Performance

The viability of artificial seeds is influenced by several factors viz. storage time, temperature level, medium selection, and aseptic conditions (Fig. 2.1c). The germinability of stored synthetic seeds increased with high temperature and declined in cold storage in *R. vesicarius* L., whereas no significant difference was noted in room storage (Nandini et al. 2014). Another decisive factor is the quality of storage during transport, which regulates physiology of synthetic seeds and in turn determines the success of plantlet conversion from artificial seeds. Higher conversion efficiency of synthetic seeds was reported at cold (4 °C) temperature as was noted in *Cleopatra tangerine* (Nieves et al. 1998) and Apple stock (Sicurani et al. 2001). A promising degree of interaction was observed between cold storage (4 °C) and storage interval up to 45 days for regrowth as well as root and shoot development as reported in olive (Ikhlaq et al. 2010). The storage of encapsulated seed is a crucial factor for transport and conservation of germplasm on which the success of synthetic seeds depends.

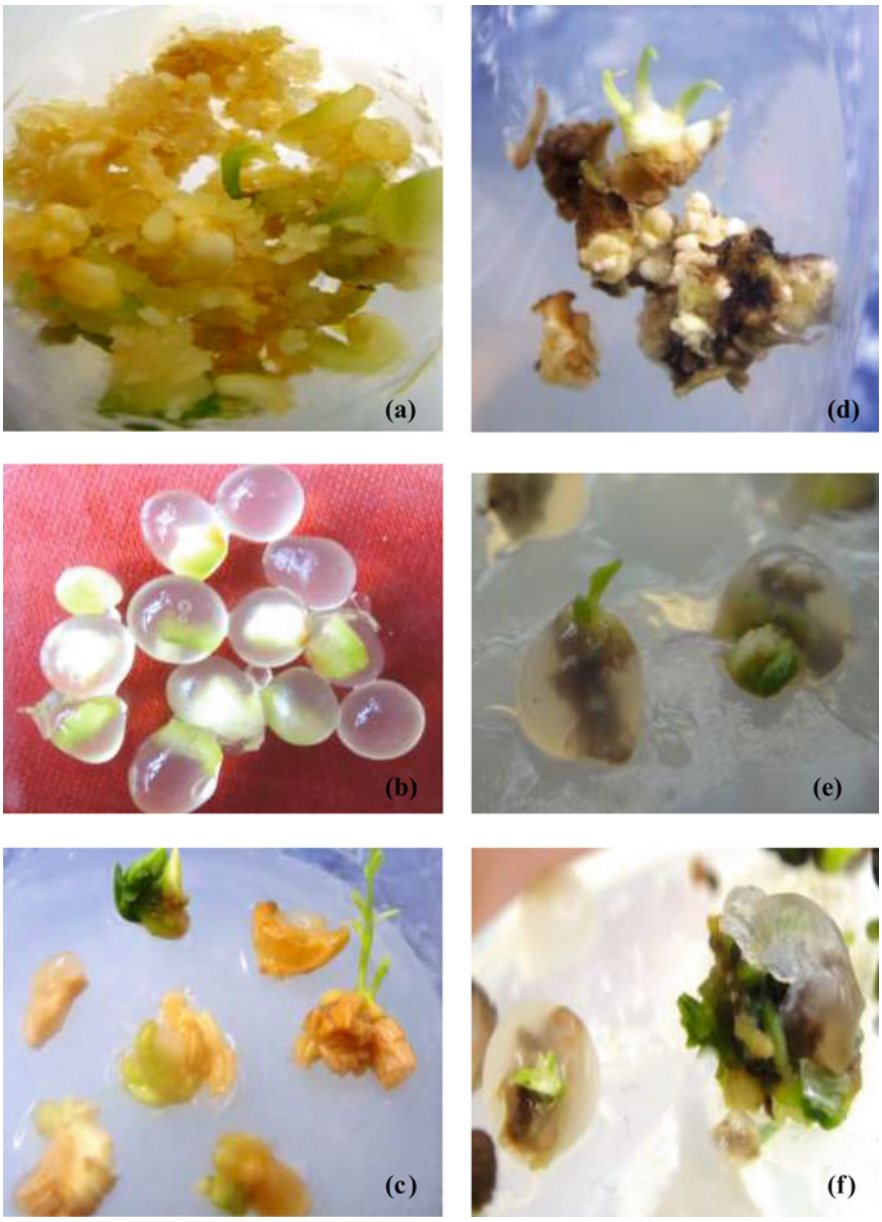

Fig. 2.1 (**a**) Somatic embryos in *Catharanthus roseus,* (**b**) encapsulation of embryos, (**c**) emergence of plantlets from matrix in *C. roseus,* (**d**) somatic embryos in *Caladium bicolor,* (**e**) encapsulation of embryos, and (**f**) emergence of plantlets from matrix in *C. bicolor*

2.7 Advantages of Synthetic Seeds

- The synthetic seeds may be used as an alternative planting stock especially to forest and other economically important plant materials (Asmah et al. 2011).
- The use of artificial seeds offers additional avenues for direct planting to greenhouse or field, thus several intermediate steps may be bypassed (Reddy et al. 2012).
- Artificial seeds have merits in plants (cross-pollinated) where sexual incompatibility-based issues, like F1 seed setting and germination, are a major challenge (Latif et al. 2007).
- The synthetic seeds have multiple advantages including uniformity in production, higher scale-up capacity, potential for automation production process. Handling is simple and even during transportation; long-term preservation of valuable germplasm is also a reality (Singh et al. 2007).
- Synthetic seeds are exploited nowadays in advanced cryopreservation procedures with an aim at long-term preservation of germplasm (Ruby et al. 2015).

2.8 Disadvantages of Synthetic Seeds

Despite numerous advantages, synthetic seed possesses various limitations:

- Lack of efficient root systems in plantlets derived from synthetic seeds; limitations in artificial seed production and sowing of commercially important plants/varieties in aseptic cultural conditions (Hung and Trueman 2012).
- Scale-up production of micropropagules/embryos for making capsules in gel matrix at feasible rate is a serious limitation, and micropropagation system is still another major bottleneck in many woody plant genera, required for the development of artificial seed technology (Rihan et al. 2012).
- The planting of artificial seeds directly in soil or in other substances like soilrite, organic matter, vermiculite under aseptic conditions is a practical challenge to growers of developing countries (Jung et al. 2004).

2.9 Conclusion

In summary, the development of synthetic seed protocol and optimization of plant regeneration system will help in alternative propagation method of important plant genera like *Caladium* where plants do not propagate through seeds. Synchronous, uniform, and fast SE formation and encapsulation of embryos could provide a realistic approach for germplasm preservation. The encapsulated SEs were successfully preserved at low temperature (4 °C) for 10–12 weeks; further refinement of process may improve in enabling "short and medium" term storage possibility of in vitro-cultivated tissues of other horticulture and medicinal plants. Similarly, medicinal plants are important sources of alkaloids, conservation/cryopreservation

of cell lines/embryogenic tissues with higher yield even up to medium term would extend its uses further in alkaloid enrichment study.

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Cytokinin Influence on Micropropagation System of *Dianthus caryophyllus* L.

3

Iram Siddique and Vikas Yadav

Abstract

A potent, quick and refined plant regeneration protocol was effectively established for *Dianthus caryophyllus* via nodal segment explants cut out from a 2-year-old vigorous mother plant. The three diverse cytokinins viz. 6-benzyladenine (BA), Kinetin (Kin) and thidiazuron (TDZ) were assessed as enhancements to Murashige and Skoog (MS) medium, the maximum frequency of multiple shoot bud induction was attained on MS medium augmented with 5.0 μM BA and 1.0 μM α-naphthalene-acetic acid (NAA). The in vitro raised shoots when subcultured on plant growth regulator (PGR) free MS basal medium extensively amplified the rate of shoot multiplication (44.8 ± 3.5) and shoot length (7.5 ± 0.29 cm) without showing any morphological and physiological features of degeneration even after fifth subculture passage. The in vitro raised microshoots were best rooted in half-strength MS medium augmented with 2.0 μM indole-3-butyric acid (IBA). The tissue culture raised plantlets with a completely developed shoot and root system were effectively transferred to the earthen pots having garden soil and grown in greenhouse condition with a 90% survival rate. Growth parameters of 2 months old in vitro raised plants were compared with in vivo grown seedlings of the same age group.

Keywords

Caryophyllaceae · *D. caryophyllus* · In vivo · Subculture · Tissue culture

I. Siddique (✉) · V. Yadav
Plant Biotechnology Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, India

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3.1 Introduction

Dianthus caryophyllus L. (Carnation) is a herbaceous perennial plant that belongs to family Caryophyllaceae growing up to 80 cm height, cultivated all over the world for its importance as cut flowers and other decoration purposes. The significance of this decorative flower is because of its beauty, variety of colours, wonderful long-lasting quality and varied collection of numerous cultivars (Ali et al. 2008; Kanwar and Kumar 2009). The *Dianthus* flowers are an excellent accent to garlands and carnation home-flowered arrangements. It has been used as an unusual food in salad decoration, in fruit salads and for condiment fruits, and so on. The flower heads of carnation are dried up and used in cosmetics and perfumes. *Dianthus* flowers are appraised to be an antidote, antispasmodic, cardiotonic, diaphoretic and nervine (Chopra et al. 1986).

In current years, there has been bigger attention to micropropagation practices that provide a practicable means for large scale proliferation and germplasm preservation of rare, endangered, aromatic and medicinal plants (Faisal et al. 2006; Siddique et al. 2010). These techniques could be a cost-effective means for mass propagation of the best planting material all over the year, without any seasonal limitations. Effective protocols have been published for the micropropagation of valuable plants from leaf and nodal segment explants (Van Altvorst et al. 1994, 1995; Kantia and Kothari 2002) and the stimulation of direct somatic embryogenesis from leaf, petal and anther explants (Pareek and Kothari 2003; Karami et al. 2006; XiaoPeng et al. 2008) in *Dianthus*.

As far as we know, very few reports are present on the successful utilization of TDZ for fast and effective mass multiplication of Carnation (Frey and Janick 1991; Ahmad et al. 2006b; Casas et al. 2010), and the existing protocol yielded a low number of shoots that cannot fulfil the demand of this plant on a large scale. Therefore, the present research work has been assumed to prove the relative efficiency of TDZ, BA and Kin with those of earlier reports for in vitro plant regeneration from nodal segment explants and to detect the optimal concentration of cytokinins exposure for maximum shoot multiplication and formation of complete plantlets.

3.2 Materials and Methods

3.2.1 Nodal Explants and Surface Sterilization

The nodal segment explants of *Dianthus* were taken from the nursery-grown 2 years old healthy mother plant. Nodal segments were fully splashed under running tap water for about 30 min and followed by soaking in 5% (v/v) solution of a liquid detergent Labolene (Qualigens, Mumbai, India) for about 5 min. Following repetitive rinses with sterile, single distilled water, the nodal explants were again surface sterilized with freshly prepared 0.1% (w/v) mercuric chloride (Qualigens, Mumbai,

India) for 5 min under laminar flow hood and followed by repetitive rinses with sterile double distilled water.

3.2.2 Culture Media and Culture Room Conditions

Murashige and Skoog (1962) medium composed with 3% (w/v) sucrose and 0.8% agar (w/v) or 0.25% (w/v) Gelrite was used during the experiment. Plant growth regulators (PGRs) viz. BA, Kin and TDZ either singly or in combinations with auxin (NAA) were supplemented to the MS medium as stated later. The pH of the medium was adjusted to 5.8 using 1 N NaOH or 1 N HCl earlier to autoclaving at 121 °C for 15 min. All the cultures were placed in culture room at 25 ± 2 °C under a 16/8 h light/dark control, under a photon flux density of 50 μ mol m^{-2} s^{-1} provided by 40 W white fluorescent tubes and at 55 ± 5% relative humidity.

3.2.3 Effect of PGRs on Shoot Induction and Multiplication

For induction of multiple shoot regeneration, nodal segments having axillary shoot meristem were inoculated on MS medium augmented with BA, Kin and TDZ at 0.5, 2.5, 5.0, 7.5 and 10.0 μM either singly or in combination with NAA (0.1, 0.5 and 1.0 μM). Data for shoot bud regeneration and multiplication were observed after 4 and 8 weeks of incubation.

3.2.4 In Vitro Rooting in Microshoots

Well-developed healthy microshoots (3–4 cm long) were removed and transferred on to rooting medium comprised of full and half-strength MS medium supplemented with auxin (IBA) at diverse concentrations (0.5, 1.0, 1.5 and 2.0 μM). Data were noted as the percentage response of rooting, the mean number of roots/shoots with their average length after 4 weeks of incubation.

3.2.5 Hardening and Acclimatization of Plantlets

In vitro raised plantlets with the healthy developed shoot (5–6 cm) and roots (4–5 cm) were taken out from the culture tubes and washed carefully under running tap water to remove all the traces of agar from the roots. Then, they were transferred to thermocol cups holding disinfected Soilrite and retained under diffuse light (16/8 h photoperiod) conditions. Potted plants were covered with transparent polythene bags to confirm high levels of humidity around the plants and irrigated every third day with half-strength MS salt solution up to 2 weeks. Polythene bags were removed above the cups after 2 weeks in order to acclimatize the plants to natural field conditions. After 4 weeks, completely acclimatized plants were transferred to

earthen pots having normal garden soil and kept in a greenhouse under normal day length conditions.

3.2.6 Statistical Analysis

All the experiments were carried out with 20 replicates with three repetitions each. The whole data were analysed statistically by using SPSS Ver. 14 (SPSS Inc., Chicago, USA). The significance of variances among means was accredited using Duncan's multiple range test at $P = 0.05$ and data denoted as mean ± standard error (SE).

3.3 Result and Discussion

3.3.1 Induction and Multiplication Phase

The in vitro morphogenetic results of nodal segment explants to different cytokinins tested (BA, Kin and TDZ), either singly or in combination with an auxin (NAA), are outlined in Tables 3.1 and 3.2. No axillary bud shoot induction was detected when nodal segment explants were inoculated on PGR-free MS basal medium. Although a distinctive response with respect to axillary shoot bud induction was detected on

Table 3.1 Effect of plant growth regulators on shoot regeneration from nodal explants of *D. caryophyllus* in MS Medium after 4 weeks of culture

Plant growth regulators (μM)			% Regeneration	Mean number of shoots/explant	Mean shoot length (cm)
BA	Kin	TDZ			
0.0	0.0	0.0	0.0	0.0^{j}	0.0^{g}
0.5			60	$1.9 \pm 0.31^{h,i}$	$1.9 \pm 0.31^{d,e,f}$
2.5			69	3.1 ± 0.34^{g}	$2.6 \pm 0.29^{c,d}$
5.0			80	5.3 ± 0.26^{e}	3.7 ± 0.29^{b}
7.5			71	$3.8 \pm 0.26^{f,g}$	$2.8 \pm 0.37^{c,d}$
10.0			64	$1.4 \pm 0.23^{h,i}$	1.5 ± 0.20^{f}
	0.5		55	$1.3 \pm 0.20^{h,j}$	$1.6 \pm 0.35^{e,f}$
	2.5		62	2.1 ± 0.23^{h}	$3.0 \pm 0.23^{b,c}$
	5.0		71	$3.6 \pm 0.35^{f,g}$	3.8 ± 0.25^{b}
	7.5		63	$1.9 \pm 0.17^{h,i}$	$2.7 \pm 0.23^{c,d}$
	10.0		56	1.0 ± 0.26^{i}	1.2 ± 0.08^{f}
		0.5	68	2.3 ± 0.29^{f}	$2.4 \pm 0.26^{d,e}$
		2.5	77	3.5 ± 0.40^{d}	$3.5 \pm 0.23^{b,c}$
		5.0	88	7.0 ± 0.89^{a}	4.1 ± 0.23^{a}
		7.5	70	6.2 ± 0.37^{b}	3.5 ± 0.35^{b}
		10.0	61	1.6 ± 0.49^{c}	2.2 ± 0.20^{b}

Values represent means ± SE. Means followed by the same letter within columns are not significantly different ($P = 0.05$) using Duncan's multiple range test

Table 3.2 Effect of different concentrations of NAA with an optimal concentration of BA and Kin (5.0 μM) on shoot multiplication and elongation from nodal explants of *D. caryophyllus* in MS Medium after 8 weeks of culture

Plant growth regulators (μM)	Mean number of shoots/explant	Mean shoot length (cm)
BA (5.0) + NAA (0.1)	5.9 ± 0.20^{c}	$4.4 \pm 0.29^{b,c}$
BA (5.0) + NAA (0.5)	8.7 ± 0.43^{b}	5.6 ± 0.20^{b}
BA (5.0) + NAA (1.0)	6.9 ± 0.53^{a}	3.8 ± 0.32^{a}
Kin (5.0) + NAA (0.1)	4.5 ± 0.55^{d}	4.0 ± 0.17^{c}
Kin (5.0) + NAA (0.5)	7.6 ± 0.60^{c}	4.8 ± 0.35^{c}
Kin (5.0) + NAA (1.0)	5.9 ± 0.71^{c}	$3.5 \pm 0.35^{b,c}$

Values represent means ± SE. Means followed by the same letter within columns are not significantly different ($P = 0.05$) using Duncan's multiple range test

medium augmented with a varying range of cytokinins viz. BA, TDZ and Kin (0.5–10 μM). TDZ was found more potent than other cytokinins used and a concentration of 5.0 μM was proved to be optimum for the induction of maximum percentage regeneration response (88%), the mean number of shoots/explant (7.0 ± 0.89) and average shoot length (4.1 ± 0.23 cm) (Fig. 3.2a, b). The effect of TDZ on multiple shoot regeneration has been reported in previous studies of many different ornamental plant species (Nontaswatsri et al. 2002; Sivanesan et al. 2011). The percentage response of multiple shoot regeneration, the mean number of shoots/explant and their average shoot length were gradually increased with an increase in the concentration of cytokinin from 0.5 to 5.0 μM. MS medium comprising BA induced 5.3 ± 0.26 mean shoots/explants in 80% cultures, while Kin at the similar concentration regenerated 3.6 ± 0.35 mean shoots/explant with 71% regeneration frequency (Table 3.1). All in vitro regenerated microshoots were free from basal callusing at their cut ends. The percentage of shoot regeneration frequency and the mean number of shoots/explants with their average shoot length was dropped immediately with a rise in the concentration of all the cytokinin tested beyond the optimal level (5.0 μM).

To make more improvements in shoot multiplication frequency, NAA at diverse concentrations (0.1, 0.5 and 1.0 μM) was tried with MS medium augmented with optimal cytokinin concentration (5.0 μM) of BA and Kin (Table 3.2). The combination of NAA with cytokinin triggered the regeneration of multiple shoots having healthy leaves during subculture. Out of all the cytokinin–auxin combination treatments tested, the maximum number of mean shoots/explant (8.7 ± 0.43) with an average shoot length (5.6 ± 0.20 cm) was attained at the MS + 5.0 μM BA +0.5 μM NAA. Therefore, this growth regulator combination treatment was considered as optimal for maximum shoot regeneration and multiplication among all the BA, Kin and NAA combination treatments examined. The outcomes found here exhibited reliability with other findings where BA and NAA stimulated the regeneration and proliferation of shoots in several plant species (Miller et al. 1991; Kantia and Kothari 2002; Remya et al. 2013).

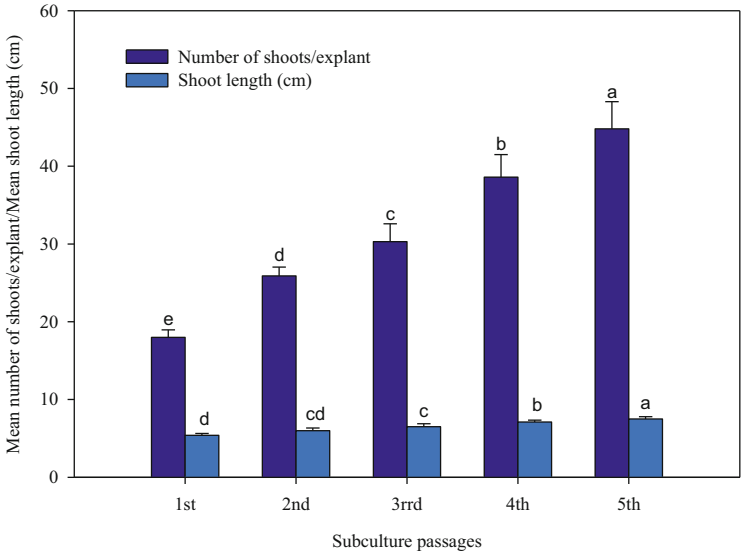

Fig. 3.1 The evaluation of the morphogenetic potential of shoot culture obtained from TDZ (5.0 μM) after being tested for five subculture passages on growth regulator free MS medium. Bars represent mean ± SE. Means followed by the same letter are not significantly different ($P = 0.05$) using Duncan's multiple range test

Shoot buds regenerated from nodal segment explants on TDZ supplemented MS medium did not lengthen properly and resulted in the formation of a rosette of regenerated shoots when continuously transferred on the similar media composition. TDZ generally induced shoot multiplication and obstructed their elongation, and hence, might be dependable with its intense cytokinin action. The difficulty of shoot elongation was solved by a subculture of aggregate shoot mass to another medium devoid of TDZ, where the further elongation of multiple shoots was taking place within 4 weeks of subculture.

The influence of different subculture passages was also assessed by proliferation and elongation of shoots on TDZ supplemented MS medium after transfer to MS basal medium free from any TDZ concentration (Fig. 3.1). The in vitro cultures that had grown-up constantly on TDZ comprising media showed fasciation and distortion of the apical shoot meristem. The damaging effect of the persistent occurrence of TDZ on the multiplication and elongation has been testified in a number of plant species (Ahmad et al. 2006a; Siddique et al. 2013). In this research analysis, the mean number of shoots/explant (44.8 ± 3.5) and average shoot length (7.5 ± 0.29 cm) (Fig. 3.2c, d) continued to proliferate up to fifth subculture passages without any mark of degeneration. A related trend of subculture passages was also reported by Tiwari et al. (2001) and Siddique and Anis (2007).

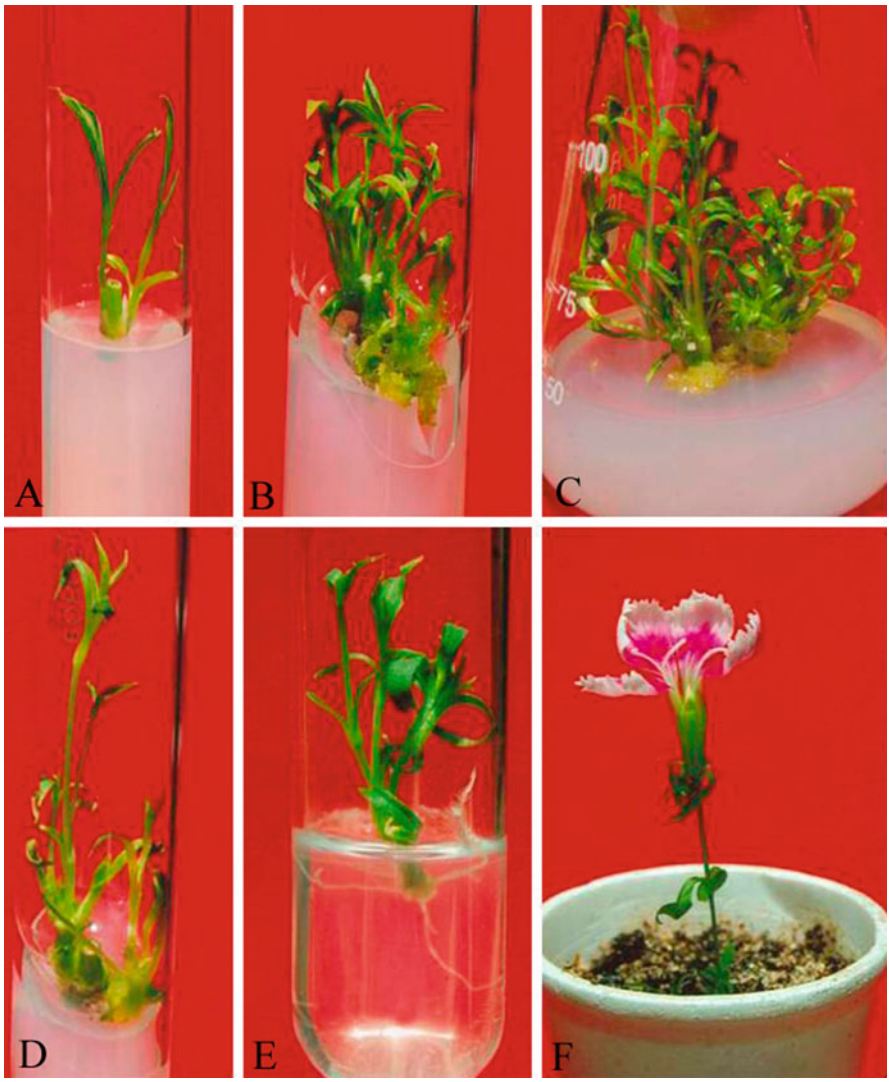

Fig. 3.2 (**a–f**) In vitro shoot multiplication and plantlet formation in *D. caryophyllus*. (**a**, **b**) Induction of multiple shoots on MS + TDZ (5.0 μM). (**c**, **d**) Shoot multiplication and elongation on MS medium devoid of TDZ. (**e**) In vitro rooting on ½ MS + IBA (2.0 μM). (**f**) In vitro flowering in an acclimatized micropropagated plant

3.3.2 Rooting of Microshoots

Healthy developed microshoots (3–4 cm) were taken out from in vitro raised cultures and placed into the full and half-strength MS medium supplemented with a diverse concentration of IBA (0.5, 1.0, 1.5 and 2.0 μM) (Table 3.3). Half-strength MS

Table 3.3 Effect of MS strength and IBA concentration on root induction from in vitro raised shoots of *D. caryophyllus* after 4 weeks of culture

Treatment	% Rooting	Mean no. of roots/ shoot	Mean root length (cm)
MS	60	1.8 ± 0.11^{d}	1.2 ± 0.14^{d}
(1/2)MS	65	$3.1 \pm 0.14^{b,c}$	2.0 ± 0.12^{c}
(1/2)MS + IBA (0.5 μM)	70	3.5 ± 0.17^{b}	2.8 ± 0.17^{b}
(1/2)MS + IBA (1.0 μM)	75	4.0 ± 0.23^{a}	3.0 ± 0.29^{a}
(1/2)MS + IBA (1.5 μM)	79	4.2 ± 0.27^{b}	3.6 ± 0.17^{a}
(1/2)MS + IBA (2.0 μM)	83	5.4 ± 0.20^{c}	4.0 ± 0.20^{b}

Values represent means ± SE. Means followed by the same letter within columns are not significantly different ($P = 0.05$) using Duncan's multiple range test

medium was better than full strength MS medium and half-strength MS medium augmented with 2.0 μM IBA induced the production of 5.4 mean roots/shoot in 83% cultures after 4 weeks of transfer in rooting medium (Fig. 3.2e). The efficacy of IBA for induction of in vitro rooting has been recorded by Siddique and Anis 2007; Anis et al. 2010.

3.3.3 Acclimatization

In vitro raised plants having 4–6 completely extended leaves and fully grown root system were effectively acclimatized in the culture room conditions by planting them in disinfected Soilrite for 4 weeks and were finally transferred into the garden soil (Fig. 3.2f). About 90% of the in vitro raised plants survived when transferred from Soilrite to garden soil and did not show any noticeable dissimilarity with respect to plant morphology and other growth parameters.

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Phenolic Compound Contents of *Hypericum* Species from Turkey

4

Hilal Surmuş Asan

Abstract

Hypericaceae is a small cosmopolite dicot family of tree, shrub, and herb. *Hypericum* is the largest genus of Hypericaceae comprising of about 484 species throughout the world. In Turkey, *Hypericum* is represented by 96 species, 104 taxa in which 45 of them are endemic. The genus *Hypericum* is known worldwide with its wide folk medicinal usage. The extracts of *Hypericum perforatum* L., the most abundant and well-known species, are now widely used in Europe as a drug for the treatment of mild-to-moderate depression. In Turkish traditional medicine, *Hypericum* L. species have been known under the names "kantaron, peygamber çiçeği, kılıçotu, kanotu, kuzukıran, and binbirdelik otu" and used for the treatment of burns, wounds, bacterial and viral infections, hemorrhoids, diarrhea, and ulcers. The pharmacological studies revealed that this species has antitumor, antidepressant, anti-inflammatory, anti-microbial, anti-viral, anti-nociceptive, and wound healing activity. The value of *Hypericum* plants has been mainly attributed to the phytochemicals, namely naphthodianthrones (e.g., hypericin and pseudohypericin), acylphloroglucinol derivatives (e.g., hyperforin and adhyperforin), flavonoids (e.g., quercetin, quercitrin, hyperoside, and biapigenin), *n*-alkanes, tannins, xanthones and essential oils they possess.

Due to their phytochemical composition and waste usage in traditional folk medicine, many studies were made on biological activities of different species extracts of *Hypericum* in Turkey and the world. Turkey is an important prevalence center of *Hypericum* species. This review will investigate the phenolic compound contents of *Hypericum* species in Turkish flora.

H. S. Asan (✉)
Science Faculty, Biology Department, Dicle University, Diyarbakır, Turkey

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Keywords

Hypericum · Phenolic compound · Hypericins · Turkey

4.1 Introduction

The genus *Hypericum* (Hypericaceae), includes 484 species, is lived mainly in varied temperate regions all over the world, and is found in forms of small trees, shrubs, and herbs. Recently, 36 taxonomic sections have been recognized within *Hypericum* genus (Crockett and Robson 2011).

Scientific studies on *Hypericum* species are concentrated as its members have yielded many bioactive compounds namely, hyperforin and adhyperforin (phloroglucinol derivatives), hypericin and pseudohypericin (naphthodianthrones), hyperoside, rutin, quercitrin, quercetin, and biapigenin (flavonoids), caffeic and chlorogenic acids (the phenylpropanes) and essential oils that show many biological activities (Kasper et al. 2010). *Hypericum* species have been used as healing agents due to their variety of medicinal properties for centuries. The best known of one is *H. perforatum* L. and extracts of *H. perforatum* L., the most prevalent and domesticated species, are now largely used in Europe as a drug for the treatment of depression (Fiebich et al. 2011). Turkey is an important geographical distribution center of *Hypericum* species. According to the most recent revision of *Hypericum* genus carried out by Guner et al. (2012), there are a total of 96 *Hypericum* species in the flora of Turkey, 46 of which are endemic. *Hypericum* species have been used traditionally as sedatives, antiseptics, and antispasmodics in Turkey. They are known under several names such as "kantaron, peygamber çiçeği, kılıçotu, kanotu, kuzukıran, and binbirdelik otu" (Bingol et al. 2011). The aim of the present study was to review and summarize noteworthy studies on the phenolic contents of Turkish *Hypericum species*.

4.1.1 The Floristic Characters of *Hypericum* L.

The floristic characters of *Hypericum* genus can be defined as (The Flora of Turkey–Vol 2) "shrubs or herbs are usually with translucent glands containing essential oils and sometimes red or black glands containing hypericin. Leaves simple, opposite or rarely whorled. Flowers bisexual, Sepals 5, Petals 5, usually yellow, often tinged red or with red veins, rarely with nectary appendages. Stamens in 5 fascicles, anti-petalous, free or 4 of them united in pairs to form 2 anti-sepalous compound fascicles, with 3-c. 125 stamens in each, rarely alternating with sterile fascicles ('fasciclodes'). Ovary superior with axile or parietal placentation, 3-5-locular or partly or completely 1-locular with 2-many ovules on each placenta. Styles 3-5, free, slender, Fruit capsular, dehiscing septicidally, usually with resin-containing vittae or vesicles in the wall, or rarely fleshy and indehiscent. The distribution and configuration of glands is important in classification. In the following account, glands are

described as marginal where they interrupt the contour of the organ, intramarginal where they occur near the margin without interrupting the contour and superficial where they occur away from the margin. Narrow glands in the ovary and capsule walls are termed *vittae*, and short swollen glands are described as *vesicles*" Davis et al. 1967).

4.1.2 *Hyperıicum* Specıes in Turkey

Turkey is an important geographical distribution center of *Hypericum* species. According to the most recent revision of *Hypericum* genus carried out by Guner et al. (2012), there are a total of 96 *Hypericum* species in the flora of Turkey, 46 of which are endemic (Table 4.1).

4.1.3 Traditional Uses of *Hypericum* Species in Turkey

Turkey is an important center for *Hypericum* species, which are known as "kantaron, binbirdelik otu, kan otu, kılıç otu, yaraotu, kuzukıran" (Bingol et al. 2011). The different parts of these plant are used in several forms like ointment, decoction, and oleate (dried herbs are kept in olive oil for 1 month and filtered). These species have been used traditionally in Anatolia for the treatment of burns, skin injuries, (bacterial and viral infections), hemorrhoids, diarrhea, and ulcers for centuries. The species that have traditional uses are being shown below (Table 4.2).

4.2 Phenolic Compounds of *Hypericum* Species

Hypericum species have many important chemical compounds possessing biological activities. These compounds are hypericin and pseudohypericin (naphthodianthrones), hyperforin and adhyperforin (acylphloroglucinol derivatives), quercetin, quercitrin, hyperoside and biapigenin (flavonoids) (Bombardelli and Morazzoni 1995; Bruneton 1995), especially 3,8-biapigenin and amentoflavone (biflavones) (Berghoefer and Hoelzl 1987, 1989), chlorogenic acid, p-coumaric acid and caffeic acid (phenylpropanes) (Nahrstedt and Butterweck 1997), and lesser amounts of tannins and xanthones.

4.2.1 Naphthodianthrones

Naphthodianthrones are typical compounds of the genus *Hypericum* (Hegnauer 1989). These compounds have an intense red color and phototoxic properties (Schey et al. 2000). Hypericins are the major components of this group of compounds. These two compounds are called hypericin (Fig. 4.1) and

Table 4.1 *Hypericum* species in the flora of Turkey

	Hypericum species	Endemic
1	*H. adenotrichum* Spach	+
2	*H. amblysepalum* Hochst.	
3	*H. androsaemum* L.	
4	*H. apricum* Kar. and Kir.	
5	*H. armenum* Jaub. and Spach	
6	*H. atomarium* Boiss.	
7	*H. aucheri* Jaub. and Spach	
8	*H. aviculariifolium* Jaub. and Spach subsp. *avicullariifolium* var. *albiflorum* Hub.-Mor.	+
9	*H. avicularifolium* Jaub. and Spach subsp. *avicullariifolium* var. *avicullariifolium*	+
10	*H. avicularifolium* Jaub. and Spach subsp. *byzantinum* (Azn.) Robson	+
11	*H. avicularifolium* Jaub. and Spach subsp. *depilatum* (Freyn and Bornm.) Robson var.	+
12	*bourgaei* (Boiss.) Robson	
13	*H. avicularifolium* Jaub. and Spach subsp. *depilatum* (Freyn and Bornm.) Robson var.	+
14	depilatum	
15	*H. avicularifolium* Jaub. and Spach subsp. *depilatum* (Freyn and Bornm.) Robson var.	+
16	*leprosum* (Boiss.) Robson	
17	*H. avicularifolium* Jaub. and Spach subsp. *uniflorum* (Boiss. and Heldr.) Robson	+
18	*H. bithynicum* Boiss.	
19	*H. bupleuroides* Gris.	
20	*H. calycinum* L.	
21	*H. capitatum* Choisy var. *capitatum*	+
22	*H. capitatum* Choisy var. *luteum* Robson	
23	*H. cardiophyllum* Boiss.	+
24	*H. cerastoides* (Spach) Robson	
25	*H. confertum* Choisy subsp. *confertum*	+
26	*H. confertum* Choisy subsp. *stenobotrys* (Boiss.) Holmboe	
27	*H. crenulatum* Boiss.	+
28	*H. cuisinii* Barbey	
29	*H. davisii* Robson	
30	*H. elegans* Steph. ex Willd.	
31	*H. elongatum* Ledeb. subsp. *apiculatum* Robson	
32	*H. elongatum* Ledeb. subsp. *elongatum*	
33	*H. elongatum* Ledeb. subsp. *microcalycinum* (Boiss. and Heldr.) Robson	
34	*H. empetrifolium* Willd.	
35	*H. fissurale* Woron	+
36	*H. formosissimum* Takht.	+
37	*H. havvae* Guner	+
38	*H. helianthemoides* (Spach) Boiss.	

(continued)

Table 4.1 (continued)

	Hypericum species	Endemic
39	*H. heterophyllum* ~~V~~Vent.	+
40	*H. hircinum* L. subsp. *albimontanum* (Greuter) Robson	
41	*H. hircinum* L. subsp. *majus* (Aiton) Robson	
42	*H. hirsutum* L.	
43	*H. huber-morathii* Robson	+
44	*H. imbricatum* Poulter	+
45	*H. kazdagensis* Gemici and Leblebici	+
46	*H. kotschyanum* Boiss.	+
47	*H. lanuginosum* Lam. var. *lanuginosum*	
48	*H. lanuginosum* Lam. var. *pestalozzae* (Boiss.) Robson	+
49	*H. lanuginosum* Lam. var. *scabrellum* (Boiss.) Robson	+
50	*H. linarioides* Bosse	
51	*H. lydium* Boiss.	
52	*H. lysimachioides* Boiss. and Noë var. *lysimachioides*	
53	*H. lysimachioides* Boiss. and Noë var. *spathulatum* Robson	
54	*H. malatyanum* Peçmen	+
55	*H. marginatum* Woron	+
56	*H. minutum* Davis and Poulter	+
57	*H. monadenum* Robson apud Poulter	+
58	*H. montanum* L.	
59	*H. montbretii* Spach	
60	*H. neurocalycinum* Boiss. and Heldr.	+
61	*H. nummularioides* Trautv.	+
62	*H. olivieri* (Spach) Boiss.	
63	*H. olympicum* L. subsp. *olympicum*	
64	*H. olympicum* L. subsp. *macrocalyx* (Freyn) Robson	+
65	*H. orientale* L.	
66	*H. origanifolium* Willd.	
67	*H. pallens* Banks and Sol	
68	*H. pamphylicum* Robson and Davis	+
69	*H. perfoliatum* L.	
70	*H. perforatum* L.	
71	*H. peshmenii* Yıldırımlı	+
72	*H. polyphyllum* Boiss. and Bal. subsp. *lycium* Robson and Hub.-Mor.	+
73	*H. polyphyllum* Boiss. and Bal. subsp. *polyphyllum*	+
74	*H. polyphyllum* Boiss. and Bal. subsp. *subcordatum* Robson and Hub.-Mor.	+
75	*H. pruinatum* Boiss. and Bal.	
76	*H. pseudolaeve* Robson	+
77	*H. pumilio* Bornm.	+
78	*H. retusum* Aucher	
79	*H. rumeliacum* Boiss. subsp. *rumeliacum*	+
80	*H. rupestre* Jaub. and Spach	+
81	*H. russeggeri* (Fenzl) R. Keller	+

(continued)

Table 4.1 (continued)

	Hypericum species	Endemic
82	*H. salsolifolium* Hand.-Mazz	+
83	*H. salsugineum* Robson and Hub.-Mor.	+
84	*H. saxifragum* Robson and Hub.-Mor.	+
85	*H. saxifragum R*obson and Hub.-Mor. subsp. *eglandulosum* Parolly and Eren	
86	*H. scabroides* Robson and Poulter	+
87	*H. scabrum* L.	
88	*H. sorgerae* Robson	+
89	*H. spectabile* Jaub. and Spach	+
90	*H. ternatum* Poulter	+
91	*H. tetrapterum* Fries	
92	*H. thasium* Griseb.	+
93	*H. thymbrifolium* Boiss. and Noë	+
94	*H. thymifolium* Banks and Sol.	
95	*H. thymopsis* Boiss.	+
96	*H. triquetrifolium* Turra	
97	*H. uniglandulosum* Hausskn. ex Bornm	+
98	*H. vacciniifolium* Hayek and Siehe	+
99	*H. venustum* Fenzl	
100	*H. vesiculosum* Griseb	+
101	*H. xylosteifolium* (Spach) Robson	

Davis et al. (1967); Ekim et al. (2000); Güner et al. (2000)

pseudohypericin (Fig. 4.2), respectively. Hypericin and pseudohypericin occur in the flowers and leaves of the *Hypericum* plants (Cellarova et al. 1994).

4.2.2 Phloroglucinols

Phloroglucinol derivatives are widely distributed in the genus *Hypericum*. Two closely related phloroglucinol compounds are hyperforin (Fig. 4.3) and adhyperforin (Hobbs 1989). These are found only in the reproductive parts (Tekelova et al. 2000).

4.2.3 Flavonoids

The major group of biologically active compounds in *Hypericum* species comprises of flavonoids. Kaempferol, luteolin, myricetin, quercetin (Fig. 4.4), hyperoside (hyperin) (Fig. 4.5), and rutin; quercitrin; and isoquercitrin are samples of flavonoid compounds (Kurth and Spreemann 1998; Hansen et al. 1999; Pietta et al. 2001; Naeem et al. 2010).

Table 4.2 The traditional uses of *Hypericum* species in Turkey

Hypericum species	Traditional uses	References
H. atomarium	Enteritis, stomach diseases, sedative	Vural (2008)
H. aviculariifolium	Urethra diseases	Keskin and Alpınar (2002)
H. aviculariifolium subsp. depilatum	Hemorrhoid, psoriasis	Baytop (1999)
Hypericum bupleuroides Gris.	Burns, intestinal disorders	Baytop (1999)
H. cerastoides	Diarrhea	Kizilarslan (2008)
H. calycinum	Anti-asthmatic, remove spasm	Aktan (2011)
H. capitatum var. capitatum	To release pain	Akan et al. (2013)
H. confertum subsp. *confertum*	Wound healing, anti-asthmatic	Bulut (2006); Buyukgebiz (2006); Mart (2006)
H. confertum var. *stenobotrys*	Stomach diseases, remove spasm	Buyukgebiz (2006); Bulut (2006); Mart (2006)
H. heterophyllum	Anti-inflammatory	Unal et al. (2008)
H. hyssopifolium and *H. hyssopifolium* subsp. *elongatum* var. *elongatum*	Hemorrhoid, remove spasm, diarrhea, sedative, anti-helminthic, antiseptic, antifungal, psoriasis	Unal et al. (2008)
H. lydium	Stomach diseases, indigestions, hemorrhoid, wound healer, menstrual disorders	Yeşilada et al. (1995); Davis (1988); Sezik et al. (2001); Yesil (2007); Altundag and Ozturk (2011)
H. montbretii	Kidney stones, eczema, stomach disorders, ulcers, hemorrhoids	Altundag and Ozturk (2011); Keskin and Alpınar (2002)
H. olympicum	For inflamed wounds, stomach ache, cuts.	Tuzlaci and Aymaz (2001)
H. orientale	Stomach diseases, sedative, hemorrhoid, psoriasis	Ezer and Mumcu (2006); Tatli et al. (2009); Sezik et al. (2001); Baytop (1999)
H. perforatum	Anti-inflammatory, anti-hemorrhagic, antifungal, antihypertensive, kidney stones, eczema, urinary diseases, cold, cardiac diseases, arteriosclerosis, bladder ailments, facial paralysis, gastritis, chest diseases, internal hemorrhage, stomach diseases, bronchitis, tuberculosis, pharyngitis, wounds, burns, cuts, herpes labialis, lip chap, diabetes, enteritis, ulcers	Demirci (2010); Cimen (2007); Kultur (2007); Sezik et al. (2001); Tuzlaci and Aymaz (2001); Ezer and Avcı (2004); Ecevit and Ozhatay (2006); Yesilada et al. (1993); Yeşilada et al. (1995); Tuzlaci and Aymaz (2001)
H. scabrum	Hemorrhoid, antispasmodic, anti-inflammatory, sedative, constipation, peptic ulcer	Unal et al. (2008); Ezer and Mumcu (2006); Tanker (1971); Yeşilada et al. (1995)

(continued)

Table 4.2 (continued)

Hypericum species	Traditional uses	References
H. ternatum	Antiasthmatic, wound healing	Bulut (2006); Mart (2006)
H. thymifolium	Stomach diseases	Mart (2006)
H. triquetrifolium	Diabetes, cardiac diseases, burns, gastrointestinal diseases	Akgul (2008); Baytop (1999)

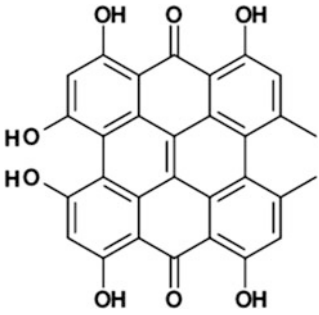

Fig. 4.1 Chemical structure of hypericin

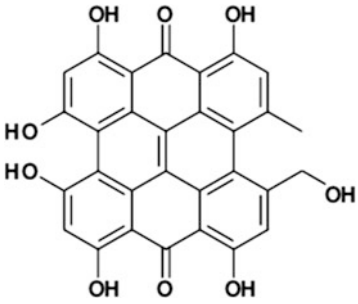

Fig. 4.2 Chemical structure of pseudohypericin

4.2.4 Biflavones

Biflavones are a rare group of flavones found in some plants. Three biflavones detected in *H. perforatum* are 3′, 8″-biapigenin, amentoflavone (Berghoefer and Hoelzl 1987, 1989), and 6′, 8″ -diquercetin (Kurkin and Pravdivtseva 2007).

4.2.5 Phenylpropanes

Caffeic, p-coumaric, ferulic, isoferulic, gentisic (Hobbs 1989; Upton 1997), shikimic acids (Bilia et al. 2001), and chlorogenic acid (Nahrstedt and Butterweck 1997) can be given as compounds in this group.

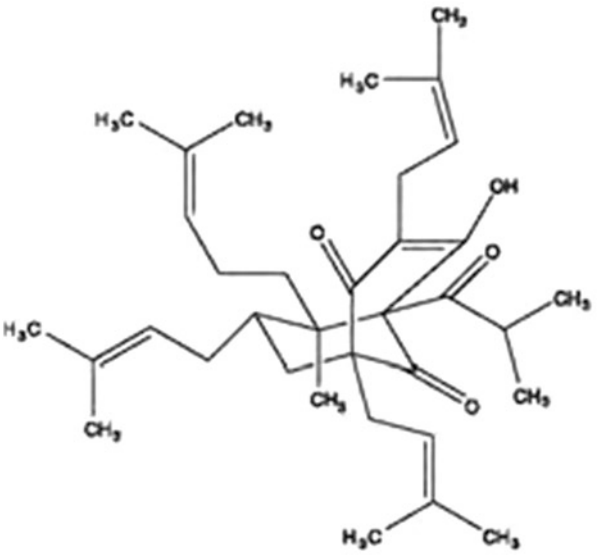

Fig. 4.3 Chemical structure of hyperforin

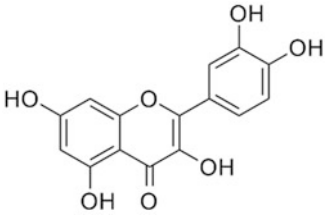

Fig. 4.4 Chemical structure of quercetin

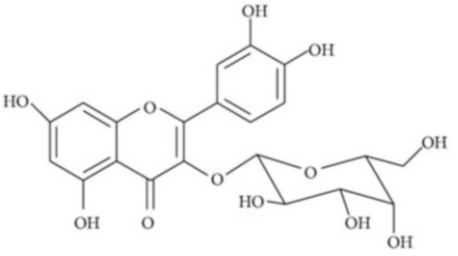

Fig. 4.5 Chemical structure of hyperoside

4.3 Phenolic Compound Content Studies on the *Hypericum* Species in Turkey

Phenolic compound contents of *H.androsaemum* L., *H.avicullariifolium* Jaub. and Spach, *H.avicullariifolium* Jaup. and Spach subsp., *depilatum, H. bithynicum* Boiss., *H. bupleuroides, H. calycinum* L., *H. heterophyllum* Vent., *H. hirsutum* L., *H. hyssopifolium* Vill., *H. hyssopifolium* L., *H. linarioides Bosse, H. lydium*

Boiss., H. montanum L., *H. montbretii* Spach, *H.nummularioides* Trautv., *H. orientale* L., *H. origanifolium* Willd., *H. perfoliatum* L., *H. perforatum* L., *H. pruinatum* Boiss., and Balansa, *H. scabrum* L., *H. triquetrifolium* Turra, *H. venustum* and *H. Xylosteifolium, Hypericum* species from Northern Turkey have been determined (Table 4.3).

Hypericin and pseudohypernicin compounds have been detected in all the species examined. Other compounds such as hyperforin, adhyperforin, hyperoside, chlorogenic acid, neochlorogenic acid, caffeic acid, 2,4 dihydroxybenzoic acid, isoquercitrin, quercitrin, quercetin, avicularin, rutin, 13,118-biapigenin, (+)-catechin, apig-7-*O*-glucoside, caffeic acid, amentoflavone, viteksin, and isoquercetin have also been identified (Table 4.3). The flavonoid vitexin was determined in *H. motbretii* for the first time in the genus *Hypericum* (Çirak et al. 2007d).

The hyperforin compound was found in *H. androsaemum, H. aviculariifolium, H. bithynicum, H. heterophyllum, H. montbretii, H. nummularioides, Hypericum orientale, H. origanifolium, H. perfoliatum, H. pruinatum,* and *H. scabrum* (Smelcerovic et al. 2008), and hypericin and pseudohypericin were detected in *H. orientale and H. scabrum* (Ayan and Çirak 2008). The presence of hypericin *in H.avicularііfolium subsp. deilatum, H. nummularoides, H. pruinatum, and H. venustum* was reported by Ayan et al. (2004) for the first time.

Smelcerovic et al. (2008) collected 17 wild-growing species of *Hypericum* plants (flowers) from various locations in Northern Turkey. They determined all extracts contained pseudohypericin, hypericin, hyperforin, rutin, hyperoside, quercitrin, and quercetin compounds. But rutin was not found in the extracts of *H. origanifolium* and *H. scabrum* plants. They reported that the greatest content of hypericin and pseudohypericin was observed in *H. perforatum* and *H. triquetrifolium* extracts. Hyperforin content was highest in the H. perforatum extract. Hyperforin was found for the first time in *H. androsaemum, H. avicularііfolium, H. bithynicum, H. heterophyllum, H. montbretii, H. nummularioides, Hypericum orientale, H. origanifolium, H. perfoliatum, H. pruinatum, and H. scabrum.* Thus, the greatest content of rutin was found in *H. montbretii* (Smelcerovic et al. 2008).

It was found that the *H. polyphyllum* Boiss. and Bal. contain several phenolic compounds pseudohypericin, hypericin, hyperforin, adhyperforin, hyperoside, chlorogenic acid, neochlorogenic acid, caffeic acid, rutin, 2,4-dihydroxybenzoic acid, 13,118-biapigenin, quercitrin, isoquercitrin, quercetin, avicularin, (−)-catechin and (−)-epicatechin (Cirak et al. 2017). Hyperforin, hypericin, pseudohypericin, chlorogenic acid, rutin, hyperoside, apigenin-7-*O*-glucoside, kaempferol, quercitrin, quercetin, and amentoflavone were detected in *H. scabrum* L. and *H. bupleuroides* Gris. It was reported that the phloroglucinol derivative hyperforin was not accumulated in *H. scabrum* (Ayan et al. 2009) (Table 4.4).

In research, *H. hircinum* L. subsp. Majus (Ainton) did not produce quercitrin, avicularin, or rutin and *H. lanuginosum* did not accumulate hyperforins, rutin, and caffeic acid in investigated hyperforin, adhyperforin, hypericin, pseudohypericin, chlorogenic acid, neochlorogenic acid, rutin, caffeic acid, hyperoside, 2,4 dihydroxybenzoic acid, isoquercitrin, quercetin, isoquercetin, avicularin, 13,118-biapigenin, (+)-catechin, (−)-epicatechin, compounds. Besides it was

Table 4.3 Studies on the phenolic compounds of *Hypericum* Species from Northern Turkey

Hypericum species	Examined phenolics	Method	References
H. androsaemum L.	Hypericin, pseudohypericin, hyperforin, adhyperforin, hyperoside, rutin, quercitrin, isoquercitrin, quercetin, chlorogenic acid, neochlorogenic acid, caffeic acid, 2,4 dihydroxybenzoic acid, avicularin, 13,118-biapigenin, (+)-catechin, (−)-epicatechin	LC/MS/MS, HPLC	Smelcerovic et al. (2008); Cirak et al. (2017)
H. avicullariifolium Jaub. and Spach	Hypericin, pseudohypericin, hyperforin, hyperoside, rutin, quercitrin, quercetin	LC/MS/MS	Smelcerovic et al. (2008)
H. montanum L.			
H. nummularioides Trautv.			
*H. avicullariifolium Jaup. and Spach subsp., depilatum**	Hypericin, pseudohypericin, chlorogenic acid, hyperoside, rutin, quercitrin, quercetin, apigenin-7-*O*-glucoside, hyperforin, isoquercetin	HPLC	Çirak et al. (2006, 2007d); Cirak et al. 2013)
H. bithynicum Boiss.	Hypericin, pseudohypericin, hyperforin, adhyperforin, chlorogenic acid, neochlorogenic acid, caffeic acid, 2,4 dihydroxybenzoic acid, hyperoside, isoquercitrin, quercitrin, quercetin, avicularin, rutin, 13,118-biapigenin, (+)-catechin	HPLC, LC/MS/MS	Cirak et al. (2016a); Smelcerovic et al. (2008)
*H. bupleuroides**	Hypericin, pseudohypericin, chlorogenicacid, hyperoside, rutin, hyperforin, quercetin, kaempferol, quercitrin, amentoflavon, apigenin-7-*O*-glucoside	HPLC	Ayan et al. (2009)

(continued)

Table 4.3 (continued)

Hypericum species	Examined phenolics	Method	References
H. calycinum L.	Hypericin, pseudohypericin, hyperforin, adhyperforin, chlorogenic acid, neochlorogenic acid, caffeic acid, 2,4 dihydroxybenzoic acid, hyperoside, isoquercitrin, quercitrin, quercetin, avicularin, rutin, 13,118-biapigenin, (+)-catechin	HPLC	Cirak et al. (2016a)
H. heterophyllum Vent.	Hypericin, pseudohypericin, hyperforin, hyperoside, rutin, quercitrin, quercetin	LC/MS/MS, HPLC	Smelcerovic et al. (2008); Ayan and Çirak (2008)
H. hirsutum L.	Hypericin, pseudohypericin, hyperforin, hyperoside, rutin, quercitrin, quercetin, adhyperforin, chlorogenic acid, neochlorogenic acid, caffeic acid, 2,4 dihydroxybenzoic acid, isoquercitrin, avicularin, rutin, 13,118-biapigenin, (+)-catechin	LC/MS/MS, HPLC	Smelcerovic et al. (2008); Cirak et al. (2016a)
H. hyssopifolium Vill.	Hypericin, pseudohypericin, hyperforin, hyperoside, rutin, quercitrin, quercetin,	LC/MS/MS, HPLC	Smelcerovic et al. (2008); Ayan et al. (2004)
H. hyssopifolium L.	Hypericin, pseudohypericin	HPLC	Ayan and Çirak (2008)
H. linarioides Bosse	Hypericin, pseudohypericin, hyperforin, hyperoside, rutin, quercitrin, quercetin,	HPLC, LC/MS/MS	Ayan and Çirak (2008); Smelcerovic et al. (2008)
H. lydium Boiss.	Hypericin, pseudohypericin, chlorogenic acid, neochlorogenic acid,	HPLC, UV spectrophotometer	Çirak (2006); Çirak et al. (2007d); Cirak et al. (2015)

(continued)

Table 4.3 (continued)

Hypericum species	Examined phenolics	Method	References
	hyperforin, adhyperforin, hyperoside, rutin, quercitrin, quercetin, apigenin-7-*O*-glucoside, 2,4 dihydroxybenzoic acid, isoquercitrin, avicularin, (+)-catechin, (−)-epicatechin, avicularin		
H. montbretii Spach	Hypericin, pseudohypericin, chlorogenic acid, hyperforin, hyperoside, rutin, quercitrin, isoquercetin quercetin, viteksin, apigenin-7-*O*-glucoside	HPLC, LC/MS/MS	Odabas et al. (2008); Ayan and Çirak (2008); Smelcerovic et al. (2008); Cirak and Radušienė (2007); Çirak et al. (2007d)
H. orientale L.	Hypericin, pseudohypericin, hyperforin, adhyperforin, chlorogenic acid, hyperoside, rutin, quercitrin, quercetin, apigenin-7-*O*-glucoside, isoquercetin, kaempferol, neochlorogenic acid, caffeic acid, 2,4 dihydroxybenzoic acid, avicularin, 13,118-biapigenin, (−)-epicatechin, rutin, amentoflavone	HPLC, LC/MS/MS	Çirak et al. (2007d, 2008, 2012, 2013); Smelcerovic et al. (2008); Camas et al. (2014a)
H. origanifolium Willd.	Hypericin, pseudohypericin, chlorogenic acid, hyperforin, hyperoside, quercitrin, isoquercetin, isoquercitrin, quercetin, rutin, apigenin-7-*O*-glucoside, caffeic acid	HPLC, LC/MS/MS	Ayan and Çirak (2008); Odabas et al. (2008); Smelcerovic et al. (2008); Çirak et al. (2007b, d)
H. perfoliatum L.	Hypericin, pseudohypericin, chlorogenic acid, hyperforin, hyperoside, quercitrin, isoquercetin, isoquercitrin, quercetin,	HPLC, LC/MS/MS	Odabas et al. (2008); Smelcerovic et al. (2008); Çirak et al. (2007a, d)

(continued)

Table 4.3 (continued)

Hypericum species	Examined phenolics	Method	References
	rutin, caffeic acid, apigenin-7-*O*-glucoside		
H. perforatum L.	Hypericin, pseudohypericin, chlorogenic acid, hyperforin, hyperoside, rutin, quercitrin, quercetin, apigenin-7-*O*-glucoside	LC/MS/MS, HPLC	Smelcerovic et al. (2008); Çirak et al. (2006, 2007c, d, 2008); Ayan and Çirak (2008)
H.pruinatum Boiss. and Balansa	Hypericin, pseudohypericin, chlorogenic acid, hyperforin, hyperoside, rutin, quercitrin, quercetin, apigenin-7-*O*-glucoside	HPLC, LC/MS/MS	Çirak et al. (2006, 2007a); Smelcerovic et al. (2008)
H. scabrum L.	Hypericin, pseudohypericin, chlorogenic acid, hyperoside, rutin, hyperforin, quercetin, kaempferol, quercitrin, amentoflavon, apigenin-7-*O*-glucoside	HPLC, LC/MS/MS	Ayan and Çirak (2008); Ayan et al. (2009); Smelcerovic et al. (2008)
H. triquetrifolium Turra	Hypericin, pseudohypericin, hyperforin, chlorogenic acid, hyperoside, rutin, quercitrin, quercetin, rutin, amentoflavone, kaempferol, apig-7-*O*-glucoside	LC/MS/MS, HPLC	Smelcerovic et al. (2008); Ayan and Çirak (2008); Cirak et al. (2014)
H. venustum	Hypericin, pseudohypericin, hyperforin	HPLC	Ayan et al. (2004)
H. xylosteifolium	Hypericin, pseudohypericin, hyperforin, adhyperforin, chlorogenic acid, neochlorogenic acid, caffeic acid, 2,4 dihydroxybenzoic acid, hyperoside, isoquercitrin, quercitrin, quercetin, avicularin, rutin, 13,118-biapigenin, (+)-catechin	HPLC	Cirak et al. (2016a)

found that the *H. pallens* Banks et Sol., have all of these compounds (Odabas et al. 2016).

Hypericin and pseudohypericin, chlorogenic acid, neochlorogenic acid, hyperforin, adhyperforin, caffeic acid, rutin, 2,4-dihydroxybenzoic acid, hyperoside, quercitrin, isoquercitrin, quercetin, avicularin, (−) –catechin, (−)-epicatechin, and amentoflavone compounds were investigated from *H. scabrum* L., *H. thymifolium* Banks and Sol., *H. lydium* Boiss., *H. olivieri* (Spach) Boiss., *H. linarioides* Bosse, *H. confertum* Choisy. It was found that the *H. thymifolium, H. linarioides, H. olivieri* and *H. scabrum* did not accumulate hyperforin and adhyperforin and those compounds were detectable only in flowers of *H. confertum* and *H. lydium* (Camas et al. 2014b).

Cirak et al. (2016b) investigated eight *Hypericum* species; *H. capitatum var. capitatum, H. capitatum* var. luteum*, H. elongatum* var. *elongatum, H. olympicum, H. polyphyllum, H. retusum, H. salsolifolium,* and *H. spectabile*, which are native to Southern Turkey. The compounds hyperforin, adhyperforin, hypericin, pseudohypericin, hyperoside, chlorogenic acid, neochlorogenic acid, caffeic acid, 2,4-dihydroxybenzoic acid, quercitrin, isoquercitrin, quercetin, avicularin, rutin, (+)-catechin, (−)-epicatechin, mangiferin, 13,118-biapigenin, and amentoflavone were determined from these species for the first time. They reported that the caffeic acid was not accumulated in *H. capitatum var. capitatum, H. capitatum var. luteum, H. retusum, H. spectabile, H. elongatum var. elongatum,* and *H. salsolifolium, similarly H. olympicum, H. polyphyllum*, did not produce hyperforin, adhyperforin and mangiferin. It was shown that the *H. elongatum* var. *elongatum* did not produce (+)-catechin (Cirak et al. 2016b).

The detailed chemical profiles of *H. aviculariifolium* subsp. *avicularііfolium* var. *albiflorum, H. cardiophyllum* and *H. elongatum subsp. microcalycinum* were made for the first time. Hyperforin, adhyperforin, hypericin, pseudohypericin, hyperoside, chlorogenic acid, neochlorogenic acid, caffeic acid, 2,4 dihydroxybenzoic acid, quercitrin, isoquercitrin, quercetin, avicularin, rutin, 13,118-biapigenin, and (+)-catechin contents were investigated of these species. It was reported that *H. cardiophyllum* accumulated the highest hyperoside and quercetin levels (Cirak et al. 2016a) (Table 4.5).

The researches from other regions of Turkey especially on endemic species showed that *H. neurocalycinum* was rich with chlorogenic acid, rutin, kaempferol, quercetin, and amentoflavon. *H. malatyanum* was rich with hyperoside, pseudohypericin, quercitrin, and isoquercitrin (Ozkan et al. 2018). Hyperoside and isoquercetin were determined as the main compounds of the *H. capitatum* Choisy var. *Capitatum*, but rutin and amentoflavon were not detected in these species (Boga et al. 2016). Hypericin, pseudohypericin, hyperoside, chlorogenic acid, rutin, quercitrin, quercetin, kaempferol, apigenin-7-*O*-glucoside, and amentoflavone chemicals were detected at various levels in *H. adenotrichum* (Çirak et al. 2009). It was determined that the aerial plant parts of *H. leptophyllum* contain hyperoside, chlorogenic acid, quercitrine, isoquercetine, and quercetine, but they did not have hypericin, hyperforin, pseudohypericin, rutin, and kaempferol.

Table 4.4 Studies on the phenolic compounds of *Hypericum* Species from Southern Turkey

Hypericum species	Examined phenolics	Method	References
H. aviculariifolium subsp. *aviculariifolium* var. *albiflorum*	Hyperforin, adhyperforin, hypericin, pseudohypericin, hyperoside, chlorogenic acid, neochlorogenic acid, caffeic acid, rutin, 2,4 dihydroxybenzoic acid, quercitrin, isoquercitrin, quercetin, avicularin, 13,118-biapigenin, (+)-catechin	HPLC	Cirak et al. (2016a)
H. elongatum subsp. *microcalycinum*			
H. cardiophyllum			
H. capitatum var. *capitatum*	Hyperforin, adhyperforin, hypericin, pseudohypericin, hyperoside, chlorogenic acid, neochlorogenic acid, rutin, caffeic acid, 2,4 dihydroxybenzoic acid, isoquercitrin, quercitrin, quercetin, avicularin, (+)-catechin, 13,118-biapigenin, amentoflavone, mangiferin	HPLC	Cirak et al. (2016b)
H. capitatum var. *luteum*			
H. elongatum var. *elongatum*			
H. spectabile			
H. salsolifolium			
H. retusum			
H. olympicum			
H. hircinum L. subsp. Majus	Hyperforin, adhyperforin, hypericin, pseudohypericin, hyperoside, chlorogenic acid, neochlorogenic acid, rutin, caffeic acid, 2,4 dihydroxybenzoic acid, isoquercitrin, quercetin, isoquercetin, avicularin, 13,118-biapigenin, (+)-catechin, ()-epicatechin	*HPLC*	Odabas et al. (2016)
H. lanuginosum Lam.			
H. russeggeri (Fenzl)			
H. confertum Choisy	Hyperforin, adhyperforin, hypericin, pseudohypericin, hyperoside, chlorogenic acid, neochlorogenic acid, rutin, caffeic acid, 2,4 dihydroxybenzoic acid, isoquercitrin, quercitrin, quercetin, avicularin, (+)-catechin, amentoflavone	HPLC	Camas et al. (2014b)
H. thymifolium Banks and Sol.			
H. olivieri (Spach) Boiss.			
H. linarioides Bosse			
H. scabrum L.			
H. lydium Boiss.	Hyperforin, adhyperforin, hypericin, pseudohypericin, hyperoside, chlorogenic acid, neochlorogenic acid, rutin, caffeic acid, 2,4 dihydroxybenzoic acid, isoquercitrin, quercitrin, quercetin, avicularin, amentoflavone, (+)-catechin, (−)-epicatechin,	HPLC	Camas et al. (2014b); Cirak et al. (2015)
H. pallens Banks et Sol.	Hyperforin, adhyperforin, hypericin, pseudohypericin, hyperoside, chlorogenic acid, neochlorogenic acid, caffeic acid, rutin, 2,4 dihydroxybenzoic acid, isoquercitrin, quercitrin, quercetin,	*HPLC*	Odabas et al. (2016); Camas et al. (2014a)

(continued)

Table 4.4 (continued)

Hypericum species	Examined phenolics	Method	References
	avicularin, amentoflavone 13,118-biapigenin, (+)-catechin, (−)-epicatechin, isoquercetin		
H. polyphyllum	Hyperforin, adhyperforin, hypericin, pseudohypericin, hyperoside, chlorogenic acid, neochlorogenic acid, rutin, caffeic acid, 2,4 dihydroxybenzoic acid, isoquercitrin, quercitrin, quercetin, avicularin, 13,118-biapigenin, (+)-catechin, (−)-epicatechin, amentoflavone, mangiferin	HPLC	Cirak et al. (2016b, 2017)

Table 4.5 Studies on the phenolic compounds of *Hypericum* species from other regions of Turkey

Hypericum species	Region	Examined phenolics	Method	References
H. malatyanum	Eastern Anatolia	Hypericin, pseudohypericin, rutin chlorogenic acid, ısoquercitrin, quercetin, kaempferol, amentoflavon	HPLC	Ozkan et al. (2018)
H. neurocalycinum	Middle Anatolia			
H.capitatum Choisy var.*capitatum*	Western Anatolia	Hypericin, pseudohypericin, hyperforin, chlorogenic acid, hyperoside, quercetin ısoquercitrin, kaempferol, quercitrin	HPLC	Boga et al. (2016)
H. adenotrichum	Western Anatolia	Hypericin, pseudohypericin, hyperoside chlorogenic acid, rutin, quercitrin, quercetin, amentoflavone, kaempferol, apigenin-7-*O*-glucoside	HPLC	Çirak et al. (2009)
H. leptophyllum	Middle Anatolia	Chlorogenic acid, hyperoside, quercitrin, quercetin	HPLC	Camas et al. (2012)

4.4 Biological Activity of *Hypericum* Species from Turkey

Historically plants have always had important roles in the improvement of human health. People have relied on them for their therapeutic and preventive uses because they are natural substances. One of these plants is *H. perforatum*, which is of medical importance (Table 4.6).

It was revealed that some major constituents were responsible for the biological activity of *H. perforatum*. For example it was reported that anti-inflammatory, antiulcerogenic (Berghoefer and Hoelzl 1989) activity of amentoflavone;

Table 4.6 Recent studies on the biological activity of *Hypericum* species growing in Turkey

Hypericum species	Biological activities	References
H. aviculariifolium Jaub. and Spach subsp. *depilatum*	Antioxidant, antibacterial	Maltas et al. (2013)
H. capitatum var. *capitatum*	Antioxidant, antimicrobial, anticholinesterase, DNA damage inhibitor	Boga et al. (2016)
H. confertum subsp. confertum	Antimicrobial	Kunduhoglu et al. (2011)
H. heterophyllum	Cytotoxic properties, mitotic, proliferative	Öcal and Eroğlu (2012)
H. lydium	Antioxidant, antimutagenic	Boran and Ugur (2017)
H. montbretii Spach.	Immunomodulating inhibitory activity	Demirkiran et al. (2013)
	Sedative and anticonvulsant	Can and Özkay (2012)
	Antidepressant	Can et al. (2011a, b, c)
H. neurocalycinum	Antioxidant, antimicrobial	Özkan et al. (2013a)
H. pamphylicum	Antioxidant, antimicrobial	Özkan et al. (2013b)
	Memory-vitalizing	Senol et al. (2016)
H. perforatum	Antioxidant	Altun et al. (2013); Maltas et al. (2013); Eğilmez et al. (2015); Uslusoy et al. (2017a, b); Nazıroğlu et al. (2014)
	Antibacterial	Maltas et al. (2013)
	Wound healing	Kiyan et al. (2015); Dikmen et al. (2011); Han et al. (2017); Damlar et al. (2017)
	Antipsychotic	Gurok et al. (2014)
	Depression treatment	Can et al. (2011a, b, c); Dalmizrak et al. (2012)
	Antitumoral	Ocak et al. (2013)
	Antidiabetic	Can et al. (2011a, b, c)
	Antiinflammatory	Nazıroğlu et al. (2014)
	Antiapoptotic	Ocak et al. (2013)
H. salsugineum Robson and Hub.-Mor.,	Antioxidant, antibacterial	Maltas et al. (2013)
H. uniglandulosum	Antioxidant, antibacterial	Turkoglu et al. (2015)

antibacterial and wound-healing (Gurevich et al. 1971; Bystrov et al. 1975; Maisenbacher and Kovar 1992), neurotransmitter inhibitor (Nahrstedt and Butterweck 1997), antidepressant (Chatterjee et al. 1998), potential anticarcinogenic (Schempp et al. 2002), angiogenesis inhibition (Schempp et al. 2005) and antimalarial activity of hyperforin (Verotta et al. 2007); antiviral (Meruelo et al. 1988; Weber et al. 1994; Lavie et al. 1995), antidepressant activity (Nahrstedt and Butterweck 1997; Chatterjee et al. 1998; Müller et al. 2001; Eckert and Müller 2001), anti-inflammatory activity of hypericin (Panossian et al. 1996); sedative

effect of 13,118-biapigenin (Berghoefer and Hoelzl 1989); antiviral activity of pseudohypericin (Meruelo et al. 1988); in vitro MAO-inhibiting activity of quercitrin (Sparenberg et al. 1993); antidepressant, antimicrobial, antiviral, diuretic, cardiotonic, MAOA inhibitor activities of xanthones (Kitanov and Blinova 1987; Hölzl et al. 1989; Sparenberg et al. 1993; Nahrstedt and Butterweck 1997).

Numerous studies were conducted for determining the biological activities of the *Hypericum* species growing in Turkey. It was found *H. perforatum* L. has acetylcholinesterase and butyrylcholinesterase inhibition activity that related to Alzheimer's and Parkinson's disease (Altun et al. 2013). Some studies on the rats show that *H. perforatum* has hepatoprotective (Bayramoglu et al. 2014), protective effect on SCI-induced oxidative stress and apoptosis (Özdemir et al. 2016) and increased the levels of Zn and Na in serum, and Cd levels in the hair (Ekin et al. 2012). Additionally, some clinical studies revealed that *H. perforatum* shows the preventive effects on intestinal ischemia-reperfusion injury in hamsters (Ocak et al. 2014), on oxidative stress and [Ca2+] ion concentrations by modulating transient receptor potential and voltage-gated calcium channel in the patients with MS (Naziroglu et al. 2014), on DNA damage, cell viability, apoptosis and a disintegrin-like and metalloproteinase (Akyol et al. 2014). Studies revealed that *H. lydium* may be provided as a useful and safe agent for the prevention of cancer and mutations (Boran and Ugur 2017).

Hypericum species have active antibacterial components such as hyperforin and flavonoids, which probably contribute to rapid and better wound healing (Shetty et al. 2008). It is well known that flavonoids have an active antioxidant capacity for the inhibition of lipid peroxidation, which leads to the prevention of cell damage and an increase in the viability of collagen fibrils (Kiyan et al. 2015).

Can et al. (2011a, b, c) showed that *H. montbretti* has antidepressant activities that reported from originating from rutin. Additionally, the anti-inflammatory and anti-nociceptive activities of *H. triquetrifolium* was known (Apaydın et al. 1999; Ozturk et al. 2002). *H. calycinum* may be used for therapeutic purposes in depression since it has the anti-depressant effect that was as potent as that of *H. perforatum* (Smelcerovic et al. 2008). The *hypericum* species, *H. lysimachioides* var. *lysimachioides*, *H. hyssopifolium* subsp. *elongatum* var. *elongatum, H. montbretii, H. venustum, H. pamphylicum,* and *H. perforatum* were found to have antioxidant properties and were investigated for antibacterial activity (Cakir et al. 2003; Eroglu 2007).

4.5 Conclusion

Our country is a significant gene center for *Hypericum species*. These species have been used for a long time in folk medicine. Several species among the 100 *Hypericum* taxa growing widely in Turkey were investigated, but there are many types of *Hypericum* species that have not yet been studied for their chemical contents. Recently, the studies focused on the genus *Hypericum*, because it is a source of a variety of compounds with different biological activities. Despite the

quantitative changes observed in the chemical compound content of plants, it was found the phytochemical contents of the species from the same section were quite similar. In our country, although several *Hypericum* species have been used in folk medicine, only *H. perforatum* exists as its pharmaceuticals in the market.

The present study could be helpful to reveal new sources of phytochemicals for potential pharmaceutical studies on the *Hypericum species* that are growing in Turkey.

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5 Morphogenesis of Cotyledon Explants and Plantlet Regeneration of Tomato (*Lycopersicon esculentum* Mill.) Under Salt Stress

M. M. A. N. Shanika and Thayamini H. Seran

Abstract

This study was aimed to determine the responses of salt stress on cotyledon callus of tomato cv. KC-1 for plant regeneration. Cotyledon explants from in vitro raised seedlings were placed on MS medium containing 1.0 mg/L BAP and 0.2 mg/L NAA or 1.0 mg/L NAA to select a suitable medium for callus formation. After 6 days of culture, green calli were initiated on the cut surfaces of cotyledon explants. Callus formation was observed in 81.2% of the explants on the medium with 1.0 mg/L BAP and 1.0 mg/L NAA. After 2 weeks of culture, cotyledon produced calli were transferred to the medium containing 1.0 mg/L BAP plus 1.0 mg/L NAA without (control) or with NaCl concentrations (20, 40, and 60 mM). Callus was evaluated after 4 weeks of subculture (i.e., after 6 weeks of initial culture) under salt stress based on callus colour, fresh and dry weights of callus and chlorophyll content of callus. Results showed that the effects of tomato KC-1 cultivar on the tested parameters had a significant effect on different salinity levels. The calli that were placed on 60 mM NaCl level were highly affected when compared with control. Calli were then transferred to the medium with 2.0 mg/L BAP and 0.2 mg/L NAA for plant regeneration. Within 4 weeks, plantlets were formed on the control treatment and 20 mM salt stressed calli and they were acclimatized under ex vitro conditions.

Keywords

Callus · In vitro culture · Plantlet regeneration · Salt effect · Tomato

M. M. A. N. Shanika · T. H. Seran (✉)
Department of Crop Science, Faculty of Agriculture, Eastern University, Chenkalady, Sri Lanka
e-mail: thayaminis@esn.ac.lk

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5.1 Introduction

Tomato (*Lycopersicon esculentum* Mill.) belongs to the family Solanaceae. It is important among the vegetable crops for human consumption and diverse uses in the world as it contains a considerable number of vitamins and minerals. Tomato products are carotenoid-rich foods. High dietary intake of tomato foodstuffs can reduce coronary heart disease in consequence of decreasing Low-density lipid (LDL) Cholesterol levels (Silaste et al. 2007). As a result, it is a market potential vegetable crop that is grown in the field, greenhouses and net houses not only in Sri Lanka but also in most of the countries (Bhatia et al. 2004). However, there are biotic and abiotic stresses to limit tomato production mainly under field conditions. Among them, salinity is one of the vital stresses in restrictive crop production (Mahajan and Tuteja 2005). In general, plant growth may be declined as a result of toxic salt ion accumulation in plants that can cause osmotic stress (Silveria et al. 2009). Sivritepe et al. (2003) reported that seedling growth at an early stage is more susceptible to salinity in many plants. Crop yields reduce in many plants when the electrical conductivity value of saturation soil extract increases above the threshold level value (Essington 2015). No significant yield reduction was revealed in most commercial cultivars of tomato grown in salinity level up to 2.5 dsm^{-1} (Singh et al. 2012). Tomato varieties namely KC-1, Tharindu, T-245, Marglobe, KWR, Roma, Thilina and Ravi are generally cultivated in Sri Lanka.

Biotechnological techniques have been applied to enhance the yield and quality of vegetable and other crops (Gosal et al. 2010; Ayobami et al. 2013). An effective protocol of in vitro culture technique is necessary for employing the benefits of plant tissue culture for genetic improvement of crops (Sheeja et al. 2004). Plant tissue culture technique has been effectively used to select tolerant cell lines against diverse biotic and abiotic stresses (Bhatia et al. 2004; Manoj et al. 2011). The establishment of callus is an important step for the in vitro culture studies. Exogenous plant growth regulators in culture medium play a vital role in callus induction and organ differentiation. According to a literature survey, IAA (Indole-3-acetic acid), NAA (-α-naphthalene acetic acid), 2,4-D (2,4-Dichlorophenoxy acetic acid), Zeatin and 6-BAP (6-benzyl amino purine) are commonly used in tissue cultures of tomato for callus formation and plant regeneration (Namitha and Negi 2013). This experiment was done to determine the effect of salt stress on callus formation from cotyledon explants of tomato cv. KC-1 for plant regeneration.

5.2 Materials and Methods

This research was done in 2015–2016 at the Tissue Culture Laboratory, Department of Crop Science, Faculty of Agriculture, Eastern University of Sri Lanka. Tomato seeds (KC-1 cultivar) used in this research work were obtained from the Horticultural Crop Research and Development Institute, Sri Lanka. The tomato seeds were dipped in 70% (v/v) ethyl alcohol for 1 min and then surface sterilized by using 20% (v/v) Clorox™ (5.25% sodium hypochlorite) with two drops of Tween 20 for

20 min. After that, they were washed four times with sterilized distilled water and dried on autoclaved filter paper for 15 min under sterile conditions. They were then inoculated on MS (Murashige and Skoog 1962) basal medium with 3% sucrose and 0.8% agar to germinate under in vitro conditions. In the present study, all cultures were kept at 25 ± 1 °C under white fluorescent light in a photoperiod of 16 h and 8 h dark. The light intensity of 2000–2500 lux and 70% relative humidity were maintained.

5.2.1 Callus Induction on Cotyledon Explants

In vitro raised seedlings (Fig. 5.1) were utilized to excise cotyledon explants. The cotyledon explants (0.5 cm long) were carefully detached from the seedlings under aseptic conditions subsequently they were placed (abaxial side) on MS basal medium containing 1.0 mg/L BAP and 0.2 mg/L NAA (Sherkar and Chavan 2014) or 1.0 mg/L BAP and 1.0 mg/L NAA to induce callus. The explants were observed at regular intervals for in vitro responses. This experiment was done twice and each replicate had 16 explants.

5.2.2 Salt Stress on Cotyledon Callus

From the previous work in this study, it was noted that the percentage of callogenesis was high in the cultured cotyledons on the culture medium with 1.0 mg/L BAP and 1.0 mg/L NAA. Hence, after 2 weeks of initial culture, the cotyledons producing calli on the above medium were placed on the MS media containing 1.0 mg/L BAP plus 1.0 mg/L NAA and also different concentrations of NaCl (0, 20, 40, 60 mM). The cultures were observed constantly for callus proliferation in each culture medium. After 4 weeks of culture in different salt concentrations, the colour of

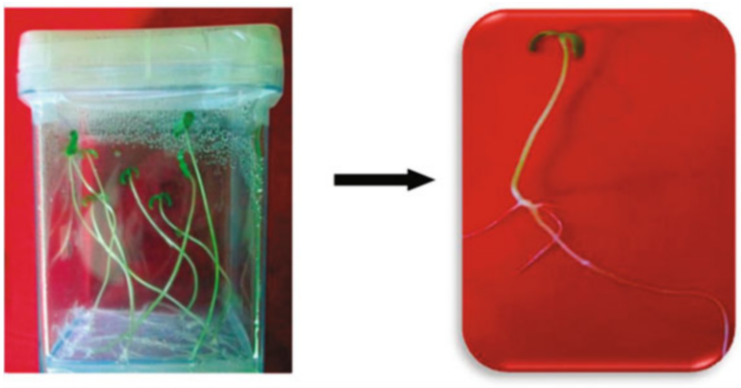

Fig. 5.1 In vitro raised 12 days old seedling

callus, fresh and dry weights of callus, water content % of callus and chlorophyll content of callus were assessed. This experiment was laid out in a completely randomized design with three replications and repeated twice.

5.2.3 Fresh and Dry Weights of Callus

After 4 weeks of culture, proliferating calli on the MS media with different NaCl concentrations were separately collected from the culture media and fresh weight of callus was measured in each salinity treatment. Thereafter, the collected callus was dried at 40 °C for 48 h and the constant weight of dried callus was determined. After measuring the fresh and dry weights of callus in each salt treatment, water content percentage of callus was estimated.

5.2.4 Chlorophyll Contents of Callus

After 4 weeks, 100 mg of calli were separately harvested from the media containing the different concentrations of NaCl. The collected calli from each treatment were placed individually in a glass vial (28 mL capacity). Subsequently, 10 mL of 80% acetone was added into each vial. Then, calli were blended with a homogenizer, and the extract was collected in a test tube to measure chlorophyll $_a$ (Chl_a) and chlorophyll $_b$ (Chl_b) contents using a UV visible spectrophotometer at 663 nm and 645 nm wavelengths respectively. The Chl_a, Chl_b and total Chl contents of calli were measured on a fresh basis using Arnon's equation (Arnon 1949).

5.2.5 Plant Regeneration from Cotyledon Callus

After 4 weeks of culture in different salt levels, the proliferating callus was subcultured on the medium containing 2.0 mg/L BAP and 0.2 mg/L NAA in line with the reports of Godishala et al. (2011) and Sherkar and Chavan (2014) for plant regeneration. In vitro plantlets were gently washed with running tap water and followed by sterile distilled water. They were then transferred to small pots containing soil and cow dung in the ratio of 1:1 (v/v). The pots were covered with a transparent polythene bag to retain high relative humidity around the plants.

5.2.6 Statistical Analysis

The data obtained in callus weights were subjected to analysis of variance using SAS 9.1.3 version. The treatment means of callus weights were compared using Tukey's Honestly Significant Difference Test at 5% significant level. A regression analysis was performed for chlorophyll contents of callus in different culture media and also

correlation coefficients were calculated to correlate the chlorophyll content with NaCl concentration incorporated into the culture medium.

5.3 Results and Discussion

5.3.1 Callus Formation on Cotyledon Explants

Cotyledon explants from in vitro raised seedlings were placed on the medium with 1.0 mg/L BAP and 0.2 mg/L NAA or 1.0 mg/L NAA to select a suitable medium for callus formation. After 6 days of culture, yellowish-green calli were mostly initiated on the cut surfaces of cotyledon explants. Callus was formed in 81.2% of the cultured explants on the medium containing 1.0 mg/L BAP plus 1.0 mg/L NAA at 2 weeks of culture. Gerszberg et al. (2016) indicated that cotyledon is the more suitable explants to induce shoot for plant regeneration. Sherkar and Chavan (2014) observed a high percentage of callus formation in the cultured cotyledon explants on the media with 1.0–4.0 mg/L BAP and 0.2 mg/L NAA, however, shoot regeneration was not obtained. Properties of callus initiation in several explants, mainly associated with the quantity of endogenous plant growth regulators and their uptake, types of auxin and cytokinin used, as well as action mode of hormones (Gupta et al. 2010). The different types and concentrations of plant growth regulators have been used for in vitro plant regeneration of tomato. In vitro plantlets can be regenerated from callus through organogenesis or somatic embryogenesis procedure as stated by Verma et al. (2011) and other researchers.

5.3.2 Salinity Stress on Cotyledon Callus

After 2 weeks of culture, cotyledons produced calli were placed on the medium containing 1.0 mg/L BAP and 1.0 mg/L NAA without (control) or with NaCl concentrations (20, 40, and 60 mM) to assess their salt tolerance. After 4 weeks on salt media (i.e., after 6 weeks of initial culture), fresh and dry weights, water content and chlorophyll content of calli were measured. Moreover, the colour of callus in each medium was observed. The results showed that salt stress affected the tested parameters of callus derived from cotyledon of tomato KC-1 cultivar. Callus formation on different NaCl treatments after 4 weeks (after 6 weeks of initial culture) is shown in Fig. 5.2. Most of the researchers used callus to evaluate its physiological and biochemical responses to salt stress (Shibli et al. 2007; Ghane et al. 2014; Alharby et al. 2016).

5.3.3 Salt Stress on Colour of Callus

In this experiment, the colour of calli was observed at a regular interval. Callus colour was changed by increasing NaCl concentrations. After 2 weeks of culture,

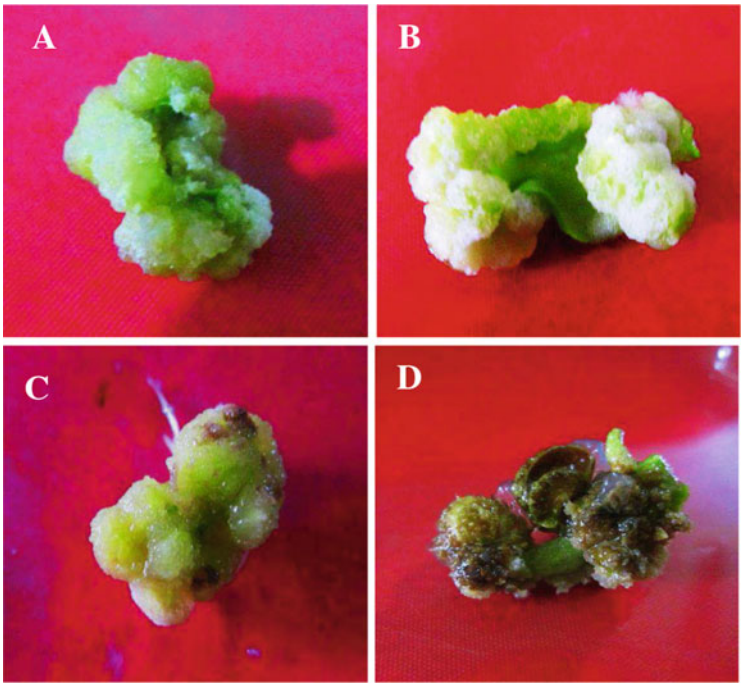

Fig. 5.2 Effects of salt stress on cotyledon calli after 4 weeks of culture in NaCl treatments (**a**–**d**) (Note: Callus cultures on MS media with 1.0 mg/L BAP + 1.0 mg/L NAA supplemented with 0 mM (**a**) 20 mM (**b**) 40 mM (**c**) and 60 mM (**d**) NaCl concentrations)

yellowishgreen colour calli were gradually turned to greenish-brown colour in the medium with high salt concentration (60 mM). Yellowish-green to green colour was mostly observed on calli in the control treatment (0 mM NaCl) but the colour of callus was brownish-green in the 40 mM NaCl treatment after 4 weeks of culture. The colour change of calli with increasing salt content is in harmony with the studies in potato (Rahnama and Ebrahimzadeh 2004), sugarcane (Gandonou et al. 2006) and rice (Rattana and Bunnag 2015) response to salinity stress. Bouiamrine and Diouri (2012) stated that change in colour of cell mass to brownish colour was generally exploited as a sign of tissue culture in tolerance to osmotic stress.

5.3.4 Salt Stress on Weights of Callus

The results showed that after 4 weeks of culture, the fresh weight of callus in the control (0 mM NaCl) treatment was significantly ($P < 0.05$) higher (0.74 g) than that in salt-treated media (Fig. 5.3). The lowest fresh weight (0.28 g) of callus was recorded in the 60 mM NaCl concentration. Furthermore, it was noted that the fresh weight of callus was slowly decreased in the 40 mM NaCl as compared to the 20 mM NaCl concentration but there was no remarkable variation between them.

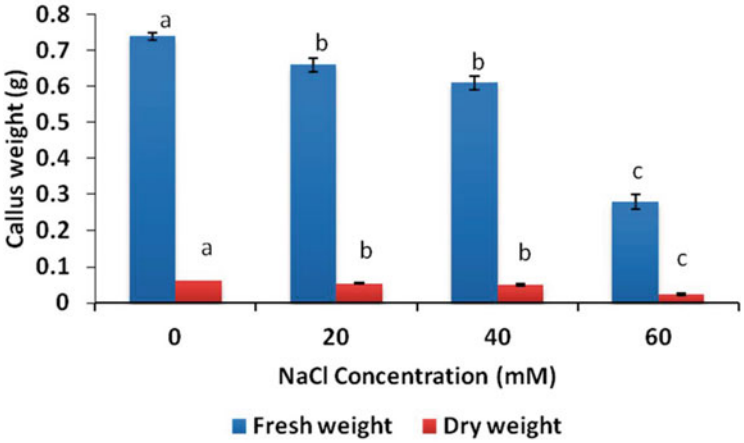

Fig. 5.3 Effect of different salinity levels on fresh and dry weights of cotyledon callus after 4 weeks of culture (Means followed by the same letter in each bar are not significantly different according to Tukey's test at 5% significant level)

In contrast, the fresh weight of callus was considerably declined on high NaCl concentration (60 mM NaCl). It may be due to salinity stress on water uptake for its growth. The dry weight of callus was also high in the control treatment with an average of 0.062 g while a dry weight of callus was low in the 60 mM concentration with an average of 0.023 g. No significant difference was noted on the 20 and 40 mM salt concentrations.

The results showed that the dry weight of calli on control treatment was considerably ($P < 0.05$) varied from those in the other treatments. It was significantly low value in 60 mM NaCl medium among the tested treatments. Water and nutrient contents are associated with the salt concentration in plant tissue (Martínez-Ballesta et al. 2006; Gao et al. 2013). Yunita et al. (2014) mentioned about the reduction of callus growth due to osmotic effects and also indicated that more energy is needed for its metabolism. This is in harmony with Abbas et al. (2012) revealed that callus growth declined at the highest salinity level and osmotic stress inhibits the absorption of mineral nutrients by the cells. Plant growth regulators in the cells take part in a vital role in regulating plant responses to abiotic stress (Upreti and Sharma 2016). As a result of osmotic stress, the addition of salt ions in the cell mass inhibits the synthesis of growth regulators that are necessary for cell differentiation and plant growth (Gupta and Huang 2014; Al-Hattab et al. 2015).

In the present study, water content % of callus in salt-treated media (0–60 mM NaCl) was about 92%. The results exhibited that salt stress was not significantly affected by water content of callus. High osmotic stress from high salt level controlled plant cells to uptake water and some mineral nutrients in the culture medium (Cicek and Cakirlar 2002). Al-Khayri and Al-Bahrany (2004) noted a progressive decline in callus growth as increasing water stress. Queiros et al. (2007) stated that the water content is reduced as a result of the high osmotic stress

of the medium supplemented with high salt concentration. Salinity at higher levels has a result in plant metabolism (Hasanuzzaman et al. 2013). The decrease in water content of callus was observed in *Carthamus tinctorius* (Soheilikhah et al. 2013) and *Stylosanthes guianensis* (Veraplakorn et al. 2013) due to osmotic stress.

5.3.5 Salt Stress on Chlorophyll Content of Callus

The obtained results revealed that there was a significant relationship between NaCl concentration and total chlorophyll content of calli ($P < 0.001$). The value of total chlorophyll content of the callus was higher (31.8 μg/g) in the control treatment than other media under in vitro salt stress whereas it was lower (16.4 μg/g) in the medium containing 60 mM NaCl level (Fig. 5.4). The findings are supported by EL-Meleigy et al. (2004) who reported that chlorophyll contents in the entire tomato genotypes tested under in vitro conditions were declined due to salinity stress. The total chlorophyll contents were negatively correlated with NaCl levels in the culture media ($r = -0.942$). The reduction of leaf chlorophyll content under high salinity stress might be due to the activity of the chlorophyll degrading enzyme, chlorophyllase (Megdiche et al. 2007; Noreen and Ashraf 2009).

As compared to the control treatment, chlorophyll a and chlorophyll b in tomato calli were decreased due to salt stress in the present study. It was also noted that reduction percentage of both chlorophyll contents were increased with increasing NaCl concentrations added to the culture media. Moreover, the decline in chlorophyll a was lower than that in chlorophyll b content of callus cultured in 60 mM NaCl medium. This effect has also been stated by Delfine et al. (1999) in spinach and EL-Meleigy et al. (2004) in tomato. Aliu et al. (2015) mentioned that the highest strength of salinity generally reduced the contents of both chlorophyll a and b over the control. Screening of different genotypes for salt tolerance can be performed

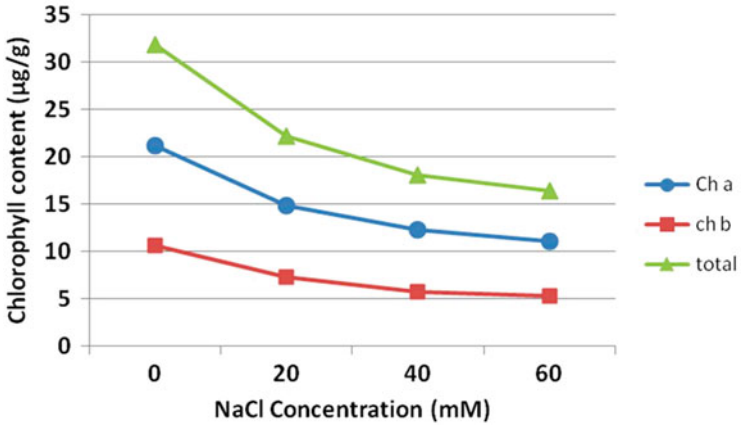

Fig. 5.4 Effect of NaCl concentrations on chlorophyll contents of calli

under in vitro conditions by using NaCl. In vitro screening of salt-tolerant genotypes has been reported in mulberry (Vijayan et al. 2003) and tomato (Rashed et al. 2016).

5.3.6 Plantlet Regeneration

In this experiment, the green nodules on the surface of cultured calli were noted after 1 week of culture. It was a sign of formation of embryoids like structures from the calli which was transferred to the medium with 2 mg/L BAP plus 0.2 mg/L NAA (Fig. 5.5). They were eventually developed into plantlets after 4 weeks. It was observed in calli under 0 mM and 20 mM salt stress. The nodule structures on the surface of the calli were also observed on 40 mM salt-stressed calli after 4 weeks. Somatic embryogenesis can be employed in mass plant propagation and transformation studies as stated by Yadav and Tyagi (2006). Godishala et al. (2011) stated that embryogenic callus was formed from cotyledon explants of tomato cultured on medium containing 2.0–4.0 mg/L BAP plus 0.2–0.5 mg/L IAA and also induction rate of somatic embryogenesis was highest on the medium with 3.5 mg/L BAP and

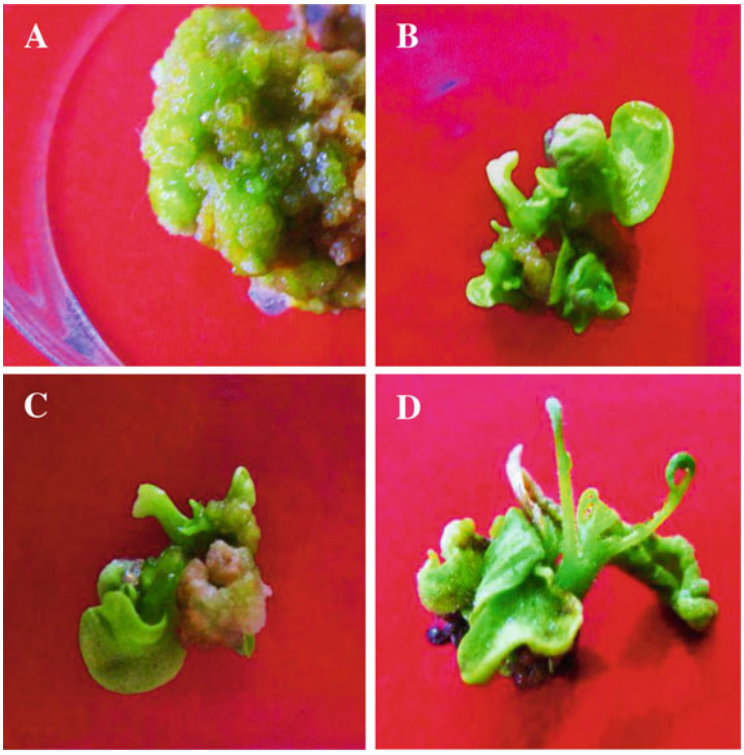

Fig. 5.5 Morphogenesis of 20 mM salt stressed callus cultured on MS medium containing 2.0 mg/L BAP plus 0.2 mg/L NAA after 1 week (**a**) and 2–3 weeks (**b–d**)

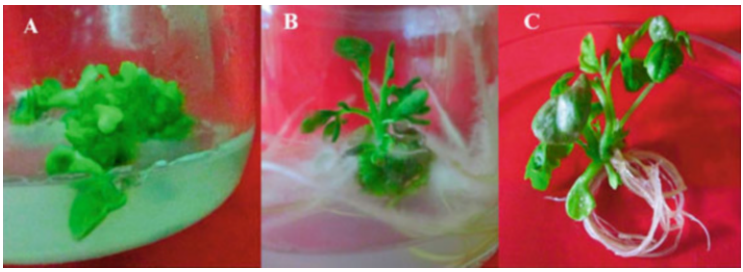

Fig. 5.6 Plantlets (**b**, **c**) regenerated from non-salt stressed callus (**a**) cultured on MS medium supplemented with 2.0 mg/L BAP plus 0.2 mg/L NAA after 4 weeks of culture

0.5 mg/L IAA. On the contrary, Durrani et al. (2017) informed that the calli exhibited greening but shoots did not appear on calli on the medium with 3 mg/L BAP and 0.3 mg/L NAA. In the present study, exogenous supply of BAP and NAA induced nodular structures on cotyledon callus for plant regeneration.

After 5 weeks, shoot elongation and root formation were observed on the medium with 2.0 mg/L BAP and 0.2 mg/L NAA (Fig. 5.6). Generally, a low concentration of BAP alone or in combination with auxin has been used for shoot elongation of plant species. But the relatively high concentration of BAP stimulated shoot bud development (Sathyagowri and Seran 2013; Lavakumaran and Seran 2014). Best callusing and shoot regeneration was noted from internode explants cultured in MS medium with 2.0 mg/L BAP and 0.2 NAA mg/L NAA (Sherkar and Chavan 2014; Shanika et al. 2018). Mohamed et al. (2010) reported that 2.0 mg/L BAP was better for growth traits of tomato among 0–4 mg/L concentrations of BAP. In the present study, the plantlets were collected from the in vitro culture and were then transferred to small pots containing a mixture of soil and cattle manure. Subsequently, they were acclimatized.

5.4 Conclusion

The cotyledons were responded well on MS medium fortified with 1.0 mg/L BAP plus 0.2 mg/L NAA. The present study revealed that by increasing the NaCl concentrations on the callus, the dry and fresh weights of callus and chlorophyll contents of callus was decreased, when cultured on the medium containing 1.0 mg/L BAP and 1.0 mg/L NAA. Tomato was more sensitive to salinity on callus formation with a significant reduction for salinities above 40 mM NaCl and a considerable reduction was observed on callus formation above this level. Formation of embryoids like structures was observed on the control treatment and 20 mM salt-stressed calli on the medium with 2.0 mg/L BAP and 0.2 mg/L NAA. In vitro plantlet formation was noticed on the same MS medium. This may lead to the mass production of plantlets within a short period. To increase the general tolerance

mechanism against salt stress, future study is needed for the crop improvement program.

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Callus Culture Systems in *Salix* L.: The Limited Database

6

Afsheen Shahid and Mohammad Anis

Abstract

Salix L. is the largest genera of family Salicaceae. Commonly known as 'willows', the genus *Salix* is a large contributor to the energy crop systems, an important source of natural 'aspirin' (Salicin and derivatives) and a large number of active constituents with medicinal properties. The dioecious nature of the tree and different geographical distributions of the male and female counterparts renders it difficult to propagate via seeds. Several studies have been published on the clonal micropropagation of *Salix* L. in the past 40 years; however, only four studies reported the callus culture systems. The presented review focus on the limited knowledge of callus induction protocols in *Salix* L. throughout the genera and the importance of pursuing this line of study.

Keywords

Micropropagation · Willow · Medicinal Plants · Salicaceae

6.1 Introduction

'Plant tissue culture is a tool that is an adjunct to studies on whole plants'—Donald K Dougall.

A. Shahid (✉)
Chair of Tree Physiology, Institute of Forest-Sciences, Albert-Lüdwigs University, Freiburg, Germany

M. Anis
Department of Botany, Aligarh Muslim University, Aligarh, India

I. Siddique (ed.), *Propagation and Genetic Manipulation of Plants*,
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Plant tissue culture systems, either for the purpose of clonal propagation or in vitro secondary metabolite production, play a pivotal role in the area of conservation biology. Tissue culture systems provide a fighting chance for the dwindling biodiversity, making it possible to upscale the clonal plantlet production for endangered and threatened plant species. Another important aspect of tissue culture is saving the medicinally important plant species from overexploitation and endangerment. The tissue culture protocols have been established by various working groups for almost all the medicinally important plant families, some getting more attention than the other. Induction and establishment of plant callus cultures with the aim of secondary metabolite accumulation depend on various factors.

Callus culture systems have been designed and established in a large number of plants. The early tissue culture strategies were all focused on cell elongation, division and dedifferentiation to achieve a free growing cell mass to be directed in desired line of indirect plant regeneration, somatic embryogenesis, de-novo metabolite production and elicitation of pre-existing active constituents. Callus masses are the raw material for various established and emerging tissue culture branches.

> 'Despite advances in the field of organic chemistry, plants are still an important commercial source of chemical and medicinal compounds'—Dodds and Roberts (1985)

Plants produce a vast array of primary and secondary product with secondary biosynthetic pathways branching at different developmental stages of the plant tissue and organs. Some secondary products are of universal occurrence throughout the plant kingdom while some can be specific to a particular plant family, genus, species or sometimes the natural intraspecific hybrids. Within the single plant species, the synthesis, distribution and accumulation pattern of one secondary metabolite may also vary. Isolated cell systems and callus culture systems provide a working base to study the differential response of specific plant organs from different species, genus and families for secondary metabolite production, biotransformation and even finding novel more potent substitutions of pre-established active constituents from various plant species. Callus culture forms the basis of all the advanced research, be it the genetic manipulation, metabolic engineering, biotransformation, metabolite production or secondary embryogenesis/adventitious regeneration.

6.1.1 *Salix* L. (The Willow)

Salix L. is the type genus and by far the largest genera of family Salicaceae (Azuma et al. 2000), which includes a total of 55 genera in the family and 772 recognized species (Table 6.1). Still, about one-third of the genetic biodiversity of the family is unrecognized and unpublished. Salix L. includes approximately 377 species (The Plant List 2013) widely distributed in the northern hemisphere including subtropical, temperate and cold regions (Chen et al. 2010) and Indo-Malesia and mountains of China in the Indian subcontinent. It is known to be a taxonomically difficult genus.

Table 6.1 List of genera in family Salicaceae including number of species listed

S. No.	Genera	No. of species
1	*Abatia*	8
2	*Aberia*	1
3	*Ahernia*	1
4	*Aigeiros*	1
5	*Azara*	9
6	*Banara*	17
7	*Bartholomaea*	1
8	*Bembicia*	1
9	*Bennettiodendron*	1
10	*Bivinia*	1
11	*Calantica*	1
12	*Carrierea*	2
13	*Casearia*	64
14	*Chosenia*	1
15	*Craepaloprumnon*	1
16	*Dovyalis*	9
17	*Euceraea*	3
18	*Flacourtia*	7
19	*Gossypiospermum*	2
20	*Guidonia*	8
21	*Hasseltia*	2
22	*Hasseltiopsis*	1
23	*Hecatostemon*	1
24	*Homalium*	33
25	*Idesia*	1
26	*Itoa*	1
27	*Laetia*	9
28	*Lasiochlamys*	1
29	*Ludia*	4
30	*Lunania*	2
31	*Macrohasseltia*	1
32	*Macrothumia*	1
33	*Neopringlea*	2
34	*Neoptychocarpus*	2
35	*Neosprucea*	4
36	*Olmediella*	1
37	*Oncoba*	14
38	*Phyllobotryon*	1
39	*Pineda*	1
40	*Pleiarina*	1
41	*Pleuranthodendron*	4
42	*Poliothyrsis*	1
43	*Populus*	87

(continued)

Table 6.1 (continued)

S. No.	Genera	No. of species
44	*Prockia*	3
45	*Pseudoscolopia*	1
46	*Ryania*	6
47	***Salix***	**377**
48	*Samyda*	6
49	*Scolopia*	9
50	*Tetrathylacium*	2
51	*Tisonia*	6
52	*Toisusu*	1
53	*Trimeria*	1
54	*Xylosma*	45
55	*Zuelania*	1

The infrageneric classification of *Salix* L. is highly controversial (Chen et al. 2010) even though several classifications have already been proposed and modified on the basis of morphology, phenology and molecular taxonomy for it (Chao et al. 1998; Leskinen and Alström-Rapaport 1999; Skvortsov 1999; Ohashi 2001). The application of *Salix* L. as the main source of herbal NSAIDs (non-steroidal anti-inflammatory drugs) in the medicine industry makes it an interesting choice for regulated secondary metabolite production in vitro along with micropropagation strategies for clonal propagation and conservation of elite genotypes and phenotypes.

Salix L. is a very diverse and useful genus with ecologically and medicinally important species. There are several species of *Salix* L. which are listed (IUCN Red List) as endangered (*S. kusani*), vulnerable (*S. xanthicola, S. megnifica, S. floridana, S. tortosina,* etc.) or near –threatened (*S. libani, S. tirrenico, S. crataegifolia, S. canareinsis, S. ionica,* etc.) all over the globe. Still, there are handful of reports available for conservation, sustainable biochemical utilization (in vitro metabolite production) and replantation of the species (via clonal propagation) using tissue culture methods.

6.1.2 Tissue Culture in *Salix*

Since the beginning of plant tissue culture methods, various workers studied the different aspects of tissue culture in *Salix* L. All the studies conducted in the area have been summarized in Table 6.2 and can be broadly categorized in two lines of investigation.

6.1.2.1 Micropropagation/Direct Organogenesis In Vitro

Various species of *Salix* L. have been selected by tissue culturists for micropropagation studies (Table 6.2). The earliest successful attempts for direct organogenesis were recorded by Bhojwani (1980) using nodal explants of hybrid

Table 6.2 Tissue culture studies on various species in genus *Salix*, for the past four decades

S. no.	Species	Tissue culture strategy	References
1	*S. matsudana X alba (NZ1002)*	Direct organogenesis	Bhojwani (1980)
2	*S. alba* *S. fragilis* *S. viminalis*	Direct organogenesis	Chalupa (1983)
3	*S. babylonica*	Direct shoot regeneration	Dhir et al. (1984)
4	*S. schwerinii* *S. Dasyclados* *S. caprea* *S. viminalis* *S. caprea (hybrid)*	Direct shoot regeneration	Bergman et al. (1985)
5	*S. viminalis*	**Callus culture and somatic embryogenesis**	Grönroos et al. (1989)
6	*S. exigua*	**Callus cultures**	Stoehr et al. (1989)
6	*S. viminalis*	**Protoplast culture**	Vahala and Eriksson (1991)
7	*S. caprea*	Direct shoot regeneration	Neuner and Beiderbeck (1993)
8	*S. fragilis X lispoclados*	Ovary culture	Agrawal and Gebhardt (1994)
9	*S. tarraconensis*	Direct shoot regeneration	Amo-Marco and Lledo (1996)
10	*S. caprea*	Direct shoot regeneration	Liesebach and Naujoks (2004)
11	*S. nigra*	Direct adventitious shoot regeneration	Lyyra et al. (2006)
12	*S. pseudolariogyne*	Direct regeneration	Park et al. (2008)
13	*S. tetrasperma*	Direct regeneration	Khan et al. (2011); Khan and Anis (2012)
14	*S. viminalis* *S. cinerea*	**Callus culture**	Skálová et al. (2012)
15	*S. viminalis*	Direct regeneration	Regueira et al. (2018)
16	*S. tetrasperma*	**Callus cultures**	Shahid and Anis (2018)
17	*S. tetrasperma*	Synthetic seeds	Khan et al. (2018)
18	*S. retusa* *S. alpina*	Direct shoot multiplication	Chornobrov et al. (2019)

willow (*Salix matsudana X S. alba* NZ-1002), while Chalupa (1983) successfully reported the direct organogenesis in three different species of *Salix* (*S. alba, S. fragilis, S. viminalis*) from nodal segments and axillary bud induction and proliferation in vitro. Later in the decade, Dhir et al. (1984) reported successful shoot proliferation under the effects of potential cytokinin, that is, BAP followed by NAA supported rooting and later acclimatization of the plantlets. Following the example of these early successful tissue culture studies, Bergman et al. (1985)

attempted direct shoot regeneration via axillary bud induction in five different species of *Salix* (*S. schwerinii, S. dasyclados, S. caprea, S. viminalis, S. caprea*) under the influence of BAP augmentation.

Salix caprea (Goat willow) is the most studied species of *Salix* for tissue culture and micropropagation. After Bergman et al. (1985), Neuner and Beiderbeck (1993) and Liesebach and Naujoks (2004) also reported the direct shoot regeneration from nodal segments of *S. caprea*.

Agrawal and Gebhardt (1994) took advantage of the in vitro regeneration strategies to establish the intrageneric hybrid between *S. fragilis* (female) X *S. lispoclados* (male) using ovary culture. The hybrid seedlings were grown in vitro and shoot multiplication was achieved using the nodal segments from them. This study revealed the positive outcomes of Woody Plant Medium augmented with balanced auxin to cytokinin ratio. In 1996, Amo-Marco and Lledo used in vitro direct shoot regeneration for the clonal propagation and conservation of *S. tarraconensis*, an endemic and threatened species of *Salix*. Another important species of *Salix* is *S. nigra* (Black willow) which is the largest and the most commercially important species of willows in North America. Lyyra et al. (2006), using immature inflorescence explants, successfully implemented the direct adventitious shoot formation. This only reported study of tissue culture studies in *S. nigra*. Khan et al. (2011) and Khan and Anis (2012) extensively studied the in vitro regeneration methods for *S. tetrasperma* (Indian willow) using different explants and an array of phytohormone and growth media combinations. For *S. tetrasperma*, a conservation protocol was established by Khan et al. (2018) using in vitro synthetic seeds made of aseptic nodal segments instead of somatic embryos. This study utilized the micropropagation and regeneration protocols established in previous studies by Khan et al. (2011) and Khan and Anis (2012). In the most recent study, Regueira et al. (2018) used liquid immersion techniques for enhanced in vitro multiplication and acclimatization of *S. viminalis* axillary shoots successfully. An incomplete tissue culture study was published by Chornobrov et al. (2019) on direct shoot regeneration of two endangered *Salix* species, *S. retusa* and *S. alpina*. The protocol was incomplete due to the lack of rooting and acclimatization of multiplied in vitro axillary shoots.

6.1.2.2 Callus Cultures

However, a large number of species have been selected for direct micropropagation studies in vitro (Table 6.2), the number of species studied for callus cultures are limited and the successful callus culture establishment in the genus are even lesser. There are very few reports available on successful callus induction in genus *Salix* L. In most instances, the utilization of tissues from both vegetative and reproductive origin was studied and compared for their callus induction and successful proliferation capabilities. The first attempts have been made by Grönroos et al. (1989) for the purpose of indirect organogenesis. They successfully utilized isolated pistils from female catkins of *S. viminalis* for callus culture production followed by somatic embryogenesis. Later in the same year, Stoehr et al. (1989) utilized developing leaves of mature *S. exigua* for successful callus culture establishment. The leaf

explants were inoculated on WPM medium supplemented with high auxin and low cytokinin. However, they reported efficient callus growth but after 7 weeks of incubation, the study indicated the slow growth rate of callus cultures. In the early 1990s, Vahala and Eriksson (1991) studied the protoplast isolation and regeneration from the suspension cultures in *S. viminalis* and *S. shwerinii* using the callus culture protocol from Grönroos et al. (1989); however, the protoplast recovery for fusion or genetic transformation and re-establishment of viable callus cultures was unsatisfactory. They observed the formation of several microcalli, but without the regeneration potential. Skálová et al. (2012) reported successful callus cultures in few species of *Salix*. The group studied various species of *Salix* for the purpose of direct and indirect regeneration of plant tissue from vegetative as well as reproductive organs. The direct organogenesis was successful in most cases, but the tissue culture of reproductive organs showed minimal results. Callus cultures were established from *S. viminalis* leaves as well as isolated anthers, anthers of *S. caprea* and microcalli were observed from ovule cultures of *S. viminalis* and leaf culture of *S. cinerea*. They used combinations of various basal medium, growth regulators and additives for various regeneration studies. For callus culture, induction-modified MS medium augmented with NAA and BA was reported to be most beneficial, but the callus induction efficiency was found to be insignificant. The most recent callus establishment was reported by Shahid and Anis (2018), using inflorescence explants of *Salix tetrasperma* Roxb., also known as 'Indian Willow', to establish non-organogenic callus cultures for secondary metabolite enhancement, in particular, anthocyanin production and elicitation. In this study, MS medium augmented with 2,4-D (1.0 μM) was reported to be optimum for maximum callus proliferation efficiency.

Over an expanse of four decades, only five studies were published for callus cultures in few species of genus *Salix* and four-fifth of these were focused on revival of the differentiated state of the tissue and plant regeneration either via shoot regeneration or somatic embryogenesis and only one-fifth on the secondary metabolite production and enhancement.

6.1.3 Future Strategies

The first requirement for genetic manipulation of a plant species in vitro is an efficient callus induction protocol, followed by regeneration of plants from callus. Biotransformation and enhanced/novel metabolite production also require successful and reproducible callus culture protocols. *Salix* L., the type genus and largest genera of family Salicaceae, lacks both the direct and indirect regeneration protocols in vitro in more than 80% of the species. Very few species have been studied so far and little accomplishments have been made towards novel biotechnological studies in the genera.

Salix L. is an important genus of family Salicaceae and includes a large number of species. It is a potential source of 'salicin' and salicylate derivatives. Till date the genus has been ignored by the scientific community as an alternative herbal source of 'aspirin' – a potent anti-inflammatory drug over chemically synthesized drug.

More focus is demanded in future, to establish an herbal drug line derived from the plant. Various enhancement and biotransformation studies need to be conducted for effective industrial production of the herbal active constituents from *Salix* L., which all need the base of successful and reproducible tissue culture protocols. Establishment of callus culture systems is a prerequisite to initiate an industrial-scale suspension culture system. Various species of the genus need to be screened for in vitro callus culture establishment and the upregulation of 'salicin' and other salicylate derivatives.

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7 An Efficient Plant Regeneration System Via Leaf Derived Callus of *Solanum melongena* L.

Hina Khan

Abstract

An efficient plant regeneration protocol developed of economically important vegetable crop, *Solanum melongena* L., through leaf-derived callus. Leaf explant from 20 days old aseptic seedling used for callus induction on MS (Murashige and Skoog) medium augmented with different concentration of 2,4-D and 2,4,5-T. A light green friable callus from leaf explant was obtained in 7.5 µM (2,4-D) which did not have regenerative potential. Differentiation of adventitious shoot was obtained on culturing the friable callus on MS medium augmented with various concentration of BA and Kinetin alone or in combination. The maximum number of adventitious shoots (65.5 ± 2.02) was obtained from leaf-derived callus on MS basal medium augmented with 2.0 µM BA and 0.1 µM kinetin. The potentiality of differentiation of nodular callus was influenced through callus age. For the induction of roots, the regenerated shoots cultured on MS medium supplemented with 0.5 µM Indole-3-acetic acid (IAA) or on hormone-free MS medium. The in vitro regenerated plantlets were acclimatized and shifted into the field. The in vitro regenerated plantlets were comparable to the parent plants. The developed plant regeneration protocol is a valuable tool in biotechnology for the improvement of eggplant crops in the plant breeding system.

Keywords

Adventitious shoots · Eggplant · Friable callus · Plant regeneration · *Solanum melongena*

H. Khan (✉)
Department of Botany and Environmental Science, Sri Guru Granth Sahib World University, Fatehgarh Sahib, Punjab, India

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7.1 Introduction

Eggplant (*Solanum melongena* L.) is a dominant agronomically crop and cultivated worldwide and provided nutritive as well as medicinal values. The highest production of Solasodine was detected in the young fruits of *S. melongena* (Prajapati et al. 2004) that is the important alternative source of steroidal drug production in pharmaceutical industry. Biotechnology techniques are most important for plant improvement requires the establishment of an efficient improved protocol for plant regeneration. Several reports on somatic embryogenesis and micropropagation reported in eggplant (Kamat and Rao 1978; Matsuoka and Hinata 1979). It is possible that plant tissue culture techniques may add to the solution of several agronomic problems in this crop. Plant regeneration from callus cultures has been reported from a variety of explants in eggplant (Alicchio et al. 1982; Swamy et al. 1988; Rotino et al. 1992; Jyoti et al. 1994; Miyoshi 1996; Franklin et al. 2004; Hossan et al. 2007; Ray and Hassan 2011; Roumiana et al. 2012). Since the information on organogenesis through the process of differentiation of the callus from different explants is not sufficient at hand, the present studies were attempted with the aim of inducing high frequency plant regeneration through de-differentiated callus tissues for its improvement.

7.2 Materials and Methods

7.2.1 In Vitro Seedlings

S. melongena L. seeds (cv. Pusa Purple Round) were soaked overnight and seeds were kept under tap water for half an hour to remove sand particles. Under the laminar air flow, the washed seeds were sequentially decontaminated with 5% (v/v) Teepol (10 min) and 0.1% (w/v) $HgCl_2$ (5 min) and finally five times rinsed with double distilled water. To optimize seedling, the disinfected seeds were cultured on Murashige and Skoog (1962) medium. For callus induction and differentiation, cotyledonary leaves were excised from aseptic seedling and cut up into smaller segments (1–1.5 cm) and cultured on MS medium containing different concentration of auxins 2,4-D, 2,4,5-T and NAA.

7.2.2 Culture Medium and Conditions

Murashige and Skoog (MS) medium contained vitamins, sucrose 3% (w/v) and agar 8% (w/v). The pH of media was fixed at 5.8 with 0.1 N NaOH before autoclaving at 121 °C for 20 min. The culture tubes were kept in the culturing room under appropriate temperature (25 ± 2 °C), photoperiod (16-h), illumination (50 μ mol m^{-2} $s^{-1)}$ provides with fluorescent tubes.

7.2.3 Production of Callus

For callus induction, MS medium alone or with different concentrations (5, 10, 15, 20 and 25 μM) of each 2,4-D, 2,4,5-T and NAA were used. Four weeks after incubation, well-developed callus production was recorded as the percentage of callus induction from leaf explants.

7.2.4 Shoot Differentiation

Light green friable callus was subcultured on MS basal medium (control medium) or MS medium augmented with BA and Kinetin alone or in combination (0.5, 1, 2 and 3 μM) for differentiation of shoot buds. The mean number of shoots and length of shoots induced from the nodular callus were evaluated after 4 weeks in culture.

7.2.5 In Vitro Rooting

For the development of roots from regenerated shoots, single shoot was excised and cultured on hormone-free MS medium or with different concentration of IAA (0.5, 1, 1.5, 2 and 2.5 μM). Data were noted later 3 weeks of culturing of shoots as percentage of rooting from the regenerated shoots, mean number and length of roots.

7.2.6 Acclimatization

For the hardening, the plantlets were excised from the rooting medium and washed with tap water and transferred to paper pots containing sterilized soilrite and covered with perforated polythene bags to maintain high humidity and kept in culturing room and irrigated at 2 days intervals with ½ strength liquid MS medium and bags were removed subsequently later 4 weeks. The saplings were shifted to the earthen pots comprising soil and sustained in green house.

7.2.7 Data Analysis

All the experimentations were repeated twice and 12 tubes were used for every single treatment. The influence of diverse treatments used in each experiment was measured and data were statistically evaluated via SPSS 10 (SPSS Inc., Chicago, USA). Duncan's test at $P = 0.05$ used for the analysis of range values among means.

7.3 Results and Discussion

Differentiation of callus was observed from the margin of leaf on MS medium contained with 10 μM 2,4-D within 15 days of culture. It was observed that the entire leaf segments transformed into callus in 4 weeks (Table 7.1, Fig. 7.1a). Initiation of callus was not perceived on MS (control medium). The light green, friable callus was ineffectual for persuading morphogenesis. Similar results were observed in *S. melongena* L. (Gleddie et al. 1985; Nigra et al. 1989). On medium containing 2,4,5-T generally the least callus formation and growth was observed, while media containing NAA induced callus at low frequency and grew relatively slow with the formation of roots as consistent by Alicchio et al. (1982). Differentiation of adventitious shoots from the friable callus achieved on MS medium augmented with BA and Kinetin alone or in combination. The production of adventitious shoots from the nodular callus on MS medium supplemented with Kinetin was lesser as compared to BA (Table 7.2). Similar morphogenic changes where BA was more effective than kinetin for shoot proliferation have been reported by Gleddie et al. (1985). Highest number of shoots obtained on media comprising 2.0 μM BA and 1.0 μM kinetin (Fig. 7.1b), where the shoot mean number from callus (72.3 ± 2.02) and mean shoot length (4.3 ± 0.29) was noted (Table 7.2, Fig. 7.1c). Superiority of BA and Kinetin combination for induction of shoot has been reported in *Capsicum annuum* (Soniya and Nair 2004). The number of shoots per culture was 2–72 from leaf-derived calluses, respectively (Table 7.2). The different response could be due to the different concentrations of auxin and cytokinins used in the MS medium (Saxena et al. 1997; Patra et al. 1998; Faisal and Anis 2003, 2005). The regenerated adventitious shoots inoculated on hormone-free MS medium or with different concentration of IAA (0.5, 1, 1.5, 2 and 2.5 μM) for induction of roots. Maximum number of roots (8.3 ± 0.23) from regenerated

Table 7.1 Effectiveness of auxins on inducing of callus from leaf explants after 4 weeks in culture of *S. melongena*

Auxin	Concentration (μM)	% Response
		Leaf
2,4-D	0	0
	5	64
	10	100
	15	87
	20	77
	25	65
2,4, 5-T	5	28
	10	50
	15	52
	20	54
	25	64
NAA	5	35
	10	30
	15	24
	20	43
	25	36

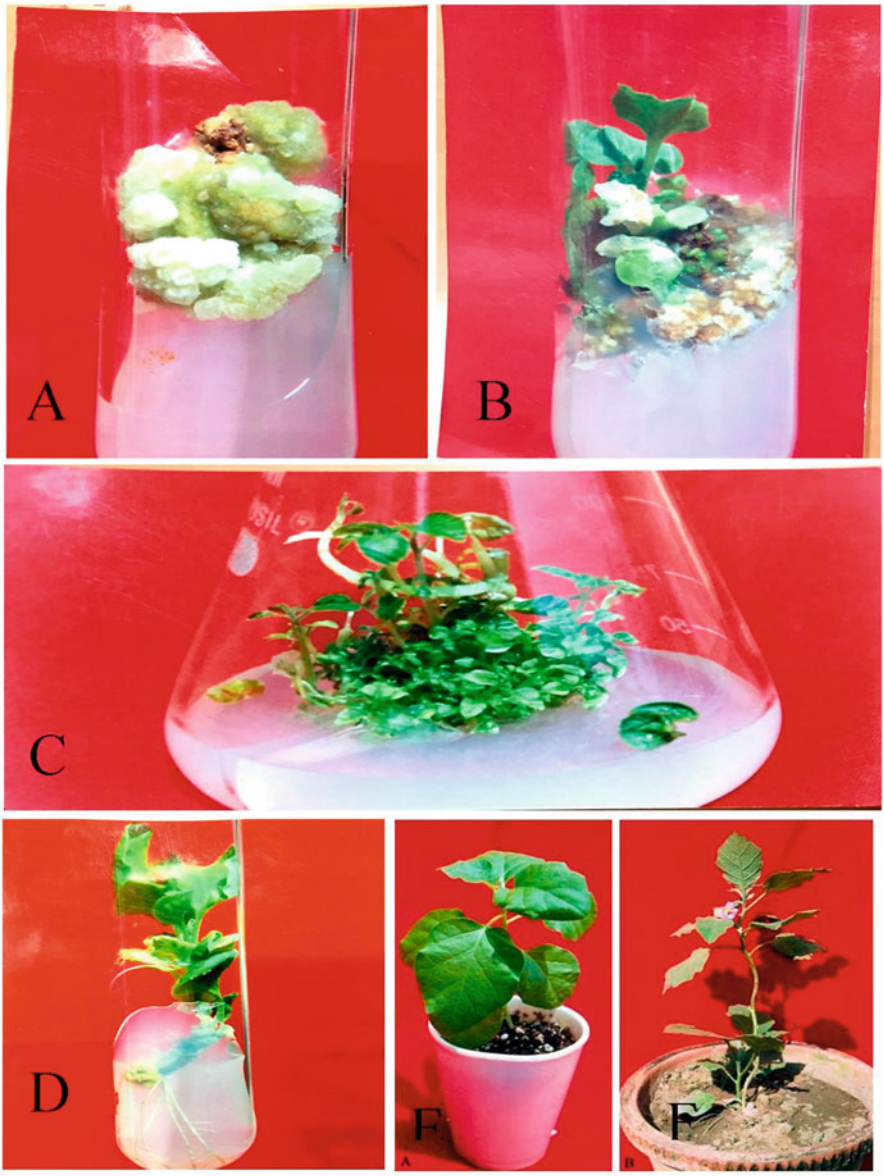

Fig. 7.1 Induction of callus, shoot regeneration and complete plant establishment from leaf explant of *Solanum melongena* L. (**a**) Callus induction from leaf explant on MS + 2,4-D (10 μM). (**b**) Shoot differentiation from organogenic callus on MS + BA (2.0 μM) + Kinetin (1.0 μM). (**c**) Elongation of shoots on MS. (**d**) Rooting of in vitro regenerated shoot on MS medium. (**e**, **f**) A plant acclimatized to greenhouse conditions

Table 7.2 Effect of BA and Kinetin for shoot regeneration from leaf-derived callus of *S. melongena* after 4 weeks of culture

Treatments (μM)		Leaf explants	
BA	Kinetin	Mean number of shoots/callus	Mean shoot length (cm)
0	0	0	0
0.5	0	$5.3 \pm 0.02^{e,f}$	1.5 ± 0.15^{g}
1.0	0	$6.6 \pm 0.33^{e,f}$	$2.0 \pm 0.19^{d,e,f}$
2.0	0	15.0 ± 0.17^{d}	3.4 ± 0.21^{b}
3.0	0	8.4 ± 0.41^{e}	$1.6 \pm 0.28^{f,g}$
0	0.5	2.1 ± 0.16^{f}	$2.3 \pm 0.11^{d,e}$
0	1.0	2.2 ± 0.20^{f}	1.3 ± 0.17^{g}
0	2.0	2.6 ± 0.32^{f}	$1.8 \pm 0.15^{e,f,g}$
0	3.0	3.5 ± 0.32^{f}	$1.6 \pm 0.12^{f,g}$
2.0	0.5	21.6 ± 0.76^{c}	$2.5 \pm 0.26^{c,d}$
2.0	1.0	72.3 ± 2.02^{a}	4.3 ± 0.29^{a}
2.0	2.0	35.3 ± 1.19^{b}	$3.1 \pm 0.20^{b,c}$
2.0	3.0	33.2 ± 1.01^{b}	$2.3 \pm 0.17^{d,e}$

Mean values signify means ± SE. In a column means represent by the identical letter are not considerably different via the Duncan's multiple range test at $P = 0.05$

Table 7.3 Influence of control medium (MS) and IAA for production of roots from regenerated shoots of *S. melongena* after 3 weeks of culture

Treatments (μM)	% Rooting	Mean no. of roots/shoot	Mean root length (cm)
MS	90	8.3 ± 0.23^{a}	3.6 ± 0.24^{a}
MS + IAA (0.5)	50	4.6 ± 0.20^{b}	3.3 ± 0.28^{a}
MS + IAA (1.0)	35	3.6 ± 0.17^{c}	3.0 ± 0.17^{b}
MS + IAA (1.5)	30	2.5 ± 0.20^{d}	2.9 ± 0.18^{b}
MS + IAA (2.0)	20	2.5 ± 0.20^{d}	2.5 ± 0.20^{c}
MS + IAA (2.5)	15	2.0 ± 0.28^{e}	2.1 ± 0.17^{c}

Mean values signify means ± SE. In a column means represent the identical letter are not considerably different via the Duncan's multiple range test at $P = 0.05$

shoot and highest rooting percentage (90%) were obtained on hormone-free MS medium within 3 weeks of culture. Roots were prominent with several root hairs (Table 7.3, Fig. 7.1d). These observations indicate that a particular level of auxin was indeed necessary to induce rooting which was readily available endogenously (Minocha 1987). Although IAA induced rooting, percentage of rooting at 0.5 μM IAA was comparatively low (50%) and roots were thin and delicate. Similar observations were reported in *S. melongena* (Jyoti et al. 1994).

Rooted plantlets were transferred into plastic pots comprising sterilized soilrite for acclimatization (Fig. 7.1e, f). After transplantation in the greenhouse about 85% of plants survived where they attained normal flowering and fruiting.

7.4 Conclusion

In conclusion, *S. melongena* L. used as a model plant due to its high morphogenetic potentiality in tissue culture and useful for the establishment of efficient plant regeneration from leaf-derived callus provides platform for the improvement of crop varieties through biotechnological approaches and somaclonal variations.

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8

Selection of Elite Genotype of a Multipurpose Forest Tree (*Pterocarpus marsupium* Roxb.) from Naturally Grown Populations in Central India

Anees Ahmad, Iram Siddique, and Ankita Varshney

Abstract

In the present study, a total of 50 trees of *Pterocarpus marsupium* were phenotypically identified on the basis of vitality and growth form of stems at ten population sites of different forest regions of central India. Planting materials (fruits/seeds) were collected and aseptically cultured on optimized ½ MS (Murashige and Skoog, Physiol Plant. 15:473–497, 1962) medium in combination with 0.5 μM GA_3 (gibberellic acid) for 21 days and data on in vitro seed germination characteristics were interpreted statistically. Selection of elite genotype of *P. marsupium* was accomplished by the integration of both biotechnological and statistical approaches. Based on the parameters evaluated, the region of central India, Anuppur (MAA) accessions were found to be fast-growing as it showed maximum values of in vitro germination parameters such as percentage germination (PG, 91.3%), speed germination (SG, 29.0 seeds day^{-1}), coefficient germination (CG, 8.23%), mean germination time (MGT, 12.15 days), seedling height (SH, 10.31 cm), vigor index (VI, 941.7), seedling fresh weight (SFW, 4.77 g per 10 seedlings), seedling dry weight (SDW, 0.90 g per 10 seedlings), chlorophyll content (2.03 mg g^{-1} FW of *Chl* a), 0.84 mg g^{-1} FW of *Chl* b, total chlorophyll (2.87 mg g^{-1} FW of T*Chl*) and carotenoids (0.70 mg g^{-1} FW of *Car*), after 21 days of seed germination. Whereas Chhindwara (MCD) accessions were found to be the slowest growing compared to others and exhibited minimum values, that is, 50.3% of PG, 9.7 seeds day^{-1} of SG, 7.37% of CG, 13.56 days of MGT, 6.27 cm of SH, 315.8 of VI, 3.15 g per ten seedlings of SFW, 0.31 g per ten seedlings of SDW, 1.20 mg g^{-1} FW of *Chl* a, 0.47 mg g^{-1} FW of *Chl* b, 1.65 mg g^{-1} FW of T*Chl* and 0.37 mg g^{-1} FW of *Car*, after 21 days of seed germination. The present study demonstrated the successful selection of healthy vegetation which could be used as a planting source material for developing

A. Ahmad (✉) · I. Siddique · A. Varshney
Plant Biotechnology Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, India

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in vitro propagation techniques for obtaining large scale young plantlets, which can be reintroduced in the wild within a comprehensive conservation project.

Keywords

Conservation · Gibberellic acid · In vitro germination · Percentage variation · Woody legume

Abbreviations

B_5	Gamborg medium
Car	Carotenoids
CG	Coefficient germination
Chl	Chlorophyll
GA_3	Gibberellic acid
MGT	Mean germination time
MS	Murashige and Skoog medium
PG	Percentage germination
PGR	Plant growth regulator
PV	Percentage variation
SDW	Seedling dry weight
SFW	Seedling fresh weight
SG	Speed germination
SH	Seedling height
VI	Vigor index
WPM	Woody plant medium

8.1 Introduction

Forest trees provide valuable resources to humankind for economical, environmental, and industrial development, as the human life is sustained directly and indirectly by these resources, supplying a wide range of goods and ecological services essential for survival and prosperity. Medicinal trees are major resources of traditional medicine system and pharmaceutical industries. They provide a big source of revenue and health security of poor communities in developing countries. *Pterocarpus marsupium* Roxb. is one of the most valuable multipurpose forest tree of India commonly known as "Indian Kino or Bijasal." The tree species has received a lot of attention in recent years in experimental as well as conservational studies because it has several evidences of having potential bioactivity. It is used to cure several diseases in traditional ayurvedic and unani medicine system for many centuries (Maurya et al. 2004; Mohire et al. 2007). It also possesses several pharmacological activities like antidiabetic (Dhanabal et al. 2006), antimicrobial and antioxidant (Chakraborty et al. 2010), cardiotonic (Mohire et al. 2007),

anti-inflammatory (Sanders et al. 2005), antioxidant and analgesic (Tippani et al. 2010), anticancer activity (Remsberg et al. 2008), and cyclooxygenase (COX-2) inhibition activity (Hougee et al. 2005). Besides, the National Medicinal Plant Board (NMPB 2018) of India estimated the annual trade value of this tree as 200–-500 metric tonnes/year in India, and each 10–15 years old mature tree produces about 0.5–0.6 tonne of dry heartwood which cost around US $1200–1500 in international trade market. Its heartwood can be utilized for making herbal drug products as well as bridge, boat, and small-scale construction materials (Barstow 2017).

The only source of propagation of the species is seeds contained in winged fruits/pod whose germination is very low due to hard fruit coat, short viability, poor pod set, and pathogenic infection (Anis et al. 2005; Ahmad et al. 2018). Furthermore, species have been overexploited due to its economic trade use by pharmaceutical and herbal drug industries, local tribes for medicine and timber, and this has resulted in its fast disappearance from natural stands (Chand and Singh 2004; Husain et al. 2010). Therefore, in view of restricted distribution, uncontrolled exploitation, and its inherent qualities, there is an urgent need to conserve this valuable forest tree through various biotechnological methods for large-scale plantlets production. However, before developing any propagation protocol one most important aspect is the identification of healthy and elite vegetation of targeted species. For this purpose, we have chosen phenotypically healthy population patches from different geo-climatic region of central India based on their abundance in particular area with straight bole and disease-free.

In the present study, attempts have been made to identify elite genotype among the ten naturally grown populations in the central India by comparing various parameters such as (a) in vitro seed germination such as percentage germination (PG), speed germination (SG), coefficient germination (CG), and mean germination time (MGT), (b) seedling growth like seedling height (SH), vigor index (VI), seedling fresh (SFW), and dry weight (SDW), (c) photosynthetic pigments content, that is, chlorophyll (*Chl* a/b), total chlorophyll (T*Chl*), and carotenoids (*Car*) in in vitro germinated seedlings. The study may help in the identification and selection of superior planting materials prior to the development of an in vitro propagation protocol to be used in tree improvement program.

8.2 Materials and Methods

8.2.1 Study Areas and Plant Material Collection

The winged fruits (Fig. 8.2a) of *P. marsupium* were collected from ten naturally grown forest populations of central India (Table 8.1) between February and March 2016. Each population has more than five tree accessions (or according to availability) and distance between subsequent collected accessions within population was approximately more than 200 m. About 500 g were collected from each tree accession and transported to laboratory in polybags at room temperature, where seeds (Fig. 8.2b) were mechanically excised from the fruits with the help of stripper

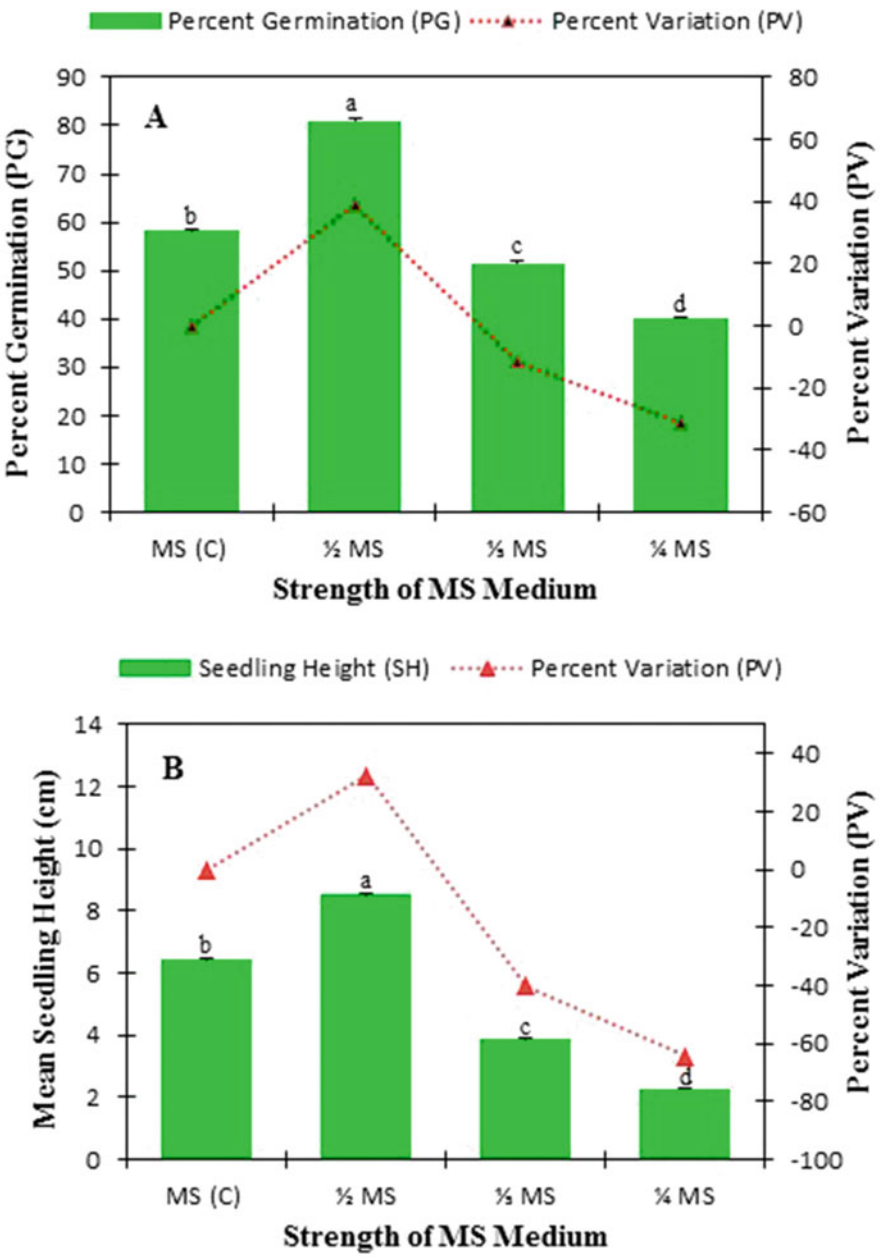

Fig. 8.1 Effect of various strength of MS medium augmented with GA_3 (0.50 μM), after 21 days of seed germination. (**a**) Percentage response of seed germination (**b**) seedling height. Full strength MS medium with optimum dose of GA_3 was considered as control and percentage variation (*PV*) of each treatment was calculated with reference to control (*C*). Bars represent the mean ± SE. Bars denoted by the same letter within response variables are not significantly different ($p = 0.05$) using Duncan's multiple range test

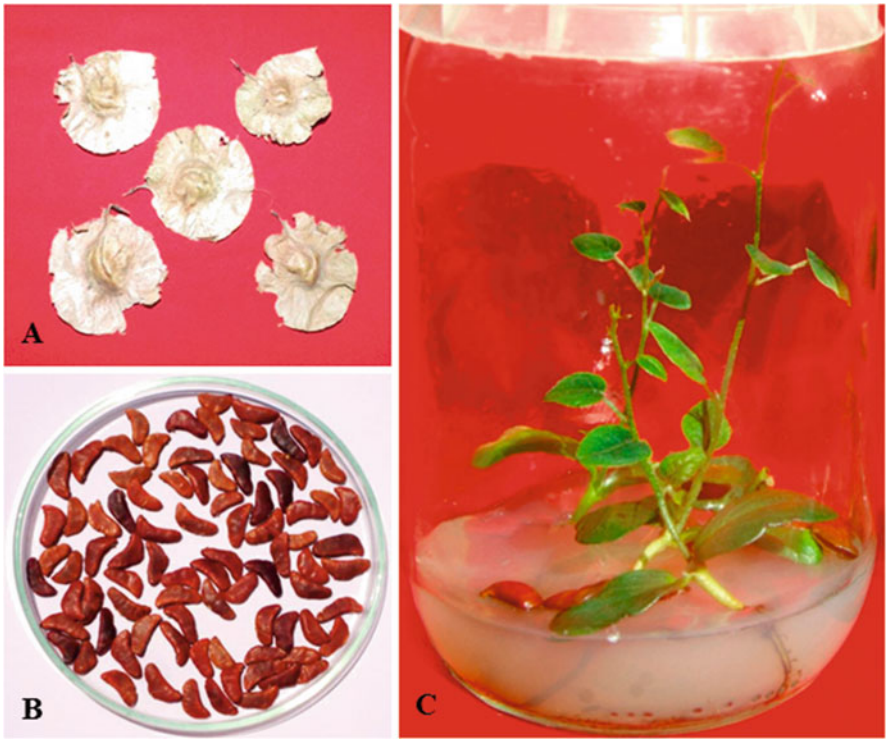

Fig. 8.2 In vitro germination of *Pterocarpus marsupium* seeds collected from Raigarh (CRK) forest region. (**a**) Winged fruits; (**b**) excised seeds; (**c**) 21-day-old seedlings germinated on ½ MS + 0.50 μM GA_3

cutter and thoroughly mixed together in a single lot to obtain compound samples of each population and stored at room temperature until further study.

8.2.2 Establishment of Aseptic Seed Culture Conditions

Surface sterilization was carried out using a two-step procedure. In the first step, seeds were washed thoroughly in running tap water for 15 min, immersed in 1% (w/v) Bavistin® solution for 8 min followed by washing with running tap water for 10 min and later treated with 5% (v/v) Labolene (Qualigens, India), a detergent for 10 min to remove adherent particles from the surface. Thereafter, seeds were kept in sterilized distilled water for imbibition for 24 h. In the second step, the seeds were sterilized with 0.1% (w/v) $HgCl_2$ for 4 min, followed by repeated washes with sterilized distilled water. Disinfected seeds were aseptically inoculated on jam bottles (Vista Biocell, India) or culture tube (25 × 150 mm, Borosil, India) containing 50 and 20 mL sterile culture medium, respectively. The media fortified with 3% (w/v) sucrose (Thermo Fisher Scientific India) as a carbon source, 0.75%

Table 8.1 Details of naturally grown populations of *Pterocarpus marsupium* Roxb. in different forest regions of the Central India

State	Populations				Geographical position		Forest type
	Forest region		Code	Mean GBH (cm)	Latitude (N)	Longitude (E)	
Madhya Pradesh	1	Mandla	MMK	125.0	22° 55′ 58″	80° 12′ 53″	Dry deciduous
	2	Jabalpur	MJH	104.0	22° 50′ 52″	79° 46′ 16″	Dry deciduous
	3	Anuppur	MAA	120.0	22° 40′ 31″	81°43′ 32″	Semidry deciduous
	4	Hoshangabad	MHP	130.0	22° 39′ 23″	78°23′ 03″	Dry deciduous
	5	Betule	MBG	128.0	22° 11′ 18″	77° 29′ 01″	Dry deciduous
	6	Seoni	MSS	122.0	21° 53′ 15″	79° 31′ 48″	Dry deciduous
	7	Chhindwara	MCD	138.0	21° 52′ 35″	78° 43′ 45″	Dry deciduous
	8	Balaghat	MBS	132.0	21° 51′ 46″	80° 21′ 01″	Dry deciduous
Chhattisgarh	9	Surguja	CSA	133.0	23° 03′ 48″	83° 17′ 25″	Dry deciduous
	10	Raigarh	CRK	128.0	22° 23′ 25″	84° 21′ 44″	Semidry deciduous

Where *GBH* Girth at breast height

(w/v) agar (Thermo Fisher Scientific India) as a gelling agent. The pH of media was adjusted to 5.8 with 1 N NaOH prior to autoclaving at 121 °C and 1.06 kg cm^{-2} pressure for 19 min. All culture tubes were incubated at 24 ± 2 °C under 16/8 h (light/dark) cycle with a photosynthetic photon flux density (PPFD) of 50 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes (Philips, India) with 55 ± 5% relative humidity, regularly checked by thermohygrometer (Testo, India Pvt. Ltd.).

8.2.3 Culture Media for Seed Germination and Seedling Growth

Different basal media such as MS, WPM (Lloyd and McCown 1980), and B_5 (Gamborg et al. 1968) singly or in combination with GA_3 at different concentrations (0.1, 0.25, 0.5, 1.0, or 2.0 μM) were examined to detect the best medium where maximum seed germination response was obtained. Various strength (full, ½, $^1/_3$, and ¼) of optimized medium in combination with optimum dose of GA_3 were also evaluated for further improvement in in vitro seed germination. The MS medium without GA_3 was considered as control treatment for calculating percentage variation (PV) of each treatment. Hundred seeds of each treatment and five seeds per jam bottle were inoculated. All experiments were repeated three times. Data on percentage germination and plantlet height were recorded after 21 days of seed culture.

8.2.4 Selection of Elite Genotype

Identification of elite genotype of *P. marsupium* was accomplished by the integration of statistical and biotechnological approaches. The surface-sterilized seeds of ten collected populations were aseptically cultured on optimized seed germination ½ MS medium containing 0.5 μM GA_3. Performance of in vitro seed germination characteristics such as in vitro seed germination, seedling growth, and seedlings photosynthetic pigment contents of ten populations was considered as biological markers. Hundred seeds of each population were cultured in three repeated experiments. All the germination parameters were recorded at 3 days intervals and continued up to 21 days (3, 6, 9, 12, 15, 18, or 21 days; Fig. 8.3). Data on germination characteristics were interpreted statistically after 21 days. The slow-growing population was considered as a control population and percentage variation (PV) of each population was calculated with reference to control. The selection of superior population was done by following parameters using different statistical expressions:

8.2.4.1 Parameters Related to In Vitro Seed Germination

(a) Percentage germination (PG): Percentage germination was calculated according to ISTA (1999) using following formula

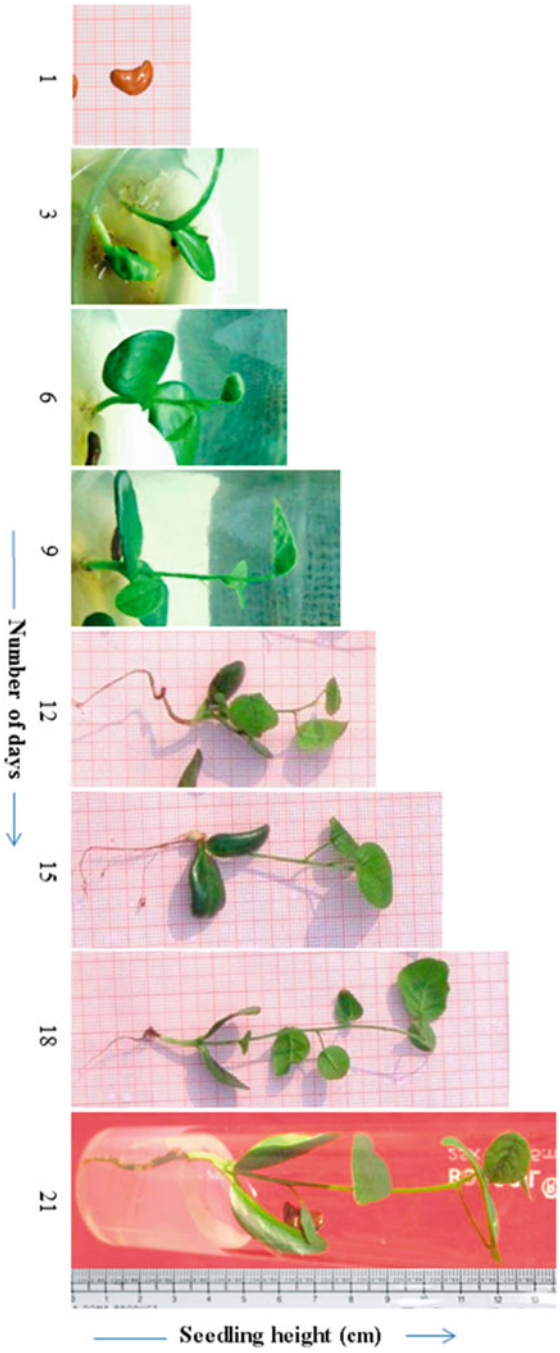

Fig. 8.3 In vitro germination of *Pterocarpus marsupium* seeds collected from Amarkantak (MAA) forest region on ½ MS medium supplemented with GA_3 (0.50 μM) at different days

$$\text{PG} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds cultured}} \times 100.$$

(b) Speed germination (SG): It was calculated by the using formula given by Maguire (1962).

$$\text{SG} = n_1/d_1 + n_2 - n_1/d_2 + \cdots n_x - (n_x - 1)/d_x,$$

where, n = number of germinated seeds, d = number of days, x = number of days to final count.

(c) Coefficient germination (CG): It was calculated by the following formula given by Copeland (1976).

$$\text{CG} = \frac{n_1 + n_2 + \cdots n_x}{n_1 d_1 + n_2 d_2 + \cdots n_x d_x} \times 100,$$

where, n = number of germinated seeds, d = number of days corresponding to n, x = number of days to final count.

(d) Mean germination time (MGT): It was calculated according to Ellis and Roberts (1981) formula.

$$\text{MGT} = \sum fx / \sum f,$$

where, f = seeds germinated on day x.

8.2.4.2 Parameters Related to Seedling Growth

(a) Seedling height (SH): Seedling height (cm) was measured after 21 days of seed culture. The mean height of 10 randomly selected seedlings of each population was recorded.

(b) Vigor index (VI): The value of vigor index was intended according to Abdul-Baki and Anderson (1973) by multiplying germination percentage and average height of ten randomly selected seedling of each population after 21 days of seed culture.

$$\text{VI} = \text{PG} \times \text{Mean seedling height}.$$

(c) Biomass content: mean fresh weight of ten randomly selected seedling of each population was recorded and then kept in shade for 24 h followed by oven drying at 85 °C for 48 h. Thereafter, dried seedlings were weighed to estimate the dry matter production, and the mean values were expressed in gram (g) per ten seedlings.

8.2.4.3 Parameters Related to Photosynthetic Pigment Content

The chlorophyll (*Chl* a/b) and carotenoids (*Car*) pigments were extracted from 21-day-old leaf tissues of ten randomly selected seedling of each population, following the MacKinney (1941) method. About 0.2 g fresh leaves were grounded in 80% (v/v) acetone solution with the help of mortar and pestle and filtered with filter paper (Whatman No. 1). The obtained extract was diluted to a final volume of 10 mL and quantification was performed by spectrophotometer (US-1700 Pharma Spec, Shimadzu, Kyoto, Japan). Optical density (OD) for chlorophyll solution was read at 645, 663 nm and for carotenoids at 480 and 510 nm wavelengths, and calculated using standard formulae. These pigments were expressed as milligram per gram of fresh weight (mg g^{-1} FW). Assessment of photosynthetic pigments and spectrophotometrical readings was repeated thrice. The experiments were conducted three times and data recorded at zero days considered as control for calculating percentage variation.

8.2.5 Statistical Analysis

All experiments were based on randomized block design and repeated three times with ten replicate for each treatment. The data on various parameters were subjected to one-way analysis of variance (ANOVA) using SPSS version 16 (SPSS Inc., Chicago, USA). The significance of differences among means was calculated using Duncan's multiple range tests at $P = 0.05$, and results were expressed as the mean with values parenthesized as percentage variations (PV). The PV was calculated by the following formula

$$\mathrm{PV} = \frac{\mathrm{Treatment} - \mathrm{Control}}{\mathrm{Control}} \times 100.$$

8.3 Results

8.3.1 Optimization of In Vitro Seed Germination Medium

Different basal media (MS, WPM, or B_5) single or in combination with various concentrations of GA_3 was examined for inducing maximum percentage of seed germination and seedling growth of randomly selected CRK (Raigarh) accessions. It showed maximum 58.3% (55.1% over control) seed germination with an average seedling height of 6.43 cm (106.0% over control) recorded on full strength MS containing 0.50 μM GA_3 after 21 days of seed culture (Table 8.2). After selection of appropriate basal media, various strengths of MS medium were also evaluated in combination with optimum concentration of GA_3 (0.50 μM) for further improvement in seed germination parameters. Of these, half-strength MS medium in combination with optimum GA_3 was found most suitable where a maximum 81% (38.9% over

Table 8.2 Effect of different media alone or in combination with GA_3 on seed germination and seedling height of *P. marsupium* after 21 days of seed culture

Media	GA_3 (μM)	Percentage germination		Seedling height (cm)	
		Mean ± SE	(PV)	Mean ± SE	(PV)
MS (C)	0.00	37.6 ± 0.57	$(0.0)^{f}$	3.12 ± 0.02	$(0.0)^{g}$
MS	0.10	40.0 ± 0.69	$(6.5)^{e}$	4.84 ± 0.03	$(55.0)^{c}$
MS	0.25	52.1 ± 0.15	$(38.5)^{b}$	5.39 ± 0.06	$(72.9)^{b}$
MS	0.50	58.3 ± 0.06	$(55.1)^{a}$	6.43 ± 0.06	$(106.0)^{a}$
MS	1.00	46.5 ± 0.12	$(23.7)^{c}$	4.95 ± 0.06	$(58.8)^{c}$
MS	2.00	41.7 ± 0.12	$(10.8)^{d}$	3.74 ± 0.04	$(19.8)^{e}$
WPM	0.00	25.3 ± 0.38	$(-32.6)^{l}$	2.05 ± 0.06	$(-34.4)^{l}$
WPM	0.10	28.4 ± 0.46	$(-24.6)^{j}$	2.56 ± 0.05	$(-17.8)^{j}$
WPM	0.25	31.1 ± 0.15	$(-17.4)^{h}$	3.75 ± 0.07	$(20.1)^{e}$
WPM	0.50	34.3 ± 0.06	$(-8.8)^{g}$	4.25 ± 0.04	$(36.3)^{d}$
WPM	1.00	29.4 ± 0.06	$(-21.8)^{i}$	3.78 ± 0.03	$(21.2)^{e}$
WPM	2.00	26.9 ± 0.12	$(-28.5)^{k}$	3.45 ± 0.04	$(10.7)^{f}$
B_5	0.00	16.0 ± 0.17	$(-57.4)^{o}$	1.38 ± 0.02	$(-55.8)^{m}$
B_5	0.10	20.1 ± 0.37	$(-46.5)^{n}$	2.65 ± 0.02	$(-15.1)^{i,j}$
B_5	0.25	24.7 ± 0.23	$(-34.4)^{l}$	2.93 ± 0.05	$(-6.0)^{h}$
B_5	0.50	27.2 ± 0.23	$(-27.6)^{k}$	3.38 ± 0.04	$(8.3)^{f}$
B_5	1.00	21.0 ± 0.12	$(-44.1)^{m}$	2.76 ± 0.06	$(-11.6)^{i}$
B_5	2.00	19.4 ± 0.12	$(-48.5)^{n}$	2.24 ± 0.05	$(-28.3)^{k}$

Values in parentheses are percentage variation (*PV*). Means values ($n = 30$) followed by the same letter with in column of each treatment are not significantly different ($p = 0.05$) using Duncan's multiple test. (*C*, control)

control) of seed germination (Fig. 8.1a) with an average seedling height of 8.52 cm (32.6% over control) (Fig. 8.1b) was recorded on randomly selected CRK accessions, after 21 days of seed culture (Fig. 8.2c).

8.3.2 In Vitro Seed Germination Parameters

Seeds of ten populations were aseptically cultured on ½ MS medium containing GA_3 (0.50 μM) and data were calculated on in vitro germination, after 21 days of culture. On the basis of germination parameters, MAA population was found to be fast-growing population compared to the others as it showed significantly increased values of germination parameters such as PG of 91.3% (81.5% over control), SG of 29.0 seeds day^{-1} (199.5% over control), and CG of 8.23% (11.6% over control), after 21 days of seed culture (Table 8.3). Also, MAA population showed the lowest value of MGT as 12.15 days (−10.4% over control) than others while MCD population indicates maximum value of MGT on 13.56 days after 21 days of culture (Table 8.3). These values represent fast- and slow-spreading capacity of populations, respectively (Table 8.3). On the other hand, MCD population was found to be

slowest growing population compared to others and exhibited minimum values, that is, PG as 50.3%, SG as 9.7 seeds day^{-1}, and CG as 7.37% (Table 8.3).

8.3.3 Seedling Growth Parameters

Different parameters of seedling growth were evaluated in different populations after 21 days of seed germination. Among the different populations, MAA was found to be fast-growing seedlings as it showed significantly increased values of different parameters such as an average SH of 10.31 cm (64.3% over control), VI of 941.7 (198.2% over control), SFW 4.77 g (51.3% over control) per ten seedlings, and SDW of 0.90 g (187.2% over control) per ten seedlings after 21 days of seed germination (Table 8.4; Fig. 8.3). On the other hand, the least growing seedlings of MCD population showed minimum values of various parameters, that is, SH of 6.27 cm, VI of 315.8, SFW of 3.15 g per ten seedlings, and SDW of 0.31 g per ten seedlings, after 21 days of seed germination (Table 8.4).

8.3.4 Photosynthetic Pigment Contents

The comparative analysis of different photosynthetic pigments chlorophyll (*Chl* a/b, T*Chl*, and *Car*) was evaluated in 21-day-old seedlings of different population. The photosynthetic pigments content of MAA population showed a significantly higher concentration than seedlings of other populations (Table 8.5). Seedlings of MAA populations showed maximum values of *Chl* a 2.03 mg/g of FW (68.9% over control), *Chl* b 0.84 mg/g of FW (80.7% over control), T*Chl* 2.87 mg/g FW (73.3% over control), and *Car* 0.70 mg/g of FW (90.9% over control) whereas minimum values of *Chl* a 1.20, *Chl* b 0.47, T*Chl* 1.65, and *Car* 0.37 mg/g of FW were recorded in seedlings of MCD population (Table 8.5).

8.4 Discussion

Forest tree species are extremely important for the world economy as well as for maintaining and conservation of nature and natural resources. Many countries, including India, are facing acute problems in maintaining forest tree resources because of their uncontrolled exploitation through anthropogenic activities and more recently climate change. These activities have resulted in the sharp decline in the population of many plant species. According to International Union for Conservation of Nature (IUCN 2018), *P. marsupium* is an internationally threatened tree species as it is being continuously overexploited by several agencies and now has reached at the edge of extinction. The mass clonal propagation of elite species is necessary to meet growing demands for forest products and reforestation as well as application in tree improvement programs (Husain et al. 2010; Ahmed et al. 2017).

Table 8.3 Seed germination parameters evaluated after 21 days of seed culture of different populations of *P. marsupium*

	Populations	PG (%)		SG (seeds day^{-1})		CG (%)		MGT (days)	
S. N.	code	Mean ± SE	(PV)	Mean ± SE	(PV)	Mean ± SE	(PV)	Mean ± SE	(PV)
1	MMK	53.0 ± 0.58	(5.3)^{i}	11.0 ± 0.05	(13.4)^{i}	7.52 ± 0.02	(1.9)^{i}	13.30 ± 0.04	(−1.9)^{b}
2	MJH	62.0 ± 0.58	(23.2)^{f}	14.8 ± 0.03	(52.8)^{f}	7.76 ± 0.01	(5.3)^{f}	12.88 ± 0.02	(−5.0)^{e}
3	MAA	91.3 ± 0.33	(81.5)^{a}	29.0 ± 0.19	(199.5)^{a}	8.23 ± 0.01	(11.6)^{a}	12.15 ± 0.01	(−10.4)^{j}
4	MHP	65.0 ± 0.58	(29.1)^{e}	16.4 ± 0.16	(69.0)^{e}	7.83 ± 0.00	(6.1)^{e}	12.78 ± 0.00	(−5.8)^{f}
5	MBG	55.0 ± 0.58	(9.3)^{h}	12.1 ± 0.16	(25.4)^{h}	7.58 ± 0.01	(2.8)^{h}	13.20 ± 0.02	(−2.7)^{c}
6	MSS	72.3 ± 0.33	(43.7)^{d}	19.8 ± 0.11	(104.5)^{d}	7.97 ± 0.01	(8.1)^{d}	12.55 ± 0.01	(−7.4)^{g}
7	MCD (C)	50.3 ± 0.33	(0.0)^{j}	09.7 ± 0.05	(0.0)^{j}	7.37 ± 0.01	(0.0)^{j}	13.56 ± 0.03	(0.0)^{a}
8	MBS	78.0 ± 0.58	(55.0)^{c}	22.6 ± 0.11	(133.4)^{c}	8.06 ± 0.01	(9.3)^{c}	12.41 ± 0.01	(−8.5)^{h}
9	CSA	60.0 ± 0.58	(19.2)^{g}	13.5 ± 0.04	(39.8)^{g}	7.69 ± 0.02	(4.2)^{g}	13.01 ± 0.04	(−4.1)^{d}
10	CRK	81.0 ± 0.58	(60.9)^{b}	24.2 ± 0.04	(149.4)^{b}	8.10 ± 0.01	(9.9)^{b}	12.34 ± 0.02	(−9.0)^{i}

Values in parentheses are percentage variation (*PV*). Means ± SE values ($n = 3$) or three repeats of 100 seeds of each population followed by the same letter within column of each population are not significantly different ($p = 0.05$) using Duncan's multiple test. (*C*, Control; *PG*, percentage germination; *SG*, speed germination; *CG*, coefficient germination; *MGT*, mean germination time)

Table 8.4 Seedling growth parameters evaluated in different populations of *P. marsupium* after 21 days of seed germination

	Populations	SH (cm)		VI		SFW (g per 10 seedlings)		SDW (g per 10 seedlings)	
S. N.	code	Mean ± SE	(PV)	Mean ± SE	(PV)	Mean ± SE	(PV)	Mean ± SE	(PV)
1	MMK	06.52 ± 0.01	$(3.9)^{g}$	345.6 ± 3.62	$(9.4)^{i}$	3.19 ± 0.01	$(1.4)^{h,i}$	0.34 ± 0.02	$(8.5)^{g}$
2	MJH	06.90 ± 0.04	$(10.0)^{f}$	428.0 ± 2.33	$(35.5)^{f}$	3.40 ± 0.01	$(8.0)^{f}$	0.40 ± 0.01	$(28.7)^{f}$
3	MAA	10.31 ± 0.03	$(64.3)^{a}$	941.7 ± 6.00	$(198.2)^{a}$	4.77 ± 0.02	$(51.3)^{a}$	0.90 ± 0.02	$(187.2)^{a}$
4	MHP	07.28 ± 0.03	$(16.0)^{e}$	473.0 ± 6.05	$(49.8)^{e}$	3.52 ± 0.02	$(11.9)^{e}$	0.47 ± 0.00	$(51.1)^{e}$
5	MBG	06.58 ± 0.02	$(4.9)^{g}$	362.1 ± 2.99	$(14.7)^{h}$	3.24 ± 0.01	$(2.9)^{h}$	0.34 ± 0.02	$(8.5)^{g}$
6	MSS	07.68 ± 0.02	$(22.5)^{d}$	555.8 ± 3.26	$(76.0)^{d}$	3.76 ± 0.01	$(19.4)^{d}$	0.53 ± 0.00	$(68.1)^{d}$
7	MCD (C)	06.27 ± 0.01	$(0.0)^{h}$	315.8 ± 2.06	$(0.0)^{j}$	3.15 ± 0.01	$(0.0)^{i}$	0.31 ± 0.01	$(0.0)^{g}$
8	MBS	08.05 ± 0.04	$(28.4)^{c}$	628.2 ± 5.38	$(98.9)^{c}$	3.92 ± 0.05	$(24.4)^{c}$	0.64 ± 0.01	$(103.2)^{c}$
9	CSA	06.85 ± 0.02	$(9.2)^{f}$	411.0 ± 3.31	$(30.2)^{g}$	3.32 ± 0.02	$(5.4)^{g}$	0.40 ± 0.01	$(26.6)^{f}$
10	CRK	08.52 ± 0.01	$(35.8)^{b}$	690.1 ± 5.86	$(118.6)^{b}$	4.02 ± 0.02	$(27.7)^{b}$	0.67 ± 0.01	$(113.8)^{b}$

Values in parentheses are percentage variation (*PV*). Means ± SE values ($n = 3$) or three repeats of ten randomly selected seedlings of each population followed by the same letter within column of each population are not significantly different ($p = 0.05$) using Duncan's multiple test. (*C*, control; *SH*, seedling height; *VI*, vigor index; *SFW*, seedling fresh weight; *SDW*, seedling dry weight)

Table 8.5 Estimation of photosynthetic pigments in 21-day-old axenic seedlings of different populations of *P. marsupium*

S. N.	Populations code	*Chl* a (mg/g of FW) Mean ± SE	(PV)	*Chl* b (mg/g of FW) Mean ± SE	(PV)	T*Chl* (a + b) (mg/g of FW) Mean ± SE	(PV)	*Car* (mg/g of FW) Mean ± SE	(PV)
1	MMK	1.29 ± 0.02	$(7.8)^{h}$	0.53 ± 0.03	$(14.3)^{d,e}$	1.80 ± 0.04	$(8.7)^{g}$	0.40 ± 0.01	$(9.1)^{e,f}$
2	MJH	1.58 ± 0.01	$(31.4)^{e}$	0.62 ± 0.04	$(32.8)^{c,d}$	2.18 ± 0.04	$(31.9)^{e}$	0.49 ± 0.02	$(33.6)^{c,d}$
3	MAA	2.03 ± 0.03	$(68.9)^{a}$	0.84 ± 0.01	$(80.7)^{a}$	2.87 ± 0.03	$(73.6)^{a}$	0.70 ± 0.01	$(90.9)^{a}$
4	MHP	1.69 ± 0.02	$(40.8)^{d}$	0.62 ± 0.02	$(32.1)^{c,d}$	2.31 ± 0.00	$(39.5)^{d}$	0.52 ± 0.04	$(42.7)^{b,c,d}$
5	MBG	1.36 ± 0.02	$(13.3)^{g}$	0.53 ± 0.03	$(14.3)^{d,e}$	1.88 ± 0.04	$(13.9)^{g}$	0.40 ± 0.01	$(10.0)^{e,f}$
6	MSS	1.82 ± 0.01	$(51.9)^{c}$	0.70 ± 0.01	$(50.0)^{b,c}$	2.52 ± 0.01	$(52.6)^{c}$	0.55 ± 0.02	$(50.9)^{b,c}$
7	MCD (C)	1.20 ± 0.02	$(0.0)^{i}$	0.47 ± 0.03	$(0.0)^{e}$	1.65 ± 0.02	$(0.0)^{h}$	0.37 ± 0.03	$(0.0)^{f}$
8	MBS	1.88 ± 0.02	$(56.4)^{b,c}$	0.72 ± 0.01	$(55.0)^{b}$	2.60 ± 0.04	$(57.3)^{b,c}$	0.58 ± 0.02	$(57.3)^{b}$
9	CSA	1.50 ± 0.01	$(25.0)^{f}$	0.57 ± 0.03	$(21.4)^{d}$	2.07 ± 0.03	$(25.4)^{f}$	0.45 ± 0.04	$(22.7)^{d,e}$
10	CRK	1.90 ± 0.02	$(58.1)^{b}$	0.74 ± 0.02	$(58.6)^{b}$	2.64 ± 0.04	$(59.5)^{b}$	0.59 ± 0.03	$(61.8)^{b}$

Values in parentheses are percentage variation (*PV*). Means values ($n = 3$) or three repeats of randomly selected seedlings of each population followed by the same letter within column of each population are not significantly different ($p = 0.05$) using Duncan's multiple test. (*C*, control; *Chl*, Chlorophyll; T*Chl*, total chlorophyll; *Car*, carotenoids)

Seed germination refers to the emergence of root or shoot from the seed coat, while emergence is the visible penetration of the shoot above the medium surface (Kader 2005). The seed germination experiment helps the researchers to determine if the seeds are suitable for planting. Studies regarding the seed germination are important for obtaining suitable planting materials for in vitro propagation (Ranal et al. 2009; Gairola et al. 2011; Singh and Kumar 2014; Trivedi and Joshi 2014). During in vitro germination practice, the optimization of an appropriate culture medium alone or in combination with appropriate plant growth regulators (PGRs) has provided useful information toward in vitro seed germination. In the present study, the ½ MS medium containing 0.5 μM GA_3 was found to be the most effective treatment and showed 81% of germination response in Raigarh (CRK) accessions. Augmentation of GA_3 in culture media has been reported in many plant species for improving germination along with better elongation of plantlets (Varshney et al. 2013; Hawkins 2018). It is also reported that GA_3 regulated the synthesis of α-amylase in aleuronic layer of seeds by upregulating α-amylase gene, SLN1 and GAMYB transcription factors promoting germination (Gubler et al. 2002), and DELLA-mediated inhibition of BZR1 transcription factor promotes plantlet elongation (Li and He 2013). The increased concentration of GA_3 favored to rupture the testa and endosperm. In many scientific reports, the plant species have developed a huge morphological and physiological diversity in seed types (Leugner et al. 2009; Deligoz 2012). Understanding the process of seed germination as a whole from early seed germination to the establishment of seedling can help plant breeders to choose better and healthier plant accessions (Ranal et al. 2009; Gairola et al. 2011).

In vitro germination and seedling growth parameters of our study showed that accessions of Amarkantak (MAA) were fast-growing compared to other populations. Analysis of germination can provide valuable information about the percentage rate, uniformity, and mean germination time (MGT) of seeds per day. By knowing the germination parameters, a researcher can adjust the planting rates to attain the desired plant population in the field. Thus, germination is considered to be a qualitative developmental response of populations, however, germination time varied among different accessions. The coefficient of germination gives an indication of the rapidity of germination of a population (Thomson and El-Kassaby 1993). It increased when number of germinated seed increases and time required for germination decreases (Jones and Sanders 1987; Oliveira and Garcia 2011). However, mean germination time is an accurate measure of time taken for a population seed to germinate, but does not correlate this well with the time spread or uniformity of germination (Matthews and Khajeh-Hosseini 2006, 2007). In the present study, MAA accession has attained minimum values of mean germination time while Chhindwara (MCD) accession showed maximum value of MGT. Our studies on germination, seedling height, biomass content, and vigor index of ten populations clearly correlated the growth pattern of all the accessions showing that MAA accession was fast-growing, while MCD accession showed slow growth in central Indian forest region. The study might help in the selection of superior mother plants prior to the development of an in vitro propagation protocol for producing healthy

and disease-free plantlets that can be used for reforestation and sustainable consumption purposes.

8.5 Conclusion

To conclude, half-strength MS media in combination with GA_3 was found to be most effective on enhancement of maximum seed germination with an average seedling height. On the basis of seed germination parameters and seedling growth performance, Amarkantak (MAA) population was found to be significantly fast grown and Deogarh (MCD) was a slow grown population in central Indian forest region. The order of superiority pattern of ten populations on the basis of germination parameters and seedling growth performance was recorded as MAA > CRK > MBS > MSS > MHP > MJH > CSA > MBG > MMK > MCD in this study. To our knowledge, this is the first report to demonstrate the identification/selection of elite genotype of *P. marsupium* among the naturally grown ten populations via in vitro seed germination and seedling growth performance. The study may surely help in the selection of superior mother plants prior to develop an in vitro propagation protocol to be used in tree improvement program. It may also be helpful in producing healthy and disease-free plantlets that can be used for reforestation and sustainable consumption purposes.

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9 Regulation of In Vitro Morphogenesis by Modulation of Culture Conditions in *Withania somnifera* L. Using Cotyledonary Node Explants

Nigar Fatima and M. Anis

Abstract

Optimization of culture conditions has been proposed for high-frequency shoots regeneration by in vitro propagation of cotyledonary node segments (CN) attained from 7-day-old aseptic seedlings in a highly valuable medicinal plant—*Withania somnifera* L. Micropropagation was accomplished from CN explants as bared to diverse range of cytokinins viz; 6-benzyl adenine (BA), kinetin (Kn), or 2-isopentenyl adenine (2iP) individually or in combined arrangement with various auxins namely indole 3-acetic acid (IAA), indole-3-butyric acid, or α-naphthalene acetic acid (NAA) in Murashige and Skoog medium. Bud break was dependent on cytokinin supply and the maximum rate (95%) of regeneration with highest number of shoots (13.0 ± 0.00) and shoot length (2.00 ± 0.36 cm) was evident on MS medium composed of BA (2.5 μM), after 4 weeks of incubation. However, the synergistic combination of cytokinin, BA (2.5 μM) along with auxin NAA (0.5 μM) encouraged maximum number (29.3 ± 0.23) of shoots and shoot length (5.62 ± 0.17 cm) with 90% regeneration frequency after 8 weeks of culture. After 4 weeks, the greatest number 12.6 ± 0.10 of roots with root length of 3.58 ± 0.16 cm/shoot with 95% regeneration frequency were attained on medium fortified with NAA (0.5 μM). The results highlight the potential conservation approaches for constant furnish of plant materials for both local and escalating markets as well as safety evaluation on sustainable usage of the species for medicinal purposes.

Keywords

Acclimatization · Cotyledonary node explants · Micropropagation · Morphogenesis · Plant growth regulators · *Withania somnifera*

N. Fatima (✉) · M. Anis
Plant Biotechnology Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, India

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Abbreviations

2-iP 2-Isopentenyl adenine
BA 6-Benzyladenine
CN Cotyledonary node
IBA Indole-3-butyric acid
Kin 6-Furfurylaminopurine
MS Murashige and Skoog's medium
NAA α-Naphthalene acetic acid

9.1 Introduction

Ashwagandha (*Withania somnifera* L.), a recognized medicinal plant sharing numerous health prosperity and well-being, a vital "Rasayana" marketed as "Sattvic Kapha Rasayana" (Indian ayurveda) since centuries designed for its incredible benefits (Mahima et al. 2012). A diverse spectrum of phytochemicals present in plant facilitates it to hold an extensive assortment of biological implications. Roots, stems, leaves, and fruits/berries of plant show unique medicinal properties approved to their assorted metabolites (Bharti et al. 2011; Bhatia et al. 2013). Extensive range of therapeutic activities is corroborated by the existence of steroidal lactones present in plant roots, which promote and maintain general health. Immunomodulation, anticancer and antiepileptic, memory enhancer, mood elevator, diuretic, rejuvenator, stress reliever, antiaging, antioxidant, hypoglycemic, adaptogen, and so on, are major health benefits and veterinary sciences focus on its potent function in maintaining sound health and therapeutic usages (Tiwari et al. 2014). Ashwagandha's extensive pharmacological activities prepared the plants as an essential constituent of polyherbal preparations. A number of central preparations like Asvagandharishtam, chavanaprasam, Valiya Narayana tailam, and so on, using the drugs are available in the market. However, conventional cultivation via seed is not sufficient to hold the flourishing requirement of *Withania* for medicament needs.

Withania propagates via seeds but low seed germination percentage, seed viability period, and shrinkage of innate potentials for vegetative propagation (Sen and Sharma 1991; Farooqi and Sreeramu 2004), resulted in deterioration of plant materials, making the plant at a risk of extinction in wild, hindrance in plant cultivation and depletion in market values. Pharmaceutical corporations are unearthing this medicinal herb from the nature, creating a huge pressure to its existence as well as troubling environmental stability. This plant species is also incorporated in the list of endangered species by International Union for Conservation of Natural Resources (Supe et al. 2006; Patel and Krishnamurthy 2013).

Modern plant improvement programs (biotechnological inventions) include in vitro tissue culture practices designed for aseptic plants production, introduction of novel traits into preferred plants, multiplication of elite selections, and development of appropriate cultivars in the small duration (Anis and Ahmad 2016). In vitro manipulations and propagation of plants from juvenile seedlings containing

cotyledonary node (CN) as an explant have earned significant attentions currently because of its simple accessibility, speedy response, and immense potential in favor of shoot organogenesis, somatic embryogenesis, culture of protoplast, and transformation comprehension (Tabei et al. 1991; Compton et al. 1994).

Phytohormones (auxins and cytokinins) recognized as the key signal molecules in the development and growth of plants (Moubayidin et al. 2009). Cytokinins activate the release of dormant shoot sprouts. In general, different cytokinins articulate different events in manipulating in vitro axillary shoot formation (Preece and Shutter 1991). In vitro cultures of plant cells, tissues, and organs are not exclusively autotrophic; ascertain requirements for continuous supply of energy sources (carbohydrate) in culture media which acts as energy sources and also maintains osmotic potential (Lipavaska and Konradova 2004). Moreover, various developmental processes like shoot proliferation, root induction, organogenesis, embryogenesis are highly energy-requiring procedures in plant biology, occur at the expense of carbon sources (De Klerk and Calamar 2002). pH of medium is an essential factor of physiochemical background throughout the progress of plant tissues, organs in vitro (Williams et al. 1990). Optimum pH is imperative for improved plant growth and expansion, the suboptimal pH levels leading to abnormalities during explants development (Anderson and Levinsh 2008).

Till date, only a single report is available for *W. somnifera* propagation and regeneration in vitro utilizing CN explants (Nayak et al. 2012). However, the report did not provide comprehensive studies on the collective outcomes of plant growth regulators, media types, carbon supply, and pH factors on plants regeneration system. Therefore, contemplating the need and relevance of the plant, the current experiment was designed to formulate a refined, resourceful, and reproducible regeneration system in Ashwagandha by optimizing various parameters like plant growth regulators (PGRs), media types, carbon sources, and medium pH.

9.2 Materials and Methods

9.2.1 Seed Germination and Preparation of Explants

Seeds of *W. somnifera* were procured from superior genotypes established at the Botanical Garden of the Aligarh Muslim University, Aligarh. Thorough washing of seeds was carried out for 30 min under running tap water in order to eliminate adherent particles. The seeds were treated with [5% (v/v)] freshly prepared Labolene, a liquid detergent (Qualigens, Mumbai, India), for 20 min and finally rinsed with distilled water (sterile DW). The surface sterilization was undertaken with [0.1% (w/v)] mercuric chloride ($HgCl_2$) solution (Qualigens, India) for 5 min and lastly washed five times with sterile distilled water for the removal of sterilant traces before implantation. The sterilized seeds were aseptically germinated on different strength (full, 1/2, 1/3, and 1/4) of Murashige and Skoog (1962) medium to raise aseptic seedlings. Cotyledonary nodes (7 day old) were used as explants in the experiments.

9.2.2 Culture Media and Conditions

In the experiments, Murashige and Skoog (MS) medium fortified with sucrose 3% (w/v) and a bacteriological grade agar (Hi-media) 0.8% (w/v) was employed. 5.8 medium pH was adjusted by 1 N NaOH/1 N HCl prior to autoclaving the culture media at pressure (121 °C at 1.06 kg cm^{-2}) for 20 min. The entire cultures were kept in culture room with standard environmental conditions of temperature, humidity, and photoperiod duration, which includes photoperiod (16 h), photosynthetic photon flux density (PPFD) (50 μmol m^{-2} s^{-1}), 40 W fluorescent tubes (Phillips, India), 50–60% relative humidity under 24 ± 2 °C.

9.2.3 Induction and Multiplication Shoot

Seven-day-old aseptic CN explants containing axillary buds were placed on different concentrations (1.0, 2.5, 5.0, 7.5, and 10.0 μM) of various cytokinins (BA, Kn, and 2iP) alone or else in mixture with diverse auxins (IAA, IBA, and NAA) at a range of concentrations (0.5, 1.0, and 2.0 μM) in MS medium for shoot bud initiation and regeneration. Multiplication of shoot and stable/constant establishment, the regenerating tissues were recurrently subcultured on the regenerative medium [MS + BA (2.5 μM) + NAA (0.5 μM)] after every 6 weeks.

9.2.4 Effect of Different Media, pH, and Carbon Sources

CN segments were cultured on diverse basal media specifically MS (Murashige and Skoog 1962), B_5 medium (Gamborg et al. 1968), L_2 (Phillips and Collins 1979), and WPM (Lloyd and McCown 1981), carbon sources (glucose, sucrose, and fructose) along with media pH (4.2, 5.2, 6.4, and 7.0) containing most favorable concentration of BA (2.5 μM) plus NAA (0.5 μM) for greatest shoot regeneration and proliferation. Subculturing of cultures was carried out onto fresh media at 3 weeks interval. Explants frequency, quantity of shoot/explants with shoot length after 8 weeks of culture conditions were recorded regularly.

9.2.5 Rooting and Acclimatization

In vitro rooting was carried out through the excision and transfer of (3–4 cm) long microshoots on rooting medium containing various auxins applications (0.1–2.0 μM) viz., indole-3-acetic (IAA), indole-3-butyric acid (IBA), and α-naphthalene acetic acid (NAA). The rooted plantlets were potted in plastic pots containing sterile soilrite™ (Keltech, India Ltd., Bangalore). The potted plantlets were covered with transparent polythene bags to guarantee elevated humidity, also watered with half-strength MS salts upto 2 weeks, and placed below 16/8 h light photoperiod environment. Toward the process of plantlets acclimatization and

hardening, the polythene bags were opened frequently. Further, following 4 weeks period, the adapted plants were shifted to earthen pots holding regular garden soil, sustained in a net house under innate light. Rooting percentage, root numbers with root length data were documented prior to relocating into soil.

9.2.6 Data Collection and Statistical Analysis

A minimum of 20 replicates/treatments with three times repetition of experiment were carried out. The periodical and morphological modification in the cultures was observed and documented at regular period. Statistical analysis of the data was prepared employing SPSS version 16 (SPSS Inc., Chicago, USA) and the significance of differences was conceded out using Duncan's multiple range test (DMRT) at $P \leq 0.05$. The outcome was articulated as: means $\pm$ SE for all the experiments.

9.3 Results and Discussion

The implementation of plant cell, tissue, and organs in culture with significant application in the regulation of morphogenesis in plants is highly dependent on the utilization of appropriate PGR regimes, explants type, different factors like media types, pH, sucrose, light, temperature, and so on (Rout et al. 2000; Ahmad and Anis 2010; Fatima et al. 2015). Explants establishment is a very crucial stage for in vitro propagation and development. Plant growth regulators (PGRs) include a large arrangement of molecules that manipulate physiological and biological processes of higher plants at low concentrations (George et al. 2008). In general, these compounds have a regulatory role in plant growth, differentiation, and development. In vitro morphogenetic outcomes are largely governed by the presence and relative abundance of PGRs supplemented in culture media through complex pathways interacting with one another via multiple levels of control and regulation.

9.3.1 Establishment of Aseptic Seedlings

The surface-sterilized seeds were inoculated on different strengths (full, 1/2, 1/3, and 1/4) of MS medium for germination. Half-strength MS medium was found to be superior over other strengths. Maximum percentage (86.2%) of germination was recorded on ½ strength Murashige and Skoog medium, after 4 weeks of incubation (Fig. 9.1). On reducing the strength to 1/3 and 1/4, the germination response was found to be declined (Fig. 9.1). Seven-day-old CN excised from aseptic sapling was utilized as explants material.

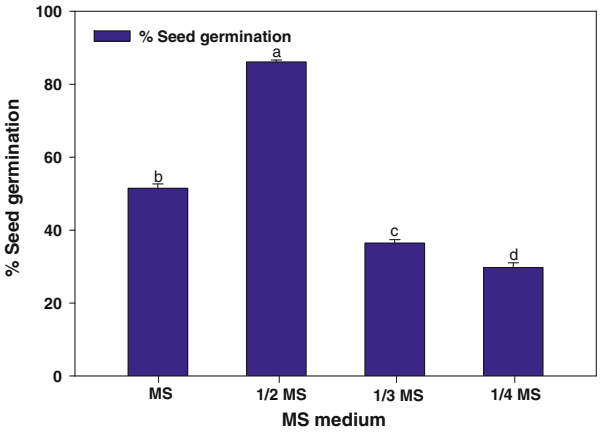

Fig. 9.1 Effect of different strength of MS medium on in vitro germination of *W. somnifera* seeds, after 4 weeks of culture. Bars represent means ± SE. Bars denoted by the same letter within response variables are not significantly different ($P = 0.05$) using Duncan's multiple range test

9.3.2 Implication of PGRs on Shoot Regeneration Outcome

CN explants did not respond morphogenetically to MS medium lacking growth regulator but once cultivated on medium complementing with different cytokinins namely BA, Kin, and 2iP (0.0–10.0 μM), a discrepancy in response was monitored (Table 9.1). The cotyledonary node induction is proved to be an efficient micropropagation method in plant as the emerging buds commencing from meristematic organs and tissues possess an immense potential for dynamic development (Yadav et al. 1990). Maximum rate of shoot sprout initiation was attained on Murashige and Skoog medium fortified with BA (2.5 μM), which gave maximum shoots (13.0 ± 0.00), (18.3 ± 0.20) with shoot length (2.00 ± 0.36 cm), (4.20 ± 0.12 cm) respectively, following 4 and 8 weeks of culture conditions (Table 9.1; Fig. 9.5a). For shoot stimulation and propagation in plant tissue culture system of numerous medicinal herbs, the superiority of BA over other cytokinins has been well discussed (Fatima et al. 2012; Faisal et al. 2018). A negative outcome with decline in regeneration frequencies and shoot bud formation from each explant is exhibited with an increase in BA concentration beyond the optimal level (2.5 μM). Cytokinin has been accounted for stimulating the activities and initiation of axillary meristems in the direction of shoot development. Medium having 2.5 μM Kin stimulated maximum shoot number (11.0 ± 0.00; 14.2 ± 0.20) with shoot length (1.36 ± 0.00; 3.50 ± 0.00 cm), after 4 and 8 weeks, respectively (Table 9.1). Whereas, 2iP augmented medium upshot in highest figure of shoots (10.8 ± 0.30; 12.4 ± 0.40) with shoot length (2.40 ± 0.22; 4.00 ± 0.00 cm) after 4 and 8 weeks of culture, respectively (Table 9.1). On elevated concentration of cytokinin, reduced shoot number and shoot length were examined. Whereas, on lowering the cytokinin application, stunted and malformed shoots were observed. The results obtained are in consonance with earlier findings in plants like *W. somnifera* (Fatima and Anis 2012) and *Cuphea procumbens* (Fatima et al. 2012), and so on. The decline in the regeneration prospective emerged owing toward a negative result of elevated applications on the cells programmed to outline vegetative buds.

Table 9.1 Effect of different cytokinins in MS medium on shoot regeneration from cotyledonary node explants in *W. somnifera* after 4 and 8 weeks of culture

Cytokinins (μM)				4 weeks		8 weeks	
BA	Kin	2iP	Regeneration (%)	Mean number of shoots	Mean shoot length (cm)	Mean number of shoots	Mean shoot length (cm)
0.0	–	–	00	0.00 ± 0.00^{g}	0.00 ± 0.00^{g}	$0.00 \pm 0.00^{\circ}$	0.00 ± 0.00^{f}
0.1	–	–	70	5.04 ± 0.24^{f}	$0.50 \pm 0.10^{g,f}$	4.00 ± 0.00^{l}	1.10 ± 0.10^{e}
0.5	–	–	75	6.56 ± 0.36^{e}	$0.66 \pm 0.00^{d,e,f}$	$6.40 \pm 0.10^{i,j}$	1.36 ± 0.15^{e}
1.0	–	–	80	$10.40 \pm 1.37^{b,c}$	$1.20 \pm 0.00^{a,b,c,d}$	13.5 ± 0.16^{b}	$3.10 \pm 0.10^{b,c}$
2.5	–	–	95	13.00 ± 0.00^{a}	2.00 ± 0.36^{a}	18.3 ± 0.20^{a}	4.20 ± 0.12^{a}
5.0	–	–	70	10.90 ± 0.53^{b}	$2.56 \pm 0.14^{a,b,c,d}$	11.3 ± 0.22^{d}	2.80 ± 0.25^{c}
10.0	–	–	70	7.00 ± 0.00^{e}	$1.94 \pm 0.00^{c,d,e,f}$	7.20 ± 0.20^{h}	2.10 ± 0.10^{d}
	0.1	–	55	4.08 ± 0.23^{f}	$1.50 \pm 0.15^{e,f}$	3.34 ± 0.18^{m}	1.36 ± 0.41^{e}
	0.5	–	80	4.80 ± 0.30^{f}	$0.88 \pm 0.42^{c,d,e,f}$	5.10 ± 0.10^{k}	1.50 ± 0.00^{e}
	1.0	–	80	9.00 ± 0.31^{d}	$1.32 \pm 0.16^{c,d,e,f}$	10.4 ± 0.18^{e}	2.00 ± 0.00^{d}
	2.5	–	92	11.00 ± 0.00^{b}	$1.36 \pm 0.00^{a,b,c,d}$	14.2 ± 0.20^{b}	3.50 ± 0.00^{b}
	5.0	–	80	$9.48 \pm 0.38^{c,d}$	$2.00 \pm 0.22^{a,b,c}$	8.44 ± 0.19^{g}	2.14 ± 0.21^{d}
	10.0	–	80	7.48 ± 0.12^{e}	$2.10 \pm 0.00^{c,d,e,f}$	6.00 ± 0.00^{j}	1.20 ± 0.12^{e}
		0.1	70	4.00 ± 0.00^{f}	$1.50 \pm 0.27^{e,f}$	2.36 ± 0.15^{n}	1.20 ± 0.13^{e}
		0.5	75	5.00 ± 0.00^{f}	$1.00 \pm 0.36^{b,c,d,e}$	4.06 ± 0.11^{l}	1.32 ± 0.19^{e}
		1.0	82	$9.76 \pm 0.56^{b,c,d}$	$1.56 \pm 0.40^{a,b}$	$6.80 \pm 0.20^{h,i}$	$3.12 \pm 0.22^{b,c}$
		2.5	90	10.80 ± 0.30^{b}	2.40 ± 0.22^{a}	12.4 ± 0.40^{d}	4.00 ± 0.00^{a}
		5.0	80	7.00 ± 0.00^{e}	2.62 ± 0.60^{a}	6.02 ± 0.12^{j}	3.30 ± 0.12^{b}
		10.0	70	4.00 ± 0.00^{f}	$2.60 \pm 0.38^{c,d,e,f}$	4.00 ± 0.00^{l}	2.20 ± 0.10^{d}

Values represent means ± standard error of 20 replicates per treatment in three repeated experiments. Means followed by the same letter are not significantly different ($P = 0.05$) using Duncan's multiple range test

Table 9.2 Effect of optimal concentration of BA along with auxins in MS medium on shoot proliferation from cotyledonary node explants after 8 weeks of culture

Plant growth regulators (μM)				Regeneration (%)	Mean number of shoot	Mean shoot length (cm)
BA	IAA	IBA	NAA			
2.5	0.1	–	–	60	7.74 ± 0.18^{k}	3.60 ± 0.10^{d}
2.5	0.5	–	–	72	16.1 ± 0.16^{e}	$5.20 \pm 0.20^{a,b}$
2.5	1.0	–	–	70	11.4 ± 0.18^{g}	$4.12 \pm 0.22^{c,d}$
2.5	2.0	–	–	68	8.50 ± 0.15^{j}	3.00 ± 0.00^{e}
2.5	–	0.1	–	70	10.5 ± 0.16^{h}	4.36 ± 0.16^{c}
2.5	–	0.5	–	80	18.3 ± 0.16^{d}	5.00 ± 0.00^{b}
2.5	–	1.0	–	70	14.6 ± 0.18^{f}	2.50 ± 0.00^{e}
2.5	–	2.0	–	70	9.72 ± 0.18^{i}	$4.04 \pm 0.24^{c,d}$
2.5	–	–	0.1	80	22.4 ± 0.18^{b}	$4.00 \pm 0.00^{c,d}$
2.5	–	–	0.5	90	29.3 ± 0.23^{a}	5.62 ± 0.17^{a}
2.5	–	–	1.0	72	20.2 ± 0.15^{c}	4.24 ± 0.26^{c}
2.5	–	–	2.0	70	14.2 ± 0.20^{f}	3.56 ± 0.36^{d}

Values represent means ± standard error of 20 replicates per treatment in three repeated experiments. Means followed by the same letter are not significantly different ($P = 0.05$) using Duncan's multiple range test

Consequently, one of the most influencing factors in multiple shoot regeneration determining the modulation of endogenous auxin to cytokinin stability. Auxins and cytokinins have been extensively reviewed with regard to their biosynthesis, catabolism, transport, physiological roles, gene expression, and signal cascade (Müller and Leyser 2011; Shabir et al. 2016). Moreover, cytokinin–auxin ratio in culture medium plays an important factor determining the pathway of morphogenesis in plants in vitro. Ninety percent regeneration frequency with highest quantity of shoots (29.3 ± 0.23)/explant and shoot length (5.62 ± 0.17 cm) was observed on MS medium containing BA (2.5 μM) along with NAA (0.5 μM) followed by 8 weeks (Table 9.2; Fig. 9.5b). MS medium supplemented with Kin (2.5 μM) along with NAA (0.5 μM) induced maximum number of shoots (25.6 ± 0.17) with shoot length (5.26 ± 0.18 cm) per explant by 8 week (Table 9.3). Whereas, 2ip (2.5 μM) in combination with NAA (0.5 μM) proved lesser numbers (20.8 ± 0.30) of shoots and shoot length (5.88 ± 0.22 cm) per explant in the same duration (Table 9.4).

Escalating NAA concentration to 1.0 μM directed a decline in shoot arrangement competency along with basal callusing. Though, a gradual reduction in shoot numbers was examined at elevated concentrations of NAA (1.0 and 2.0 μM). NAA distinctly improved the regeneration percent, number of shoots, shoot lengths, while IBA or IAA did not progress the parameters estimated. Among different auxins analyzed, NAA was established to be most efficient followed by IBA and IAA in development of shoot clusters. The promising arrangement of BA along with NAA for in vitro shoot stimulation and multiplication has been accounted in a variety of medicinal plants such as *Ziziphora tenuiror* L. (Dakah et al. 2014), *Cassia*

Table 9.3 Effect of optimal concentration of Kin along with auxins in MS medium on shoot proliferation from cotyledonary node explants after 8 weeks of culture

Plant growth regulators (μM)				Regeneration (%)	Mean number of shoot	Mean shoot length (cm)
Kin	IAA	IBA	NAA			
2.5	0.1	–	–	72	15.1 ± 0.22^{f}	$4.36 \pm 0.16^{c,d}$
2.5	0.5	–	–	80	17.5 ± 0.36^{d}	$5.00 \pm 0.00^{a,b}$
2.5	1.0	–	–	75	14.0 ± 0.00^{g}	$5.08 \pm 0.48^{a,b}$
2.5	2.0	–	–	70	12.0 ± 0.00^{h}	$4.00 \pm 0.00^{c,d}$
2.5	–	0.1	–	80	16.4 ± 0.40^{e}	$4.12 \pm 0.22^{c,d}$
2.5	–	0.5	–	85	18.1 ± 0.22^{d}	5.20 ± 0.20^{a}
2.5	–	1.0	–	75	10.0 ± 0.63^{i}	$4.72 \pm 0.32^{a,b,c}$
2.5	–	2.0	–	65	7.60 ± 0.10^{j}	3.56 ± 0.36^{d}
2.5	–	–	0.1	80	24.3 ± 0.30^{b}	$4.00 \pm 0.00^{c,d}$
2.5	–	–	0.5	95	25.6 ± 0.17^{a}	5.26 ± 0.18^{a}
2.5	–	–	1.0	85	20.0 ± 0.48^{c}	$5.04 \pm 0.24^{a,b}$
2.5	–	–	2.0	70	15.0 ± 0.00^{f}	$4.08 \pm 0.23^{c,d}$

Values represent means ± standard error of 20 replicates per treatment in three repeated experiments. Means followed by the same letter are not significantly different (P = 0.05) using Duncan's multiple range test

Table 9.4 Effect of optimal concentration of 2iP along with auxins in MS medium on shoot proliferation from cotyledonary node explants after 8 weeks of culture

Plant growth regulators (μM)				Regeneration (%)	Mean number of shoot	Mean shoot length (cm)
2ip	IAA	IBA	NAA			
2.5	0.1	–	–	62	10.0 ± 0.00^{f}	3.28 ± 0.18^{g}
2.5	0.5	–	–	78	$14.0 \pm 0.00^{d,e}$	$5.00 \pm 0.00^{c,d}$
2.5	1.0	–	–	70	13.1 ± 0.74^{e}	$4.88 \pm 0.28^{c,d,e}$
2.5	2.0	–	–	70	8.40 ± 0.40^{g}	$4.30 \pm 0.30^{e,f}$
2.5	–	0.1	–	80	13.8 ± 0.28^{e}	$4.80 \pm 0.30^{c,d,e}$
2.5	–	0.5	–	85	16.0 ± 0.00^{c}	$5.68 \pm 0.17^{a,b}$
2.5	–	1.0	–	75	15.2 ± 0.70^{c}	4.00 ± 0.00^{f}
2.5	–	2.0	–	70	12.9 ± 0.31^{e}	$4.50 \pm 0.00^{d,e,f}$
2.5	–	–	0.1	78	18.0 ± 0.00^{b}	$5.14 \pm 0.21^{b,c,d}$
2.5	–	–	0.5	90	20.8 ± 0.30^{a}	5.88 ± 0.22^{a}
2.5	–	–	1.0	80	17.5 ± 0.36^{b}	$5.38 \pm 0.23^{a,b,c}$
2.5	–	–	2.0	75	$15.0 \pm 0.00^{c,d}$	$5.04 \pm 0.24^{b,c,d}$

Values represent means ± standard error of 20 replicates per treatment in three repeated experiments. Means followed by the same letter are not significantly different (P = 0.05) using Duncan's multiple range test

angustifolia (Siddique et al. 2015) and *Ruta graveolens* (Faisal et al. 2018). Wickson (1958) previously elucidated the promising regulatory cytokinin–auxin actions with supporting functions in apical dominance in shoot rejuvenation practice as well.

9.3.3 Effect of Media Types on Morphogenesis

MS medium was set up en route for the most appropriate medium designed for initiation of highest shoot buds as well as plant regeneration than B_5, L_2 and WPM. MS medium was established as more decisive when evaluated with other media for subsequent regeneration and proliferation of shoots (Fatima et al. 2015). WPM displayed deprived response for every parameters assessed and proved lesser number of shoots (11.2 ± 0.20) per explant, while L_2 gave agreeable results as compared to MS medium with a utmost of (15.0 ± 0.00) shoots/explant (Fig. 9.2). The highest number (20.0 ± 0.00) of shoots was recorded on full strength MS medium followed by L_2, B5, and WPM media in decreasing order when observed after 4 weeks of culture (Fig. 9.2).

9.3.4 Effect of Diverse Carbon Sources

Different applications of carbon sources were added in the culture medium to evaluate their effects on shoot regeneration and growth in *W. somnifera*. However, differential responses were recorded in plant morphogenesis with varied concentrations of carbon sources. It is well established that the carbohydrate requirements depend on the culture stages and might prove variation in uptake and utilization of different carbon sources with different species. The effect of different carbon sources (sucrose, fructose, and glucose) with a most approving medium holding BA (2.5 μM) along with NAA (0.5 μM) was studied for shoot regeneration.

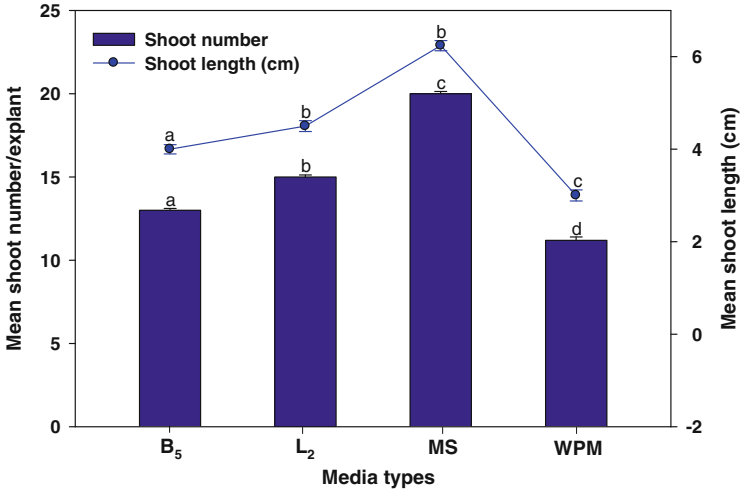

Fig. 9.2 Effect of different media types on shoot proliferation from cotyledonary node explants supplemented with BA (2.5 μM) and NAA (0.5 μM) after 4 weeks of culture. Bars represent the means ± SE. Bars denoted by the same letter within response variables are not significantly different ($P = 0.05$) using Duncan's multiple range test

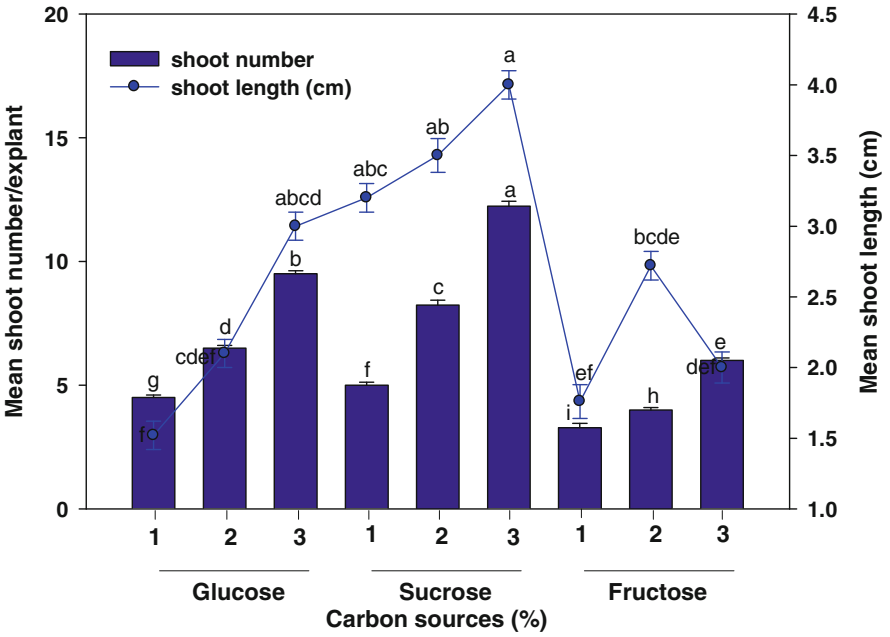

Fig. 9.3 Effect of different carbon sources on shoot proliferation from cotyledonary node explants supplemented with BA (2.5 μM) and NAA (0.5 μM) after 4 weeks of culture. Bars represent means ± SE. Bars denoted by the same letter within response variables are not significantly different ($P = 0.05$) using Duncan's multiple range test

Three percent sucrose (w/v) induced an optimum number of shoots (23.4 ± 0.40) followed by glucose (12.6 ± 0.10) and fructose (8.20 ± 0.27), respectively (Fig. 9.3).

Carbohydrate controls in vitro morphogenesis by functioning as energy resource, adjusting the osmotic potentials of culture medium, which thereby modifies cell wall possessions (extension, composition, and hardening process) (Pritchard et al. 1991). Sucrose is considered as an imperative source of carbohydrate and functions as an osmoticum in the culture medium (Hartmann et al. 1997), utilized in various energy requirement processes, and occurs at the pace of existing metabolic substrates intended for root introduction and development. Moreover, the sucrose stored in micropropagated plantlets helps in ex vitro acclimatization and physiological adaptations in TC-raised plants (Pospisilova et al. 1999). Comparable outcome in micropropagation of *W. somnifera* has been documented by Fatima et al. 2015.

9.3.5 Outcome of Different pH Levels

An optimal pH is requisite for growth and development of plant cells and tissue cultured in vitro.

It contributes significant position in uptake of nutrients, enzymatic, and hormonal actions (Bhatia and Ashwath 2005). pH controls cytoplasmic activities, which influence cell division and shoots growth in plants during morphogenesis, besides, it did not interfere in cells membrane functions and cytoplasm's buffered pH. Hence, the effect of different medium pH (4.2, 5.2, 5.8, 6.4, and 7.0) was also examined on MS medium having the optimal concentrations standardized. The highest shoots quantity and length (15.0 ± 0.00, 2.10 ± 0.10 cm) were harvested at pH 5.8, and thus was, considered as an optimum pH for maximum multiplication and elongation of shoots (Fig. 9.4).

The adjustment in pH of the medium could comprise different consequences that could manipulate performance and explants expansion (George et al. 2008). The modifications in pH of cells, tissues, and organs are owing to the ions absorbed from the medium. Furthermore, pH also manipulates the position of the solidifying agent in a medium. It was observed that higher pH level (above 6) fabricates hard medium, while a pH below 5 does not solidify the medium adequately (Bhatia and Ashwath 2005). Medium pH also influences the status of solidifying agent (Bhatia and Ashwath 2005). pH below optimal level resulted in watery medium, slight acidic in nature declines the number of shoots. Whereas, high (6.0) pH level resulted in shoots malformation and formation of callus at the base of cultures. Our findings substantiate with earlier findings reported by Fatima et al. 2015.

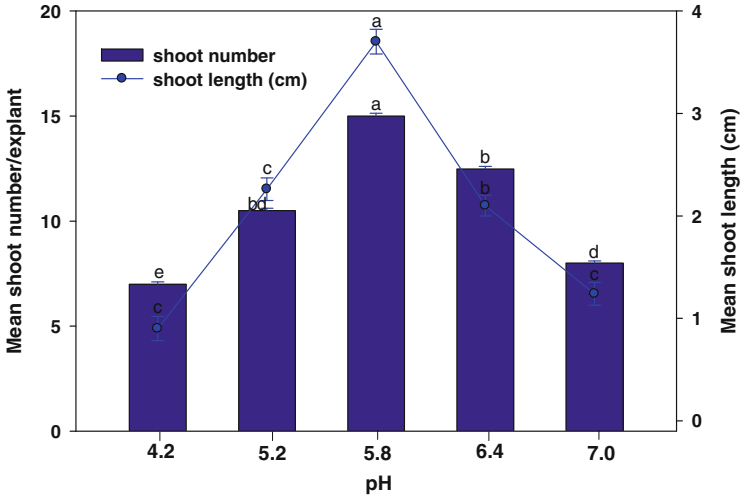

Fig. 9.4 Effect of medium pH on shoot proliferation from cotyledonary node explants supplemented with BA (2.5 μM) and NAA (0.5 μM) after 4 weeks of culture. Bars represent means ± SE. Bars denoted by the same letter within response variables are not significantly different ($P = 0.05$) using Duncan's multiple range test

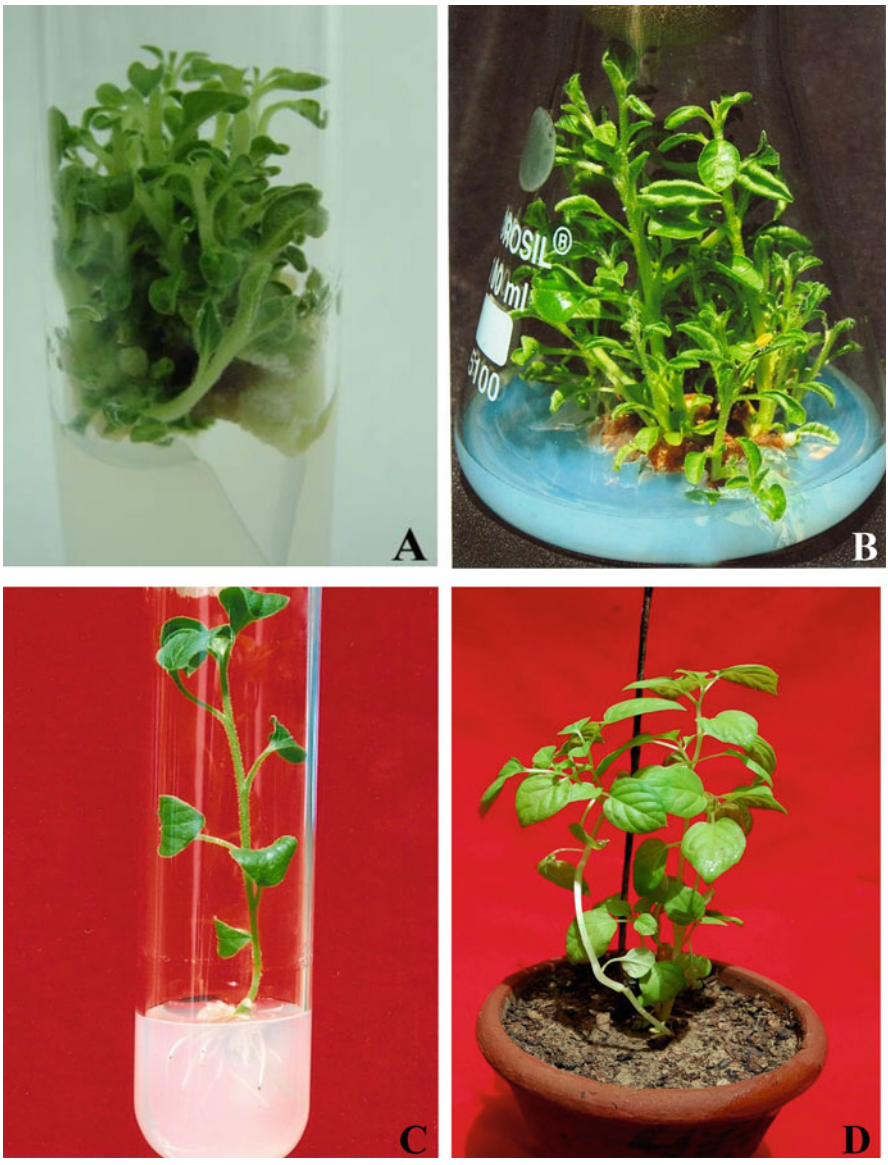

Fig. 9.5 (**a**) Culture showing shoots bud emergence and multiple shoot formation from cotyledonary node explants of *W. somnifera* on MS + BA (2.5 μM), after 4 weeks of culture condition. (**b**) Multiplication and proliferation of shoots on MS + BA (2.5 μM) + NAA (0.5 μM), after 8 weeks of culture. (**c**) In vitro rooting in micro-shoots on MS medium fortified with NAA (0.5 μM), after 4 weeks. (**d**) Well established *W. somnifera* plantlets after 3 months of transfer to field conditions

9.3.6 In Vitro Rooting and Acclimatization

Roots play an essential arrangement for the growth and buildup of water and nutrients supply to plants from the atmosphere. For *W. somnifera*, the in vitro rooting of micropropagated shoots was accomplished by auxin treatments. The rhizhogenesis occurs through induction, initiation, and expression phases. Auxins are significantly and primarily essential for these developmental stages of rooting. The maximum figure of roots (12.6 ± 0.10) and length of roots (3.58 ± 0.16 cm) per shootlet has been stimulated on MS medium fortified with NAA (0.5 μM), after 4 weeks of culture condition (Table 9.5; Fig. 9.5c). Rooting in vitro has been effectively employed for the organization of root system in numerous medicinal plants species including *W. somnifera* (Fatima et al. 2015); *Syzgium cumini* (Naaz et al. 2018); *Allamanda cathatica* (Khanam and Anis 2018), and so on.

In vitro raised plantlets have heterotrophic mode of nutrition and therefore they lack proper adjustment to the external atmospheric conditions. The micropropagated plants are kept in hardening chamber during lab to land transfer. There is a gradual series of acclimatization weeks in shades followed by lowering of air humidity (Pospisilova et al. 1998). During the 4 weeks period, plantlets having expanded leaves, healthy grown roots were effectively hardened off within the growth room. The acclimatization procedure thereby facilitated the successful transfer of TC-raised plantlets to soil (Fig. 9.5d).

Table 9.5 Effect of different auxins on in vitro root induction from microshoots of *W. somnifera* after 4 weeks of culture

Plant growth regulators (μM)			Regeneration (%)	Mean number of roots	Mean root length (cm)
IAA	IBA	NAA			
0.0			00	0.00 ± 0.00^{j}	0.00 ± 0.00^{h}
0.1			85	3.38 ± 0.16^{i}	$1.68 \pm 0.35^{f,g}$
0.5			85	7.30 ± 0.12^{e}	$2.76 \pm 0.25^{b,c}$
1.0			75	5.38 ± 0.14^{g}	$2.30 \pm 0.16^{c,d,e}$
2.0			70	4.30 ± 0.12^{h}	$1.80 \pm 0.08^{e,f,g}$
	0.1		65	$7.90 \pm 0.24^{c,d}$	$2.44 \pm 0.18^{c,d}$
	0.5		80	10.1 ± 0.18^{b}	3.04 ± 0.02^{b}
	1.0		80	$7.52 \pm 0.15^{d,e}$	$2.14 \pm 0.09^{d,e,f}$
	2.0		70	$5.38 \pm 0.16^{c,g}$	1.58 ± 0.11^{g}
		0.1	90	8.10 ± 0.18^{c}	$2.64 \pm 0.21^{b,c,d}$
		0.5	95	12.6 ± 0.10^{a}	3.58 ± 0.16^{a}
		1.0	85	10.0 ± 0.22^{b}	$2.30 \pm 0.12^{c,d,e}$
		2.0	70	6.50 ± 0.27^{f}	$1.90 \pm 0.06^{e,f,g}$

Values represent means ± standard error of 20 replicates per treatment in three repeated experiments. Means followed by the same letter are not significantly different (P= 0.05) using Duncan's multiple range test

9.4 Conclusion

In recent years, ayurveda has gathered increasing recognition of Ashwagandha for preventive and therapeutic properties, as the chemically manufactured preparations (drugs) for single target treatment prove limited achievement and unfavorable toxic effects. The proposed protocol for en masse propagation of *W. somnifera* from CN explants is found to be more promising, enviable, and remains the most consistent approach for large-scale propagation of true-to-type progenies. Moreover, a direct regeneration protocol established in plant represents a stable platform for conservation strategies, sustainable utilization of exciting germplasm, broadening the genetic base, genetic transformation, secondary metabolites production, and so on, in a short duration and limited space. The decisive function of various factors like PGRs, media types, carbon sources, and so on, at molecular, cellular, or organ/whole plant levels can be documented during the regulation of morphogenesis and manipulated for the improved and enhanced plant propagation and establishment.

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Substitution of Benzyladenine with *meta*-Topolin Improved Shoot Regeneration and Rooting in *Wedelia chinensis* (Osbeck) Merr. and Quantitative Estimation of Flavonoids

Mehrun Nisha Khanam, Saad Bin Javed, Naseem Ahmad, and Neetu Singh

Abstract

A successful regenerative method was developed for *Wedelia chinensis* by using nodal segments as explant. Nodal segments were implanted on Murashige and Skoog (MS) (Physiol Plant, 15: 473–497, 1962) medium augmented with different combinations (0.5, 1.5, 2.5, 5.0, or 7.5 μM) of benzyladenine (BA) and *meta*-Topolin (*m*T) singly as well as in combination with various auxins *viz*. Indole-3-acetic acid (IAA), indole-3 butyric acid (IBA), and α-naphthalene acetic acid (NAA) at various concentrations (0.1, 0.5, or 1.0 μM). MS medium supplemented with *m*T (2.5 μM) was most efficient in inducing axillary shoot induction and growth, producing 12.40 ± 0.22 average number of shoot with 5.39 ± 0.09 cm shoot length after 8 weeks of incubation. The highest regeneration frequency (90%) was achieved by the inclusion of low concentration of NAA (0.5 μM) with optimal *m*T (2.5 μM) in MS medium, as it produced 28.90 ± 0.27 mean number of shoots with an average of 6.01 ± 0.04 cm shoot length after 12 weeks of incubation. Further microshoots initiated rooting on ½ MS liquid medium containing 0.5 μM IBA which produced 3.60 ± 0.16 mean number of roots/shoot with 4.20 ± 0.08 cm root length after 4 weeks of incubation. In vitro raised plants with well-developed roots were acclimatized well in thermocol cups comprising sterile soilrite for 4 weeks under culture conditions. Significant enhancement in the pigment content (Chlorophyll a, b, and carotenoid) was observed during successive period (0, 7, 14, 21, and 28 days) of acclimatization. The total flavonoid content was highest in the cultures treated with *m*T (2.5 μM) + NAA (0.5 μM) producing 111.60 CE/g of flavonoid.

Keywords

Auxin · Cytokinin · Flavonoids · Micropropagation

M. N. Khanam (✉) · S. B. Javed · N. Ahmad · N. Singh
Plant Biotechnology Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, India

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10.1 Introduction

Considering the need of mankind from time immemorial, plants were the ultimate source of food, flavors, and medicine, and so on. There are many strong evidences regarding the use of plants for their medicinal values by Egyptians, Romans, and Chinese in ancient times (Cowan 1999). The use of herbal medicines is growing in the developed countries, pharmaceutical industries directly or indirectly use about 40% of compounds (secondary metabolites) obtained from plants like tannins, alkaloids, flavonoids, saponins, steroids, and so on (Rout et al. 2000) because chemically synthesized compounds are expensive than plant-based compounds (Sidhu 2011). Various health advantageous characteristics of nutritional flavonoids are identified for humans, their antiproliferative and antioxidant properties could shield the body from numerous ailments, such as cancer, inflammation, and cardiovascular disease (Nijveldt et al. 2001). Consequently, due to overexploitation of large number of medicinal plants for the extraction of secondary metabolites, some are under threat of extinction (Vines 2004; Edwards 2004). One such medicinally important species is *Wedelia chinensis* (Asteraceae), generally known as "Pilabhangara or Bringaraj." It is widely distributed in India, China, and Japan. In India, it is mainly found in the regions of Uttar Pradesh, Assam, and Arunachal Pradesh. *W. chinensis* is a perennial herb with light camphor-like odor (Agarwala et al. 2010). The plant contains acylated active constituents like germacrene, eudesmanoids, α-humulene, caryophyllene, squalene, phellandrene, *p*-cymene, sitosterol, wedelia-seco-kaurenolide, and leaves are rich in flavonoids, isoflavonoids, and wedelolactone (Rastogi and Mehrotra 1993). It was reported that the active constituents of *W. chinensis* possess suppression activity against androgen, liver damage, and development in prostate cancer cells (Lin et al. 2007). The root extracts of *W. chinensis* are utilized for tattooing, tinting gray hair, stimulating growth of hair, and has antihepatotoxic property (Lin et al. 1994). The leaf extract is used to treat cough, arthritis, gouty arthritis, cephalagia, uterine hemorrhage, menorrhagia, and skin diseases like alopecia (Meena et al. 2011; Chopra et al. 1956; Kirtikar and Basu 1975). The growth rate of Ehrlich's ascites carcinoma is inhibited by 5% ethanolic extract of *W. chinensis* plant (CSIR 1992). *W. chinensis* is conventionally propagated through seeds and stem cuttings. Being a cross-pollinated species, it shows high level of variations among seed raised progeny and thus varies in quantity and quality of active constituents (Martin et al. 2003). The collections of plants from the wild have several disadvantages. Selective harvesting of a particular species leads to the ecological imbalance in that area; moreover, there are high chances of contamination as most of the time personnel involved in collection are not experts in species identification.

Therefore, plant tissue culture technique provides an unconventional mode of plant production in large number within much shorter duration to meet the local and international demand. The technique involves the interaction of various PGRs with different types of explants for direct and indirect organogenesis. The cytokinin–auxin interaction, ratio, and concentration determine the shoot and root regeneration from explants (Hoque 2010). Recently, some investigators have reported the

dominance of an advanced class of aromatic cytokinin with hydroxylated benzyl ring (topolins) in plant tissue culture (Aremu et al. 2012). The potency of *meta*-topolin (*m*T) as compared to 6-benzyladenine (BA) on shoot multiplication was investigated. The synergistic outcome of auxins with optimum cytokinin concentration in shoot proliferation was also examined.

The available reports describing micropropagation of *W. chinensis* use BA as the cytokinin supplement (Martin et al. 2003; Agarwala et al. 2010). BA has been implicated to induce various physiological disorders causing problem in rooting and acclimatization of the regenerated shoots (Werbrouck et al. 1995). Alternatively, various analogs of BA like the Topolins can prove more effective substitute for BA. Therefore, *m*T can be used to develop much more efficient and improved protocol. However, only limited workers have explored its use and none in the case of *W. chinensis*.

The present study was designed to explore the morphogenic response of *m*T so as to assess the potency of *m*T as compared to BA on shoot multiplication for developing efficient micropropagation system for *W. chinensis* using nodal explants, physiological parameters, and estimate total flavonoids from the leaves of regenerated plantlets.

10.2 Materials and Methods

10.2.1 Explant Collection

Mature nodal explants of young shoots (first three nodes) of *W. chinensis* were harvested from a 2-year-old plant, maintained in the botanical garden of the University, Aligarh, India.

10.2.2 Explant Preparation

The young shoots comprising two to three nodes were washed for 15 min directly below the tap water followed by the application of 5% (v/v) labolene (Qualigens, India) for 5 min. Explants were again cleaned three to four times with distilled water, treated with 1% (v/v) Savlon for 1 min to remove adherent infectants, then washed again with sterilized distilled water. Explants were further externally disinfected in the laminar hood with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 2.0 min followed by three to five times washing with sterilized distilled water. Thereafter, the explants (1.0–1.5 cm) were excised from the shoots and inoculated on shoot bud initiation medium.

10.2.3 Media and Culture Condition

Murashige and Skoog (MS 1962) nutrient media with 3% (w/v) sucrose (Qualigens, India) augmented with distinct concentrations of BA and *m*T (0.5, 1.5, 2.5, 5.0 or 7.5 μM) alone or in combination with various auxins *viz*. Indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), or α-naphthalene acetic acid (NAA)) having different concentrations (0.1, 0.5, or 1.0 μM) were used. The pH of the medium was regulated to 5.8 using 1 N NaOH or 1 N HCl and then gelled with 0.8% (w/v) bacteriological grade agar prior to autoclaving at 121 °C and 15 psi for 18 min. MS medium lacking any plant growth regulators (PGRs) was used as control. All the cultures were maintained at 25 ± 2 °C under 16/8 h light and dark photoperiod in culture room, with a light intensity of 50 μmol m^{-2} s^{-1} and 55% relative humidity.

10.2.4 *In Vitro* Rooting and Acclimatization

In vitro generated healthy shoots (4–5 cm) were removed carefully and transferred onto root initiation media containing of full and half-strength MS liquid medium supplemented with various auxins (IAA, IBA, or NAA) at different concentrations (0.1, 0.5, and 1.0 μM). Data were documented on percentage response, root number, and length after 4 weeks of incubation. Well-grown rooted plants were cleaned directly with tap water to clean away the adherent media and transplanted to thermocol containers consisting of Soilrite™ (Keltech Pvt. Ltd., Bangalore). The plastic glass was then capped with plastic covers to maintain relative humidity in culture room condition and watered every 3 days. The plastic covers were removed after 2 weeks so that it accustom the plants to land requirements. After the 4 week, the plants were transplanted to earthen pots comprising garden soil and kept in a greenhouse.

10.2.5 Chlorophyll Estimation

Chlorophyll (a and b) and carotenoids in leaf tissue were estimated using the McKinney (1941) method. About 1 g of fresh leaf tissues from interveinal area was taken after 0 (juvenile leaves), 7, 14, 21, and 28 (fully expanded and mature leaves) days of hardening. The leaves were ground in 5 mL 80% acetone using motar and pestle. The extract was filtered using Whatman No. 1 filter paper. Total filtrate was collected in a test tube and final volume made 10 mL using 80% acetone. The optical density (OD) of chlorophyll was read at 645 and 663 nm wavelength, while for carotenoid, OD was recorded at 480 and 510 nm wavelength using UV–spectrophotometer (UV-Pharma Spec1700, Shimadzu, Japan).

10.2.6 Preparation of Plant Extract

The leaves from the control and regenerated plants and control (untreated) were air-dried separately, powdered to moderately coarse powder with the help of mortar and pestle. Two gram materials from each sample were extracted with 20 mL ethanol by soaking the samples overnight into it. Later, the samples were warmed in water bath at 45 °C, filtered using Whatman filter paper No. 1. The collected filtrate was concentrated under vacuum.

10.2.6.1 Estimation of Total Flavonoid (TF)

Aluminum chloride colorimetric assay was employed to measure total flavonoid content in the plant extract (Zhishen et al. 1999). Initially, measuring flask (10 mL) consisting 4 mL of distilled water was added with 1 mL of leaf extracts and standard solution of catechin (1 mg/mL), to this solution 0.3 mL 5% $NaNO_2$ was added. After 5 min, 0.3 mL solution of 10% $AlCl_3 \cdot 6H_2O$ was added into volumetric flask (10 mL). Thereafter 1 min, 2 mL of 1 M NaOH was added and the total volume was made upto 10 mL using distilled water. After thorough mixing the absorbance was measured against prepared reagent blank at 510 nm. Total flavonoid content of leaf extracts expressed as mg catechin equivalents (CE)/g of extract. All samples were analyzed in triplicates.

10.2.7 Statistical Analysis

The whole experimentation was set up in randomized block design and replicated thrice with ten replicates for each treatment. The data were subjected to analysis of variance (ANOVA) to differentiate between means using SPSS Ver 16 (SPSS Inc., Chicago, USA). All the results were articulated in mean ± standard error.

10.3 Results and Discussion

10.3.1 Effect of BA and *m*T on shoot induction

In plant tissue culture, the selection and use of cytokinin determine the efficiency in inducing shoot multiplication and growth. BA is used in many micropropagation systems because of its being economical and effective in shoot regeneration in many plant species. However use of BA also has some disadvantages like negative impact on rooting and acclimatization (Werbrouck et al. 1995). BA-derived analogs such as *meta*-Topolin used as an alternative plant growth regulator to refrain the adverse effects of BA in cultures. In *W. chinensis, m*T treatments proved significantly better to induce shoot production when compared to control and BA treatment. After 8 weeks of culture, 2.5 μM *m*T produced higher shoot number 12.40 ± 0.22 with 5.39 ± 0.09 cm mean shoot length having 80.00 ± 0.29 percentage responsive explants as compared to BA, which even at higher concentration produced lesser

Table 10.1 Effect of BA and *m*T for shoot regeneration on nodal explants after 8 weeks of incubation

Cytokinins (μM)				
BA	*m*T	Percent response	Mean shoot number	Mean shoot length (cm)
0.0	0.0	0.00 ± 0.00^{i}	0.00 ± 0.00^{i}	0.00 ± 0.00^{g}
0.5		45.90 ± 0.27^{h}	1.80 ± 0.13^{h}	1.55 ± 0.11^{f}
1.5		55.60 ± 0.22^{f}	3.60 ± 0.16^{f}	2.80 ± 0.08^{e}
2.5		66.90 ± 0.37^{d}	6.00 ± 0.21^{c}	4.05 ± 0.11^{c}
5.0		75.20 ± 0.24^{b}	8.40 ± 0.16^{b}	5.03 ± 0.04^{b}
7.5		59.90 ± 0.34^{e}	4.50 ± 0.16^{e}	3.50 ± 0.16^{d}
	0.5	59.30 ± 0.26^{e}	5.11 ± 0.17^{d}	2.65 ± 0.07^{e}
	1.5	74.50 ± 0.68^{b}	8.00 ± 0.25^{b}	3.08 ± 0.08^{c}
	2.5	80.00 ± 0.29^{a}	12.40 ± 0.22^{a}	5.39 ± 0.09^{a}
	5.0	70.30 ± 0.21^{c}	6.00 ± 0.29^{c}	4.10 ± 0.06^{c}
	7.5	49.20 ± 0.29^{g}	2.60 ± 0.16^{g}	2.50 ± 0.16^{e}

Values represent means ± standard error of readings of ten replicates per treatment in three repeated experiments. Means sharing the same letter are not significantly different ($P = 0.05$) using Duncan's multiple range test

number of shoots (Table 10.1; Fig. 10.1a). Similar findings of BA and *m*T effect on cultures were reported in *Spathiphyllum floribundum* (Werbrouck et al. 1996). The findings of their comparative study were that the key metabolite of *m*T was more stable and balanced than the main metabolite of BA, because BA had a negative influence on rooting and acclimatization as the *O*-glucoside does not degrade rapidly during acclimatization. The results showed that plants treated with *m*T generated considerably maximum root number and higher root length than those treated with BA. Limited reports are available on the use of topolins indicating that this group of cytokinins could be a new source of cytokinins with high morphogenetic activity. Kaminek et al. (1987) reported that *m*T was nearly twice as active and efficient as BA in the initiation of shoot growth in wheat plant species.

10.3.2 Effect of Cytokinin: Auxin Combination on Shoot Proliferation

The additive or interdependent influence of auxins with cytokinins on proliferation of shoots has been studied in numerous plant species (Shimizu-Sato et al. 2009). The equilibrium among auxin and cytokinin is an important regulator of in vitro organogenesis, demonstrated in the classical experiment of Skoog and Miller (1957). Among the three auxins used, NAA does not involve active uptake to pass through the plasma membrane into cells (Nordstrom et al. 2004). The optimum concentration of BA (5.0 μM) and *m*T (2.5 μM) was tested with numerous concentrations (0.1, 0.5, or 1.0 μM) of auxins. Among the tested concentration, *m*T (2.5 μM) + NAA (0.5 μM) significantly increased shoot production with 90% responsive explants, produced 28.90 ± 0.27 shoot number with 6.01 ± 0.04 cm shoot length after

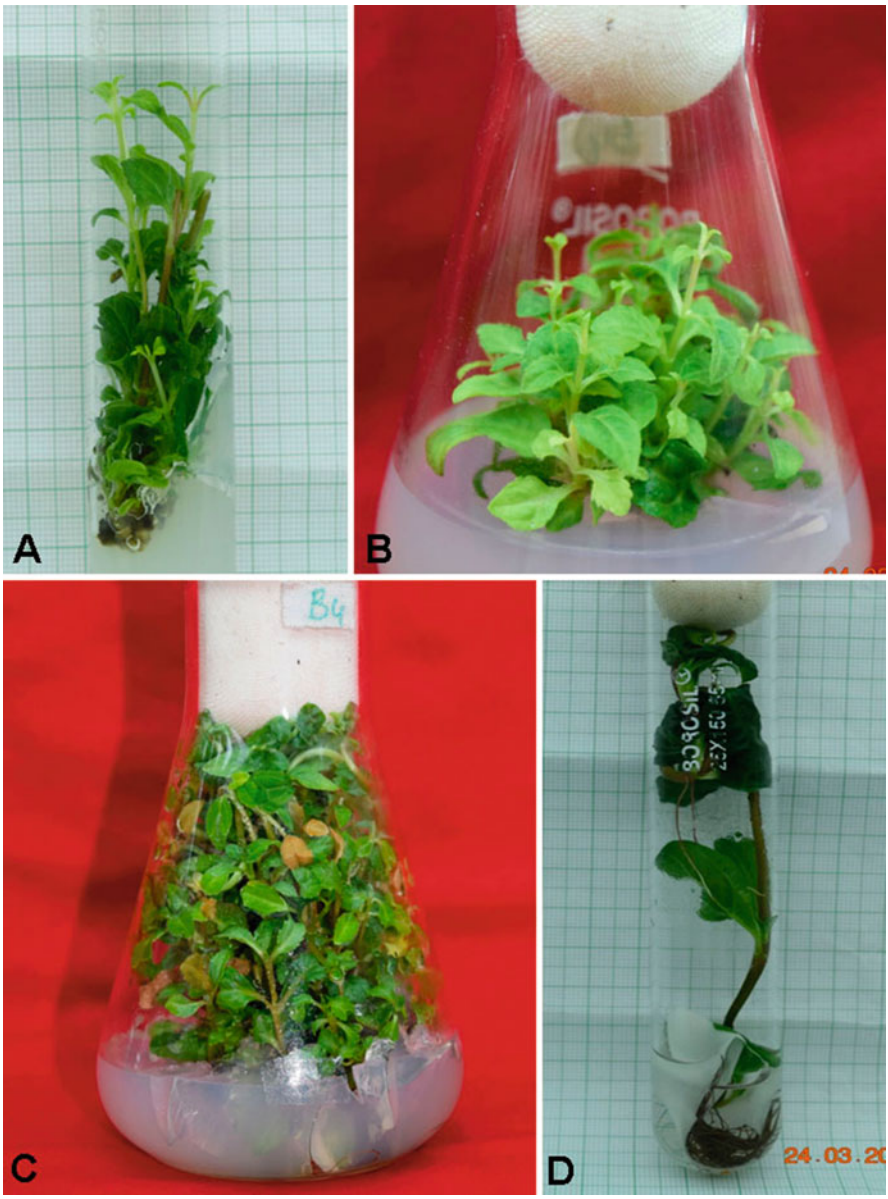

Fig. 10.1 (**a**) Multiple shoot induction from nodal explant cultured on MS medium augmented with *m*T (2.5 μM) after 8 weeks of culture. (**b**) Shoot proliferation and multiplication on MS medium augmented with BA (5.0 μM) + NAA (0.5 μM) after 12 weeks of incubation. (**c**) Mass multiplication on MS + *m*T (2.5 μM) + NAA (0.5 μM) after 12 weeks of incubation. (**d**) Rooting in the regenerated microshoots on ½ MS liquid media containing 0.5 μM IBA after 4 weeks of incubation

Table 10.2 Effect of various auxins supplemented with optimized *m*T (2.5 μM) in MS medium on nodal explants after 12 weeks of incubation

Auxins (μM)					
IAA	IBA	NAA	Percent response	Mean shoot number	Mean shoot length (cm)
0.00	0.00	0.00	0.00 ± 0.00^{j}	0.00 ± 0.00^{i}	0.00 ± 0.00^{j}
0.1			72.40 ± 0.68^{e}	15.00 ± 0.21^{d}	3.85 ± 0.03^{e}
0.5			82.10 ± 0.60^{c}	21.60 ± 0.88^{b}	4.50 ± 0.03^{c}
1.0			40.60 ± 0.22^{i}	10.60 ± 0.16^{f}	2.82 ± 0.04^{h}
	0.1		74.10 ± 0.27^{d}	12.70 ± 0.15^{e}	3.63 ± 0.04^{f}
	0.5		65.50 ± 0.37^{g}	6.70 ± 0.15^{g}	2.97 ± 0.05^{g}
	1.0		54.60 ± 0.33^{h}	4.40 ± 0.16^{h}	2.04 ± 0.01^{i}
		0.1	84.60 ± 0.38^{b}	21.00 ± 0.25^{b}	5.00 ± 0.03^{b}
		0.5	90.00 ± 0.47^{a}	28.90 ± 0.27^{a}	6.01 ± 0.04^{a}
		1.0	70.00 ± 0.47^{f}	16.90 ± 0.27^{c}	4.15 ± 0.01^{d}

Values represent means ± standard error of readings of ten replicates per treatment in three repeated experiments. Means sharing the same letter are not significantly different ($P = 0.05$) using Duncan's multiple range test

Table 10.3 Effect of various auxins supplemented with optimized BA (5.0 μM) in MS medium on nodal explants after 12 weeks of incubation

Auxins (μM)					
IAA	IBA	NAA	Percent response	Mean shoot number	Mean shoot length (cm)
0.00	0.00	0.00	0.00 ± 0.00^{i}	0.00 ± 0.00^{i}	0.00 ± 0.00^{i}
0.1			60.00 ± 0.63^{e}	6.70 ± 0.21^{f}	3.16 ± 0.08^{d}
0.5			70.00 ± 0.47^{c}	11.50 ± 0.34^{c}	$4.10 \pm 0.06^{b,c}$
1.0			53.00 ± 0.57^{g}	3.30 ± 0.21^{h}	2.67 ± 0.03^{e}
	0.1		60.00 ± 0.51^{e}	7.40 ± 0.22^{e}	4.30 ± 0.08^{b}
	0.5		55.00 ± 0.36^{f}	4.60 ± 0.16^{g}	3.25 ± 0.08^{d}
	1.0		50.00 ± 0.63^{h}	3.00 ± 0.21^{h}	2.20 ± 0.08^{f}
		0.1	76.00 ± 0.47^{b}	14.50 ± 0.16^{b}	3.97 ± 0.05^{c}
		0.5	84.00 ± 0.24^{a}	17.30 ± 0.26^{a}	5.23 ± 0.05^{a}
		1.0	65.00 ± 0.47^{d}	10.40 ± 0.22^{d}	2.50 ± 0.08^{e}

Values represent means ± standard error of readings of ten replicates per treatment in three repeated experiments. Means sharing the same letter are not significantly different ($P = 0.05$) using Duncan's multiple range test

12 weeks of culture (Table 10.2; Fig. 10.1c), signifying a synergistic effect of NAA on shoot proliferation. Other *m*T–auxins combinations proved less responsive. However, among BA (5.0 μM) + NAA (0.5 μM) combinations could only produce a maximum of 17.30 ± 0.26 mean shoots and 5.23 ± 0.05 cm shoot length in 84% cultures after 12 weeks of incubation (Table 10.3; Fig. 10.1b).

10.3.3 Rooting in Microshoots

Regenerated microshoots (5.0 cm) from nodal explants did not induce any root on full or ½ strength MS media devoid of plant growth regulators. However, the cut ends of microshoots instigated root primordial when cultured on ½ MS liquid medium augmented with auxins after 2 weeks of incubation. IBA (0.5 μM) was most persuasive in inducing rhizogenesis in 94% cultures which produced about 3.60 roots, with an average root length of 4.20 ± 0.08 cm per shoot (Table 10.4; Fig. 10.1d). The effectiveness of treating microshoots with IBA for root induction has been reported in several plant species, *Saussurea obvallata* (Dhar and Joshi 2005), *Aspilia montevidensi* (Kunst-Barosky et al. 2011), and *Grindelia chiloensis* (Wassner and Ravetta 2000). However, NAA and IAA with ½ MS media produced basal callusing at the cut end and produced lesser number of roots than IBA. Hence, IBA was found to be superior for root initiation without any induction of callus in *W. chinensis*.

10.3.4 Acclimatization

Acclimatization is the most essential step in tissue culture. Therefore, well-developed healthy rooted plantlets with six to seven leaves were transferred to pots containing sterile Soilrite™ for 4 weeks. The plantlets were positively transferred to greenhouse after acclimatization where of 94% survival rate was recorded (Fig. 10.2). All the regenerated plantlets showed normal morphology and growth similar to that of the mother plant.

Table 10.4 Effect of ½ MS medium augmented with various auxins on rooting in microshoots after 4 weeks of incubation

Auxins (μM)					
IAA	IBA	NAA	Percent response	Mean root number	Mean root length (cm)
00			0.00 ± 0.00^{j}	0.00 ± 0.00^{f}	0.00 ± 0.00^{f}
0.1			61.10 ± 0.31^{f}	0.60 ± 0.16^{e}	0.90 ± 0.25^{e}
0.5			67.70 ± 0.15^{d}	$1.60 \pm 0.16^{b,c}$	$1.85 \pm 0.41^{c,d}$
1.0			51.00 ± 0.25^{h}	0.50 ± 0.16^{e}	0.70 ± 0.26^{e}
	0.1		85.00 ± 0.25^{b}	2.10 ± 0.23^{b}	3.25 ± 0.08^{b}
	0.5		94.90 ± 0.23^{a}	3.60 ± 0.16^{a}	4.20 ± 0.08^{a}
	1.0		75.30 ± 0.21^{c}	$1.50 \pm 0.16^{b,c}$	2.40 ± 0.12^{c}
		0.1	56.00 ± 0.24^{g}	$0.90 \pm 0.31^{c,d}$	0.90 ± 0.31^{e}
		0.5	64.20 ± 0.35^{e}	$1.50 \pm 0.37^{b,c}$	$1.55 \pm 0.43^{d,e}$
		1.0	41.40 ± 0.22^{i}	0.50 ± 0.22^{e}	0.80 ± 0.32^{e}

Values represent means ± standard error of readings of ten replicates per treatment in three repeated experiments. Means sharing the same letter are not significantly different ($P = 0.05$) using Duncan's multiple range test

Fig. 10.2 Acclimatized plantlet

10.3.5 Photosynthetic Pigments

The variation in pigment concentration (Chlorophyll and carotenoid contents) was assessed in regenerated plants when transferred from in vitro to ex vitro. During the initial period of acclimatization a reduction in carotenoid and chlorophyll a and b was recorded. The pigment contents increased significantly with an increase in acclimatization period. According to Borkowska (2001), the young leaves of the in vitro raised strawberry plants acclimatized to ex vitro condition exhibited low phytochemical activity but as the period of acclimatization progresses with time, these pigments became more active in mature leaves. In the present study, the Chl a and b content were low (0.25 ± 0.04) mg g^{-1} and (0.21 ± 0.01) mg g^{-1} on 0 days of acclimatization, got increased up to (0.42 ± 0.02) mg g^{-1} and (0.34 ± 0.02) mg g^{-1} after 21 days and reached maximum (0.45 ± 0.05) mg g^{-1} and (0.37 ± 0.06) mg g^{-1} after 28 days of transfer (Fig. 10.3). Initial reduction in chlorophyll content may be due to poorly developed chloroplast and disorganized grana. Enhancement in

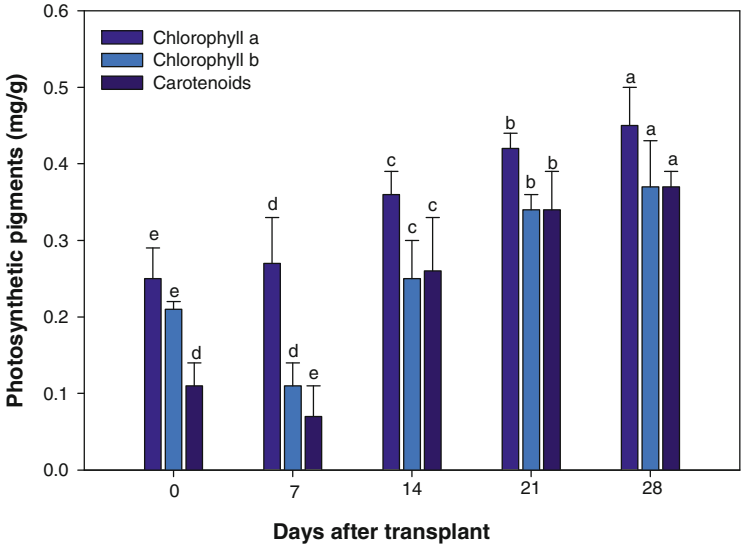

Fig. 10.3 Net photosynthetic content

pigment contents is attributed to the induction of chlorophyll synthesis enzyme required for chlorophyll biosynthesis. Similar results have been reported by Posposilova et al. (1999) and Jeon et al. (2005).

Carotenoid plays an important role in photoprotection of chlorophyll pigments under stress (Kenneth et al. 2000) which might be engendered during the acclimatization period. The carotenoid contents enhanced progressively during the transplantation period. The highest concentration (0.37 ± 0.02) mg g^{-1} was recorded after 28 days of acclimatization (Fig. 10.3). Increase in carotenoid levels is not unexpected as carotenoids are reported to be involved in protecting the photosynthetic machinery from photo-oxidative damage (Jeon et al. 2005).

10.3.6 Estimation of Flavonoids

Two grams of leaf sample collected from regenerated plantlets were screened for estimation of flavonoids. The plantlets grown on MS + *m*T (2.5 μM) + NAA (2.5 μM) gave highest yield (0.19 g or 9.5%) (Table 10.5). The method proposed by Mishra et al. (2011) and Yang et al. (2008) for extraction of flavonoids was used.

Aluminum chloride colorimetric method using spectrophotometer was employed to estimate total flavonoid content in leaf extract (Table 10.5) and expressed in milligram catechin equivalent to per gram of the extract. The content varied among treated samples and highest content (111.60 mg CE/g) was recorded in leaf tissue of plantlets raised on MS + *m*T (2.5 μM) + NAA (2.5 μM). Colorimetric reactions are speedy, easy to perform, and widely used to determine total flavonoids content in medicinal plant. This is based on the formation of the flavonoids–aluminum complex

Table 10.5 Quantification of total flavonoid content

Treatment	Extract (g)	Percent yield	Total flavonoids (CE)/g
Natural source	0.16	8.0	109.10
BA (5.0 μM)	0.17	8.5	109.51
*m*T (2.5 μM)	0.17	8.5	110.21
BA (5.0 μM) + NAA (0.5 μM)	0.18	9.0	110.73
*m*T (2.5 μM) + NAA (0.5 μM)	0.19	9.5	111.60

which has maximum absorptivity at 510 nm. The treated extract showed the presence of flavonoid catechin.

10.4 Conclusion

The overall objective of the study was to develop a system for an improved regeneration protocol of *W. chinensis* from axillary shoot buds. The micropropagation established multiplication year-round can be used for production of quality planting material at a faster rate than the available protocols in *W. chinensis*. The experiments have clearly indicated the potentiality of *m*T over BA for shoot multiplication and their health without causing any physiological disorders in rooting and acclimatization of the regenerated shoots. The in vitro culture strategies developed could be a useful tool to increase the biomass and yield of flavonoids accumulated in *W. chinensis* cultures.

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Effect of Silicon Ions on In Vitro Morphogenesis in *Rauvolfia tetraphylla* Using Shoot Tip Explants

11

Sheikh Altaf Hussain

Abstract

The study evaluates the effect of Silicon ions on in vitro morphogenesis in *Rauvolfia tetraphylla* using aseptic shoot tip explants. The explants were cultured on MS medium supplemented with varied concentrations of BA either singly or in combination with NAA. Of the tested concentrations, best response (80%) in explants for in vitro shoot production was recorded on MS medium fortified by BA (5.0 μM) and NAA (0.5 μM) with mean shoot number (9.66 ± 0.57) and shoot length (3.76 ± 0.16 cm) after 8 weeks of incubation. However, on evaluation of the morphogenic effect of different concentrations of silicon ions with optimized levels of BA and NAA, an increase in the percentage response and regeneration frequencies were observed. Of the tested combinations, MS culture medium supplemented with (5.0 μM BA + 0.5 μM NAA + 5.0 mM Na_2SiO_3) yielded 100% morphogenic response with mean shoot number (15.33 ± 0.66) and average length (5.73 ± 0.26) after 8 weeks of culture. In vitro rhizogenesis in 93% microshoots with maximum mean number (9.66 ± 0.66) of roots with mean root length (3.56 ± 0.23) per shootlet was recorded on ½ WPM medium supplemented with 0.5 μM IBA, after 4 weeks of incubation. The plantlets regenerated via this protocol were successfully acclimatized and hardened off under controlled conditions of growth chamber before their plantation to field conditions. The plantlets grew well under open sun and 90% survival rate was recorded after 12 weeks of transfer. The protocol developed can be used for propagation and conservation of rare and endangered plant assets.

Keywords

Acclimatization · Benzyl adenine · In vitro rooting · Na_2SiO_3 · WPM medium

S. A. Hussain (✉)
Plant Biotechnology Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, India

I. Siddique (ed.), *Propagation and Genetic Manipulation of Plants*,
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Abbreviations

BA	Benzyl adenine
cm	Centimetre
IBA	Indole-3-butyric acid
mM	millimole
Na_2SiO_3	Sodium meta-silicate
NAA	α-naphthalene acetic acid
μM	micro molar

11.1 Introduction

Rauvolfia tetraphylla is a potential medicinal shrub of family Apocynaceae and is commonly referred as devil pepper. The *R. tetraphylla* is a native species of tropical America and distributed in parts of Asia, Africa and India. The species is restricted to moist and warm parts of India such as Andhra Pradesh, Bihar, Howrah, Kerala, Madhya Pradesh, Orissa, Uttar Pradesh and West Bengal (Jyothi et al. 2012). Morphologically the plant body of *R. tetraphylla* is woody, evergreen and perennial and grows up to the height of 5 ft. Four unequal dark green leaves are found on each node, thus, the name *R. tetraphylla* (Anonymous 2003). It is potent source of many important alkaloids like ajmaline, alistonine, canescine, corynathene, desperdine, reserpine, tetraphyllicine and yohimbine and so on. The bioactive compound reserpine is used to cure nervous system disorders. Further it maintains blood glucose levels in diabetic patients and regulates normal blood pressure in humans (Hussain et al. 2018a). Root decoction is used for curing various intestinal infections like diarrhoea, dysentery and other helminthic diseases from pre historic times (Faisal et al. 2006; Hussain et al. 2018b). The species has been indiscriminately exploited from its natural habitat and sometime whole plants are uprooted to meet economic and medicinal gains for growing population and to satisfy increasing interest from pharmacological industries. Poor vegetative propagation methods, low seed germination and viability rates without any replantation programme have made this plant critically endangered (Faisal and Anis 2009). Therefore the need of hour is to find out the alternate propagation methods for it mass-scale replantation drive so that we can challenge all the growing demands. The in vitro micropropagation technique is the only way available for large-scale production, propagation and conservation of rare, elite and endangered plant species (Anis and Ahmad 2016). Hence, an attempt was successfully made to develop an efficient, reliable and economical in vitro regeneration protocol using aseptic nodal explants by employing tissue culture technology. The current study was undertaken to study role of silicon ions (most abundant element) on in vitro axillary shoot induction and proliferation. Silicon is known for its significant physiological role in enhancing the power of resistance under oxidative stresses caused by drought, salinity, metal toxicity and other plant diseases by modulating antioxidant enzymes (Sivanesan and Jeong 2014; Sivanesan et al. 2011).

11.2 Materials and Methods

11.2.1 Explants Source

The shoot tip explants (1.0 cm) were excised from 14 months old *R. tetraphylla* aseptic cultures which were maintained in plant biotech culture room of Aligarh Muslim University, Aligarh, India (Hussain et al. 2018a, b).

11.2.2 Inoculation and Culture Media

For in vitro shoot tip culture, the explants were cultured on Murashige and Skoogs (MS) medium (1962) enriched with various concentrations (1.0, 2.5, 5.0, 7.5 or 10.0 μM) of BA alone or in combination with various concentrations (0.1, 0.5, 1.0, 1.5 or 2.0 μM) of auxin NAA. The effect of sodium meta-silicate (Na_2SiO_3) at various concentrations (1.0, 2.5, 5.0, 7.5 or 10.0 mM) was also evaluated in the optimized culture media.

11.2.3 Culture Conditions

For all experiments, MS medium at pH 5.8, with enrichment of 3% sucrose as carbon source was used. The culture medium was autoclaved at 1.06 kg cm^2 pressure at 121 °C for 16 min after addition of 0.9% agar (Qualigens Pvt. Ltd., Mumbai, India) as solidifying agent. All inoculation and subculturing were done under sterile conditions inside laminar air flow hood and the cultures were maintained at 24 ± 2 °C for incubation in growth chamber under photoperiod of 16/8 h. The 40 W white fluorescent lamps provided desirable light intensity (60 μmol m^{-2} s^{-1}) and were periodically monitored by Lux photo metre. The relative humidity of the growth chamber was maintained at 60 ± 05% and was continuously monitored by hygrometer.

11.2.4 Subculturing

For further shoot proliferation, the cultures were subcultured after every 3 weeks onto a similar fresh culture medium.

11.2.5 In Vitro Rooting in Microshoots

Healthy microshoots (~5 cm) were excised and employed for in vitro root induction in full or half strength liquid Woody Plant Medium (WPM) supplemented with different concentrations (0.1, 0.25, 0.50, 0.75 or 1.0 μM) of IBA. The filter paper

bridges made of Whatman filter paper number 1 were used for incubating the microshoots in rooting medium for 4 weeks.

11.2.6 Acclimatization and Hardening

At the end of 4 weeks, the regenerated mini plantlets were planted in polystyrene cups filled with autoclaved Soilrite™ (Keltech Energies Ltd., Bangalore) and were immediately covered by poly bags (transparent) in order to retain and ensure proper humidity around the plant body. On alternate days, the planting substrate Soilrite was sprayed with ½ MS stock solution without organic nutrients. In order for better acclimatization, the polymembrane cover was removed from the plantlets after 2 week of transfer. Thereafter, the regenerated plantlets were potted in the earthen pots containing 3:1 mixture garden soil and manure. After proper hardening, the pots were transferred and allowed to grow under field conditions.

11.2.7 Data Collection and Statistical Analysis

The whole micropropagation cycle was repeated thrice in order to attain the reproducibility of the regenerated protocol. Data were collected on every 4 week and were analysed by statistical software (SPSS *Ver*-16 Inc., Chicago, USA). The Duncan's Multiple Range Test (DMRT) was used for carrying out the significance difference among means.

11.3 Results and Discussion

11.3.1 Effect of Different Concentrations of BA on In Vitro Shoot Bud Induction

No morphogenic response was achieved in ST explants (shoot tip) on control medium (MS 1962 without PGR) even after 8 weeks. On augmentation of BA to the medium, the explants responded well for shoot initiation and multiplication. A differential response for axillary shoot bud initiation and differentiation was observed at varied concentrations. Of the tested concentrations, highest response (75%) with average mean shoot number (5.66 ± 0.88) and shoot length (3.46 ± 0.36 cm) per explant was recorded on MS medium fortified with 5.0 μM BA after 8 weeks of culture (Table 11.1). Similar observations have been reported in many woody species where BA was effective in promoting maximum shoot bud initiation and proliferation like *Euphorbia cotinifolia* (Perveen et al. 2013) and *Cryptolepis sanguinolenta* (Monney et al. 2016).

Table 11.1 Effect of BA, NAA and Na_2SiO_3 on in vitro shoot bud induction and proliferation on aseptic shoot tip explants of *R tetraphylla*, after 8 weeks of incubation

BA (μM)	NAA (μM)	Na_2SiO_3 (Mm)	Percentage response	Mean no of shoots/ explant	Mean shoot length (cm)
0.0	0.0	0.0	0.0	0.00 ± 0.00^{l}	$0.0\ 0 \pm 0.0^{j}$
1.0			33	1.66 ± 0.33^{k}	1.43 ± 0.23^{i}
2.5			49	$3.00 \pm 0.57^{i,j}$	2.24 ± 0.17^{h}
5.0			75	$5.66 \pm 0.88^{g,h}$	$3.46 \pm 0.36^{e,f}$
7.5			70	$4.33 \pm 0.88^{h,i}$	$2.93 \pm 0.22^{f,g}$
10.0			66	3.66 ± 0.33^{i}	2.20 ± 0.13^{h}
5.0	0.1		73	$6.33 \pm 0.33^{f,g}$	2.83 ± 0.08^{g}
–	0.5		80	9.66 ± 0.57^{d}	$3.76 \pm 0.16^{d,e}$
–	1.0		78	8.33 ± 0.66^{e}	3.58 ± 0.24^{f}
–	1.5		75	$7.66 \pm 0.33^{e,f}$	$3.36 \pm 0.14^{e,f}$
–	2.0		70	$6.66 \pm 0.33^{f,g}$	$3.66 \pm 0.13^{e,f}$
5.0	0.5	1.0	85	11.00 ± 0.57^{d}	$4.33 \pm 0.10^{c,d}$
–	–	2.5	88	13.66 ± 0.33^{b}	5.16 ± 0.38^{b}
–	–	5.0	100	15.33 ± 0.66^{a}	5.73 ± 0.26^{a}
–	–	7.5	95	$12.66 \pm 0.66^{b,c}$	$4.66 \pm 0.17^{b,c}$
–	–	10.0	90	$11.33 \pm 0.37^{c,d}$	$4.43 \pm 0.21^{c,d}$

Effect of MS medium augmented with different concentrations of BA, NAA and Na2Sio3 on in vitro shoot bud induction and proliferation on aseptic shoot tip explants of R. tetraphylla after 8 weeks of culture

11.3.2 Effect of Optimized Concentration of Benzyl-Adenine with Auxin NAA

On evaluation of the combined effect of the optimized concentration of BA with different concentrations of NAA, an enhanced shoot multiplication rate was observed. On accessing the combination effects, 80% explants responded on MS medium fortified with BA (5.0 μM) and NAA (0.5 μM) with maximum mean number (9.66 ± 0.57) of shoots having an average length of 3.76 ± 0.16 cm, after 8 weeks of incubation (Table 11.1). The synergistic outcome of higher level of BA with lower doses of NAA positively influenced the shoot regeneration frequencies by increasing the shoot number up to an optimal combination and thereafter a decreasing trend in shoot growth was recorded. The observed results are in accordance with prior reports (Ahmad et al. 2013; Perveen et al. 2013; Hussain et al. 2020) where BA and NAA combinations promoted higher shoot frequencies per explant than singly supplemented BA.

11.3.3 Effect of Silicon Ions with Optimized Concentrations of BA and NAA

On evaluation of morphogenic effect of various concentrations (1.0–10.0 Mm) of silicon ions in the form of sodium meta-silicate with optimized levels of BA and NAA, a synergistic increase shoot number was observed. Among tested combinations, MS medium augmented with (5.0 μM BA, 0.50 μM NAA and 5.0 mM Na_2SiO_3) yielded 100% response (Fig. 11.1a, b) and produced good quality shoots with a maximum of 15.33 ± 0.66 shoots per explant with an average length of 5.73 ± 0.26 cm, after 8 weeks of incubation (Table 11.1). The growth parameters increased up to the thresh hold level and above that retarded shoot growth was observed. All the cultures were healthier and possess larger internodes and leaves than BA or BA + NAA combination grown cultures. These results are in accordance with the results obtained in different plant species where Si has played a significant role for shoot growth and multiplication such as rice (Islam et al. 2005); reed (Mathe et al. 2012) and *Ajuga multiflora* (Sivanesan and Jeong 2014). Further no physiological disorder like hyperhydricity, shoot tip necrosis or shoot fasciation was observed in silicon mediated cultures even after 1 year while as in the BA or BA + NAA regenerated cultures these effects were evident only after 4 months of culture. The possible reason for the better growth in silicon-mediated cultures may be due to the maintenance of stability among different constituents of the culture medium by detoxifying the free radicals generated during the tissue culture process (Ma 2004; Prabagar et al. 2011; Sivanesan and Jeong 2014). Overall, our results indicate that in vitro morphogenic response and efficiency of micropropagation system depends upon the medium composition and their manipulation in right way.

11.3.4 Rooting of Regenerated Shoots

No root induction was observed on control medium, however, on enrichment of different concentrations of IBA promoted initiation of in vitro rooting after 1 week of incubation. The 93% microshoots rooted in ½ WPM enriched with 0.5 μM of IBA, and yielded maximum number (9.66 ± 0.66) of roots per shootlet with an average length of 3.56 ± 0.23 cm, after 4 weeks of incubation (Table 11.2). Half strength WPM medium in combination with IBA was found more effective for rhizogenesis (Fig. 11.1c). The results obtained are in corroboration with the established micropropagation protocols where IBA was found effective due to its stability and easy uptake by plant tissues for better in vitro rhizogenesis in plant species like *R. tetraphylla* (Hussain et al. 2018a, b) and *Allamanda cathartica* (Khanam and Anis 2018); *Tecoma stans* (Hussain et al. 2019a).

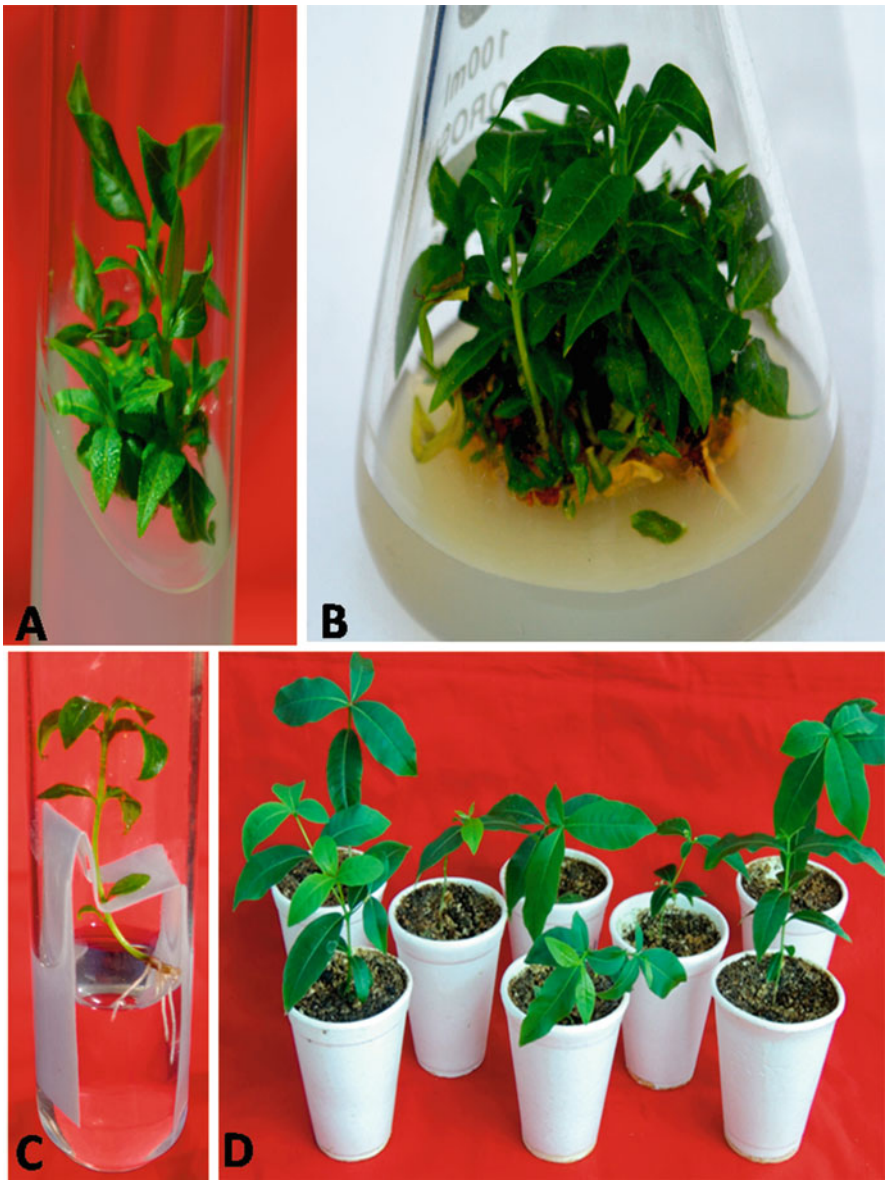

Fig. 11.1 (**a**) Shoot bud induction and multiplication on MS + (5.0 μM) BA + (0.5 μM) NAA + (5.0 mM) Na_2SiO_3 after 3 weeks of incubation. (**b**) Shoot proliferation after 8 weeks of incubation on MS + (5.0 μM) BA + (0.5 μM) NAA + (5.0 mM) Na_2SiO_3. (**c**) In vitro rooting in microshoots after 3 weeks of incubation on ½ WPM + 0.50 μM IBA (liquid medium). (**d**) Micropropagated plantlets after 4 weeks of acclimatization

Table 11.2 Effect of IBA on in vitro rooting in regenerated microshoots with full and half strength WPM after 4 weeks of incubation

IBA(μM)				
WPM	½ WPM	Percentage response	Mean root no/shootlet	Mean root length (cm)
0.00	0.0	00	0.00 ± 0.00^{g}	0.00 ± 0.00^{h}
0.10		53	1.33 ± 0.33^{f}	0.76 ± 0.13^{g}
0.25		67	3.66 ± 0.33^{e}	$1.26 \pm 0.12^{e,f}$
0.50		79	7.33 ± 0.66^{b}	$3.16 \pm 0.16^{a,b}$
0.75		71	5.66 ± 0.88^{d}	2.30 ± 0.20^{c}
1.00		65	$4.33 \pm 0.33^{d,e}$	$1.96 \pm 0.14^{d,e}$
	0.10	61	$4.66 \pm 0.33^{c,d,e}$	$1.83 \pm 0.16^{c,d}$
	0.25	75	$6.33 \pm 0.33^{b,c}$	2.26 ± 0.23^{c}
	0.50	93	9.66 ± 0.66^{a}	3.56 ± 0.23^{a}
	0.75	87	7.66 ± 0.66^{b}	2.83 ± 0.20^{b}
	1.00	81	$5.33 \pm 0.66^{c,d,e}$	$2.12 \pm 0.16^{d,e}$

Effect of full and half strength woody plant medium, supplemented with different concentrations of IBA on in vitro rooting in regenerated microshoots of *R. tetraphylla* after 4 weeks of incubation

11.3.5 Acclimatization and Hardening in Micropropagated Plants

The efficiency of micropropagation system for commercial purposes depends upon cost, production and survival rate of the plantlets under field conditions. The ex vivo transfer of tissue culture raised plants in critical phase where plantlets have to compete with new environment and the other biotic stresses (Hussain et al. 2019b; Naaz et al. 2018, 2019). Thus, all efforts are focused on ex vitro establishment of regenerants in order to reduce the stresses and mortality rate among regenerants. The micropropagated plants were acclimatized under culture room conditions successfully with 90% survival rate (Fig. 11.1d). After 4 weeks of proper hardening, the regenerated plants were planted in earthen pots filled with garden soil and thereafter pots were established in the green house where they grew well under full sun. All the tissue culture raised plants were morphologically alike with the mother plant (explant donor plant) and showed the similar growth pattern.

11.4 Conclusion

The model system developed will provide a novel concept for understanding the beneficial role of silicon in physiological and biochemical functions in plants at molecular level. The protocol established produced could be useful in enhancing and understanding the biomass production of pharmaceutically important alkaloids accumulated in *R. tetraphylla* cultures. Thus, for gaining more success in the plant tissue culture branch of plant biotechnology addition of Si with the culture medium may resolve and excel out the problems related to micropropagation.

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Conflict/Argument of Interests The author affirms that there is not any conflict of interest.

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TDZ-Induced Efficient Micropropagation from Juvenile Nodal Segment of *Syzygium cumini* (Skill): A Recalcitrant Tree

12

Afshan Naaz, Iram Siddique, and Altaf Ahmad

Abstract

An assessment was carried out to examine the TDZ (a potential cytokinin) efficacy in the induction of multiple shoot regeneration in *Syzygium cumini* from juvenile nodal (N) explants and cotyledonary node (CN) explants. TDZ in different concentration was used as a supplement to the MS basal media. Two factors viz. concentration and exposure duration to TDZ have multifaceted effect on multiple shoot induction and regeneration. Continuous presence of culture in TDZ inhibited shoot elongation and even leads to shoot fasciation. On transferring the TDZ exposed shoot clumps (4 weeks old) on hormone-free medium increased multiple shoot regeneration by threefold. Best TDZ concentration was recorded to be 5.0 μM in nodal explants while 2.5 in cotyledonary node explant. Maximum percentage response in CN explant was 75% with maximum mean shoot numbers (4.9 ± 0.67) and mean shoot length (2.90 ± 0.23 cm) while highest percent response was observed as 79% with maximum shoot numbers (4.17 ± 0.81) and maximum shoot length (2.87 ± 0.23 cm) in juvenile nodal (N) explants. The shoot clumps from CN (2.5 μM, TDZ) and juvenile nodal (5.0 μM, TDZ) when transferred onto secondary medium (hormone-free basal medium) produced mean shoot length (4.27 ± 0.34 cm) and (4.56 ± 0.35 cm) whereas mean shoot numbers noted to be (16.23 ± 0.21) and (18.34 ± 0.35), respectively. These shoot cultures are free from any discrepancy. Well-developed shootlet (3.0 cm) was successfully rooted in vitro in MS ½ and IBA with highest percent response in CN and juvenile nodal recorded to be 83% and 82% with mean root numbers (6.33 ± 0.10) and (5.99 ± 0.11) respectively and mean root length (7.19 ± 0.21 cm) and (6.79 ± 0.25 cm). The survival rate of transferred plant was nearly 70%.

A. Naaz (✉) · I. Siddique · A. Ahmad
Plant Biotechnology Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, India

I. Siddique (ed.), *Propagation and Genetic Manipulation of Plants*,
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Keywords

Carry-over effect · Secondary medium · TDZ exposure · Cotyledonary · Juvenile nodal · Acclimatization · Exposure duration

12.1 Introduction

TDZ is highly potent plant growth regulator mimicking cytokinin-like activity (Mok et al. 1982) being 20 times more effective in breaking bud dormancy (Wang et al. 1986) including recalcitrant and woody trees where other cytokinins are ineffective (Huetteman and Preece 1993). Chemically it is phenyl urea derivatives, synthesized by German Schering Corporation for defoliation of *Gossypium hirsutum* (Suttle 1985). Morphogenesis has been described as a process that occurs in three distinct stages of development, namely (a) acquisition of competence, (b) induction or determination, and (c) differentiation (Christianson and Warnick 1983). The induction process on competent tissues leads to the formation of morphogenic cells, which become determined on the morphogenic pathway. The above separation is ultimately crucial in the scaling up and mechanization of micro-propagation systems. In plant tissue culture, it has been established as a potent plant growth regulant of a wide array of plant species including trees and medicinal plant species for the development of sustainable attainable morphogenetic system. The mechanism of TDZ action is partially related to the inhibition of cytokinin degradation by cytokinin oxidase, resulting in an increased level of endogenous cytokinin (Hare and Van Staden 1994), by easy translocation of auxin within the tissue (Murthy et al. 1998) accompanied by high acid–phosphate level (Mok et al. 1987). Induction of callogenesis, embryogenesis, and caulogenesis could all be achieved by augmenting TDZ with basal media followed by transferring the culture clumps into secondary medium of desirable choice. Shoot regeneration in TDZ exposed media is concentration-dependent. It is highly stable and biologically active at lower concentration (1–5 μM). Higher concentration of TDZ implicit many morphological defects like tissue deformity, improper intermodal growth, vitrification or fasciations, and so on, in a micropropagation system, significantly affecting height of newly formed shoot.

Syzygium cumini is an evergreen tropical tree belonging to family Myrtaceae. It is one of the most widely used medicinal plant of which all of the part are being used for therapeutic use or in many industries related to food and beverage, shipping, construction, wood, and so on, and is highly recommended for rejuvenation of forestry program. Pharmaceutical research and literatures across the world show that seeds, bark, leaf extract, and fruits of the plant have great therapeutic value and are being used in Indian subcontinent as an alternative medicine for curing of diabetes mellitus (Ravikumar et al. 2012) since ages as mentioned in *Charak samhita* (ancient medical book guidance). The steroids and saponins obtained from leaf sap help in the treatment of Alzheimer disease. The plant extract is beneficial in its action for killing of cancer cells (Swami et al. 2012) with reference to the antineoplastic activities. Owing to its high medicinal value and industrial

demand economical cultivation of elite species of Indian blackberry is underway in many parts of Maharashtra particularly Vidarbha regions (Sirsat et al. 2019). There is mass-scale production of Jamun seedling of elite and economical species under government-sponsored entrepreneurship program that could be possible to generate additional revenue. However, it still lacked a proper protocol for economical and large-scale production. *S. cumini* suffers from very low seed viability and poor germination in its natural habitat (Dent 1948) and the seeds are very much prone to fungal infection (shelf life is hardly 3–4 days). Propagation through stem cuttings is an alternative, but not feasible for obtaining large quantity of planting materials. In this context, it is necessary to standardize a suitable protocol for clonal propagation of this species. Forest trees in general have proved to be difficult for mass propagation by tissue culture.

In this present communication, a reliable sustainable protocol for micropropagation from nodal and cotyledonary explants procured from 15 days old in vitro raised seedling has been presented. Very limited reports are there for the micropropagation of *S. cumini*. Given the fact that so far in vitro regeneration studies using TDZ has not been reported in *S. cumini*. The present study was aimed at working out a method of micropropagation using nodal and cotyledonary segments in response to different media and different concentrations of PGRs (TDZ, BA) for maximum plantlet production. Besides this, studies have been conducted to see the role of carryover effect on rooting acclimatization and antioxidant activity. Exposure duration of TDZ has also been taken into consideration as it had earlier been proved the damaging effect of extreme exposure on morphogenetic potential under in vitro conditions.

12.2 Materials and Methods

12.2.1 Establishment of Aseptic Culture

Shoot multiplication of *S. cumini* was evaluated by varying different parameters like type of basal media (MS and WPM), cytokinins (TDZ), and auxins (NAA and IBA) singly or in combinations during multiplication and rooting phase. In the first set of experiment, MS medium and WPM were used to identify the suitable basal media. The experimental materials (nodal explants and cotyledonary node (CN) from 15 days old seedling grown under in vitro condition in the culture room (as described by Naaz et al. 2019) were procured. The CN and nodal segments were cut (1.5 cm) from the in vitro raised seedlings under laminar flow following by inoculation into autoclaved flask (Borosil 200 mL, wide mouth) and directly inoculated into basal media with desirable hormonal concentration of TDZ (0.1–7.50 μM). MS (Murashige and Skoog 1962) and WPM (Lloyd and McCown 1981) basal media supplemented with 3% (w/v) sucrose and gelled with 0.8% (w/v) bacteriological grade agar (Qualigens, India) augmented with TDZ (0.1–7.5 μM) with the pH maintained to 5.8 using 1 N NaOH or HCl before autoclaving at 121 °C for 17 min. The explants (nodal) and CN were cultured in 50 mL flask (Borosil,

India) each containing 30 mL of medium. All the cultures were maintained at 24 ± 2 °C in a culture room illuminated by two cool white fluorescent lamps (40 W, Phillips India) with an irradiance of 50 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes (40 W, Phillips) for 16 h/day. After 2, 3, and 4 weeks of incubation, the TDZ-conditioned explants were transferred into basal medium lacking TDZ. Both the explants (nodal and CN) were inoculated in MS or WPM. Since MS response was much better than in WPM; therefore, further experiments were carried out in MS basal medium. The frequency of responding explants, the number of regenerated shoots, and shoot length were recorded after 4 weeks of incubation in the TDZ-free medium. All cultures were subcultured onto the same fresh medium after 3–4 weeks.

12.2.2 Rooting and Acclimatization

The in vitro root induction was performed by transferring the individual microshoots on full strength MS and half-strength MS medium amended with root inducing hormones namely IBA in different concentrations (1.0, 2.5, 5.0, 7.5 μM). Data on percentage of rooting and mean number of roots per shoot were recorded after 3 weeks of transplantation. All culture conditions were the same as described for the shoot bud induction. The rooted shoots were washed gently under running tap water and transplanted into 10 cm diameter thermocol cups containing autoclaved soilrite (Keltech Energies Ltd., Bangalore, India) and covered with transparent plastic bags to ensure high humidity. They were further kept at low illumination with an irradiance of 50 μmol m^{-2} s^{-1} (cool white fluorescent lamp, 40 W) for 1 week and watered regularly with half-strength MS solution lacking both vitamins and PGRs over a period of 8–10 days. The plants were then transferred to pots containing normal garden soil and maintained in greenhouse conditions.

12.3 Results and Discussion

12.3.1 Effect of TDZ on Nodal Explants

Nodal explants grown on MS medium lacking PGR did not show any morphogenic response and failed to produce shoots even after 4 weeks of incubation. However, the medium supplemented with TDZ effectively induced bud break in nodal explants at all the concentrations tested (0.1, 0.5, 1.0, 2.5, 5.0, and 7.5 μM). Explants cultured on MS and WPM basal medium alone served as control and did not promote axillary bud induction leading to necrosis. However, explants (nodal and CN) both exhibited a better response of bud break and shoot induction in MS medium to that of WPM. The recorded data indicated that MS basal medium promoted better shoot multiplication than WPM which was in contrast to earlier report by Ramashree et al. (2007). However, this result was in consonant with earlier report by Naaz et al. (2019) where MS was supposedly better than WPM. MS basal medium supplemented with TDZ

Table 12.1 Effect of TDZ on multiple shoot induction from nodal explants derived from aseptic seedlings after 4 weeks of culture

TDZ (μM)	% Response	Mean number of shoots/explant	Mean shoot length (cm)
0.0	0.0	0.0	0.0
0.1	50.23	3.56 ± 0.45^{b}	2.05 ± 0.03^{c}
0.5	68.07	$3.67. \pm 0.22^{b}$	2.10 ± 0.23^{c}
1.0	75.05	$3.89 \pm 0.62^{a,b}$	2.60 ± 0.13^{b}
2.5	75.00	4.01 ± 0.81^{a}	2.70 ± 0.22^{b}
5.0	**79.00**	**4.17 ± 0.81^{a}**	**2.87 ± 0.23^{a}**
7.5	65.26	3.50 ± 0.22^{b}	2.20 ± 0.21^{c}

Values represent means ±SE. Means followed by the same letter within columns are not significantly different ($P = 0.05$) using Duncan's multiple range test

effectively induced bud break in nodal explants at all the concentrations tested (0.1, 0.5, 1.0, 2.5, 5.0, and 7.5 μM) within 2 weeks of incubation. Shoot bud proliferation was enhanced by increasing the concentration of TDZ up to an optimal level (5.0 μM) in the MS medium beyond which there is declination in all parameters taken into consideration. The highest percent frequency (79%), maximum number (4.17 ± 0.81) of shoots per explant with shoot length (2.87 ± 0.23 cm) was obtained on MS medium containing TDZ (5.0 μM), after 4 weeks of culture (Table 12.1; Fig. 12.1a). Higher concentrations (>5.0 μM) promote callusing and fasciations of shoots in some cultures. Also, a prolonged exposure to TDZ beyond 3 weeks resulted in fasciation, stunted growth, and hyperhydric shoots and leaves (Fig. 12.1b). After an initial exposure of TDZ for 4 weeks, the shoot clumps on transferring onto secondary medium (growth regulator free) devoid of TDZ enhance shoot multiplication markedly and the culture was more healthy and vigorous without any discrepancy (Fig. 12.1c). Culture exposed to TDZ (5.0 μM) generated maximum shoots (18.34 ± 0.35)/explant with maximum shoot length (4.56 ± 0.34 cm) (Fig. 12.2). However, basal medium (WPM) along with different TDZ concentrations showed a different response. The bud break was noted at concentration of 2.5 μM and beyond. The lower concentrations only showed swollenness in the explants that later got necrosis within 4 weeks. The TDZ result was not satisfactory.

The morphogenic responses of cotyledonary node (CN) explants on MS medium supplemented with different concentrations of TDZ (0.1, 0.5, 1.0, 2.5, 5.0, and 7.5 μM) were explored and the results are summarized in Table 12.2. All the concentrations of TDZ with MS facilitated shoot bud induction. Shoot regeneration frequencies and the number of shoots per explant declined with an increasing TDZ concentration (0.5–7.5 μM) as in the case of nodal explants. Beyond the optimum level (2.5 μM) and incubation period beyond 4 weeks the culture exhibited hyperhydricity and fasciation growth cessation failing to produce healthy shoots (Fig. 12.1c). The highest regeneration frequency was recorded to be 75% with maximum mean number of shoots per explant (4.9 ± 0.67) and mean shoot length (2.90 ± 0.23 cm) (Table 12.2). Subculturing to the same medium was futile for

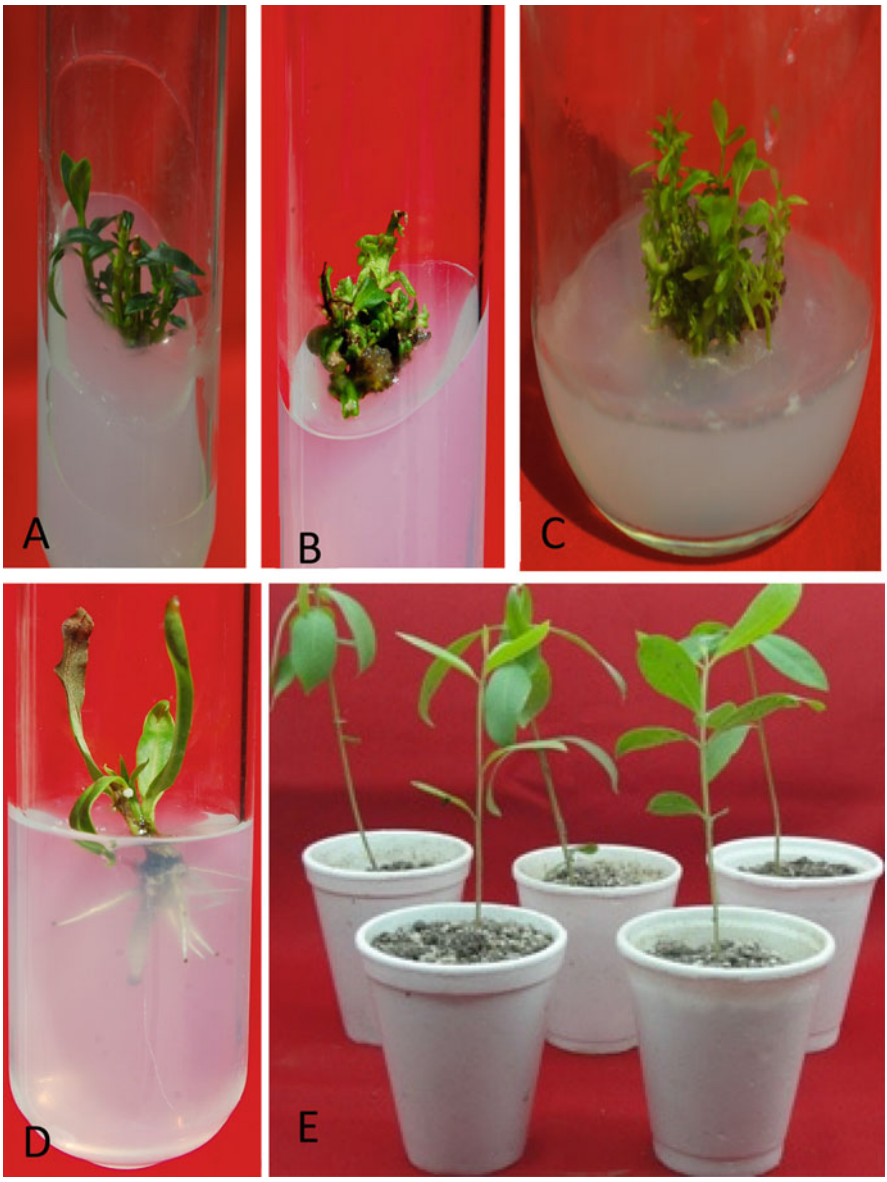

Fig. 12.1 (**a**) Induction of shoots on MS+ TDZ 2.5 (μM) in nodal explant after 4 weeks, (**b**) fasciated shoot and hyperhydricity in TDZ-mediated MS medium beyond 4 weeks of culture, (**c**) shoot multiplication and elongation on hormone-free medium after 4 weeks in CN explant, (**d**) rooting of microshoots in MS½ + IBA (**e**) acclimatized plant after 6 weeks of hardening

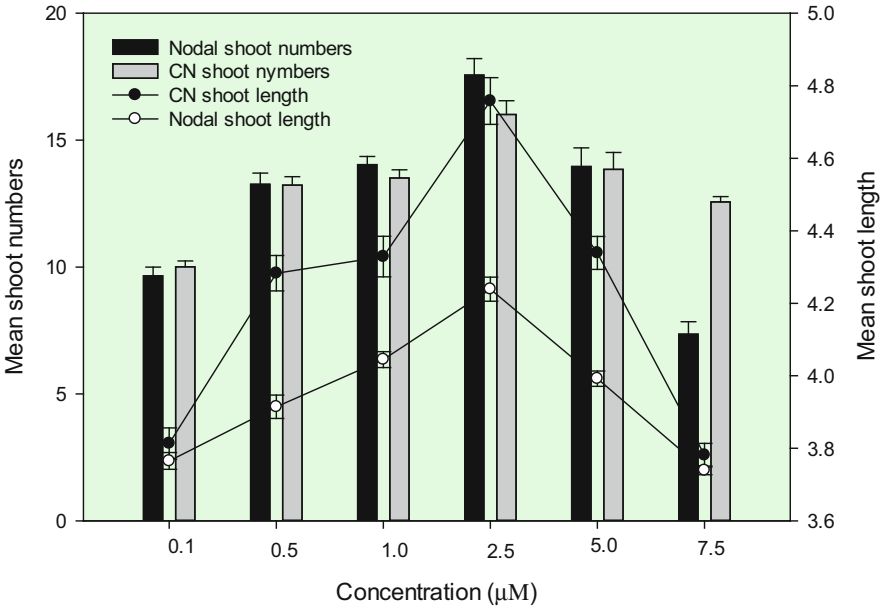

Fig. 12.2 Effect of TDZ on shoot proliferation from CN and nodal explants after 4 weeks of transfer to MS basal media (TDZ-free media). Bars represent the means ± SE. Bars denoted by the same letter within response variables are not significantly different ($P = 0.05$) using DMRT. Evaluation was made after 8 weeks of culture

Table 12.2 Effect of TDZ on multiple shoot induction from cotyledonary node (CN) explants derived from aseptic seedlings after 4 weeks of culture

TDZ (μM)	% Response	Mean number of shoots/explant	Mean shoot length (cm)
0.0	0.0	0.0 ± 0.0^{e}	0.0 ± 0.0^{d}
0.1	50.23	2.67 ± 0.34^{d}	2.05 ± 0.21^{c}
0.5	60.87	3.90 ± 0.45^{b}	2.10 ± 0.23^{c}
1.0	71.56	4.50 ± 0.22^{a}	2.60 ± 0.23^{b}
2.5	**75.04**	**4.90 ± 0.67^{a}**	**2.90 ± 0.23^{a}**
5.0	71.34	3.20 ± 0.81^{c}	2.61 ± 0.22^{b}
7.5	55.26	3.10 ± 0.22^{c}	2.20 ± 0.21^{c}

Values represent means ±SE. Means followed by the same letter within columns are not significantly different ($P = 0.05$) using Duncan's multiple range test (*DMRT*)

recovering growth in the cultures; therefore, the cultures were transferred to MS basal media devoid of TDZ. This resulted in the resurgence of shoot differentiation in the cultures. Maximum growth of shoot numbers and shoot length was recorded in cultures which were initially cultured on MS supplemented with 2.5 μM TDZ fetching about 16.23 shoots per explant with an average shoot length of 4.27 ± 0.34 cm after 8 weeks of culture (Fig. 12.2). These cultures were healthy and without any discrepancy (Fig. 12.1c). The effectiveness of TDZ in shoots bud

differentiation and subsequent regeneration has been documented in several plants including *Salix tetrasperma* (Khan and Anis 2012). This behavior is believed to be due to the potency of TDZ to increase the biosynthesis and accumulation of endogenous adenine-type cytokinins, thus, making TDZ as an effective cytokinin for the stimulation of shoot buds. The mechanism of TDZ action is partly related to the inhibition of cytokinin degradation by cytokinin oxidase, resulting in increased levels of endogenous cytokinin or may be due to enhanced accumulation and translocation of auxin (Hare and Van Staden 1994; Murch and Saxena 2001). Exposure duration also played an important role in obtaining maximum number of regenerants as reported in *Vitex trifolia* (Ahmed and Anis 2014). The explants of *S. cumini* produced the best result when cultured in TDZ medium for a maximum of 4 weeks prior to their transfer to a TDZ free basal media. A high carryover effect which enabled the shoots to continue proliferation on the hormone-free medium was also depicted by Neuman et al. 1993. The carryover effect in tissue culture is quite common especially with cytokinin. These carryover effects are visible in the form of shoot and root regeneration frequency and successful acclimatization. TDZ affects pathway of purines and cytokinin metabolism (Mok et al. 1982; Laloue and Fox 1989). It involves in modification of cell membrane, energy levels, nutrient uptake, and nutrient assimilation. Our result corroborates findings by Faisal et al. (2005).

12.3.2 Effect of Exposure Duration of TDZ

TDZ exposed culture showed time-dependency effect. On increasing the exposure duration, the highest concentration (10.0 μM) did the maximum damage to the tissues while lower concentration (less than 5.0 μM) could sustain the tissue lines even after 8 weeks exposure (data not shown). A 4 week exposure to the optimal TDZ concentration (5.0 μM) was preferred as this culture regime amounted to maximum shoot yield per explant (14.91 ± 1.10) on transfer to secondary medium (devoid of growth regulator) after 4 weeks (Fig. 12.3). An exposure duration exceeding 4 weeks elicited lower shoot induction afterwards. Up to maximum exposure duration of 6 weeks, nodal explants expressed regeneration capability to produce new shoots. The exposed tissues turned brown and necrotic after 6 weeks and no further regeneration was observed on changing the media. Moreover, continuous exposure of culture (more than 4 weeks) on TDZ led to abnormal shoot morphology, fasciation, and distortion (Fig. 12.1b) while on the other hand, shorter exposure time (2 weeks) could not elicit proper bud break and thus was incapable to produce sufficient and desired results. Our result is in congruence with Khan and Anis (2012) (Fig. 12.2).

12.3.3 Effect of Subculturing

TDZ containing media could not affect multiplication and elongation of induced shoots. Therefore these TDZ exposed shoots were further transferred in secondary

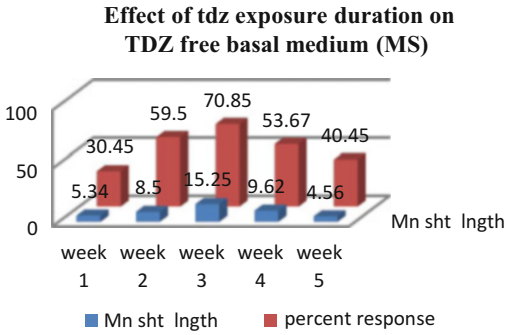

Fig. 12.3 Effect of TDZ-exposure duration on TDZ free medium (MS) on nodal explants

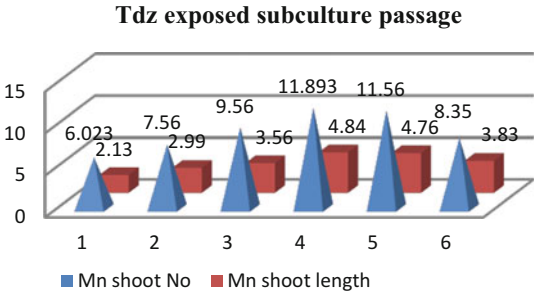

Fig. 12.4 Effect of TDZ exposure duration of nodal culture on TDZ-free basal medium (MS)

medium for their further growth and proliferation. In the present study, the optimal TDZ (2.50 and 5.0 μM) exposed explants (CN and nodal) carry a high potential of multiplication on a basal media devoid of any growth regulators. This transfer in secondary medium after TDZ exposure greatly reduced the morphological and physiological disorders and increased the regeneration potential of the explants. Our result corroborates earlier findings by Khan and Anis (2012) and Dewir et al. (2018). In the first four subculturing passages, the rate of multiplication of shoot buds was found to increase and beyond which decline in multiplication rate was noticed. The well-developed shoots were regularly removed and transferred to rooting medium (Fig. 12.4).

12.3.4 In Vitro Rooting and Acclimatization

Rooting of in vitro regenerated shoots and transplantation of the plantlets to the field is the most important, crucial, and essential step (Pospisilova et al. 1999), but difficult task in tissue culture of woody trees. In the present study, optimization of rooting response of micro shoots of *S. cumini* was achieved using different auxins at varying concentration. Translocation of IBA and IAA has a very critical role in rooting response in woody tree species due to complex mechanism involved wherein carryover effect of the previously induced cytokinins during multiplication phase

also effect rooting system of the newly developed plantlets. (Rashotte et al. 2003). The regenerated shoots measuring 3.4 cm were excised from the regenerated tissue and planted on the rooting medium. MS (full and half strength) medium without any growth regulator failed to induce root formation even after 4 weeks of transfer. To facilitate the induction of root, MS basal and half strength medium were augmented with an auxin (IBA) at different concentration (1.0, 2.5, 5.0, 7.5 μM) with 3% sucrose. Of the various treatments evaluated for in vitro rooting, the best rooting response in CN derived microplants was observed in half-strength MS along with 5.0 μM IBA, giving 83% response with average number of 6.0 roots with a length of 7.0 cm approx. (Table 12.3; Fig. 12.1d). Initiation of roots was observed after 7 days of culture. Half-strength MS showed promotory effect on rooting response in comparison with full strength MS. Whereas microshoots derived from nodal explants showed percentage response of 82% and highest shoot numbers to be six with maximum shoot length 6.79 ± 0.25 cm (Table 12.3). IBA has often been used in plant tissue culture because of its easy translocation (Davies et al. 1982; Rashottte et al. 2003). Our reports are in consonance with earlier reports in *Sterculia urens* (Hussain et al. 2008), *Balanites aegeptiaca*, and *S. cumini* (Naaz et al. 2014) who supported reduced salt concentration for better rhizogenesis.

Acclimatization of plants grown in vitro to natural conditions is a crucial step for many plant species. Due to heterotrophic mode of nutrition, lack of adaptation or exposure to the outside environment (excessive temperature and humidity, atmospheric CO_2, mineral nutrition, pH, and texture of soil) affects the regenerated plantlets. Maximum acclimatization percentage was noted to be 70% in soilrite (Fig. 12.1e). These results are in congruent with the findings of Anis et al. (2010) and Perveen and Anis (2015), where regenerants showed highest survival in soilrite. The plants appeared morphologically healthy with normal leaf, shape, and growth patterns.

12.4 Conclusion

The present communication describes the effectiveness of TDZ as potent cytokinin for bud breaking, shoot multiplication, healthy culture, and acclimatization in *S. cumini* (L.). The TDZ-exposed cultures could induce better shoot regeneration on being transferred to hormone-free basal medium thereby protecting the juvenile shootlet from carryover effect of the hormones. There are also better survival and adaptation of the juvenile plants to the harsh weather and high sunlight and humidity outside. The protocols could be efficiently used for large-scale regeneration of other woody and recalcitrant species to produce disease-free clones without any seasonal variation. Ex vitro acclimatization is the final step that owes a major influence on any micropropagation system. In many cases, this is a major bottleneck and needs to be approached carefully, considering carryover effects and stress tolerance from the in vitro phase toward the external environmental condition. Numerous protocols have exemplified that TDZ can successfully replace the commonly used CKs (BA, KN, Zeatin, 2ip) in many plant tissue culture protocols and can even produce better

Table 12.3 Effect of IBA on root regeneration from nodal explants on ½ MS medium after 4 weeks of culture

Treatments (μM)	IBA (μM)	Response (%)		Mean root number		Mean root length (cm)	
		Nodal (N)	CN	Nodal (N)	CN	Nodal (N)	CN
½ MS	0.0	0.0 ± 0.0^{e}	0.0 ± 0.0^{e}	0.0 ± 0.0^{e}	0.0 ± 0.0^{e}	0.0 ± 0.0^{e}	0.0 ± 0.0^{e}
½ MS	1.0	$75.05 \pm 0.67^{d,e}$	$73.09 \pm 0.43^{d,e}$	2.50 ± 0.10^{d}	2.67 ± 0.11^{d}	5.20 ± 0.17^{b}	4.80 ± 0.19^{b}
½ MS	2.5	80.02 ± 0.41^{b}	80.12 ± 0.23^{b}	4.10 ± 0.15^{b}	4.04 ± 0.25^{b}	6.03 ± 0.18^{c}	5.63 ± 0.28^{c}
½ MS	**5.0**	$\mathbf{83.05 \pm 0.18^{a}}$	$\mathbf{82.75 \pm 0.23^{a}}$	$\mathbf{6.33 \pm 0.10^{a}}$	$\mathbf{5.99 \pm 0.11^{a}}$	$\mathbf{7.19 \pm 0.21^{a}}$	$\mathbf{6.79 \pm 0.25^{a}}$
½ MS	7.5	78.03 ± 0.54^{c}	77.73 ± 0.22^{c}	2.45 ± 0.15^{c}	2.56 ± 0.25^{c}	$6.05 \pm 0.23^{b,c}$	$6.01 \pm 0.13^{b,c}$

Values represent means ±SE. Means followed by the same letter within columns are not significantly different ($P = 0.05$) using Duncan's multiple range test (*DMRT*)

results where the routinely used CKs are not much of success and have inhabitant effect at various steps in woody tree species. The effectiveness of TDZ lies in the fact that they can play pivotal roles on some physiological response or disorders. Beside shoot multiplication carryover effect should be taken into consideration as accumulation of hormones in the tissue culture shootlets inducing toxicity, which inhibits proper growth of cultures as well as rooting and acclimatization process as a whole. Such an approach will elucidate us solving many constraints associated with physiological and developmental problems in vitro of woody tree species.

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