J. Crop Sci. Biotech. 2010 (March) 13 (1) : 13 ~ 20
DOI No. 10.1007/s12892-009-0127-3

RESEARCH ARTICLE

High Frequency Direct Plant Regeneration, Micropropagation and Shikonin Induction in *Arnebia hispidissima*

Minakshi Pal, Ashok Chaudhury*

Department of Bio & Nano Technology, Guru Jambheshwar University of Science & Technology, Hisar-125001 (Haryana), India

Received: September 22, 2009 / Accepted: November 26, 2009

Abstract

The data presented herein reports a rapid and efficient method for direct plant regeneration at high frequency without intervening callus formation from shoot tip (93%) and nodal segment (60%) cultured on MS media supplemented with 0.5 mg l^{-1} KIN, 0.25 mg l^{-1} BAP, 0.1 mg l^{-1} IAA and 100 mg l^{-1} CH. Conversely, leaf and internodal explants were poorly responsive. Adventitious shoot buds arose not only from the cut ends but all along the surface of the explants leading to the formation of clusters with multiple shoots. Multiple shoots upon transfer to MS media supplemented with 2.0 mg l^{-1} IBA induced efficient rooting (80%). *In vitro* flowering was observed when tissue culture-raised plantlets were maintained for extended period in culture. Shikonin was induced in roots of regenerated plants which often exudates in the culture medium was quantified spectrophotometerically by recording absorbance at 620 nm and estimated to be 0.50 mg g^{-1} fresh weight of tissue at the end of the 50 days of culture. The regenerated plants were successfully acclimatized, hardened, and transferred to soil in green house for micropropagation. The protocol developed here will be very useful for the supply of *Arnebia hispidissima* all year as a raw product necessary for obtaining Shikonin for the cosmetic, dyeing, food, and pharmaceutical industries.

Key words: *Arnebia hispidissima*, callus, direct plant regeneration, *in vitro*, shikonin

Abbreviations: BAP-6-benzylaminopurine; CH-Casein hydrolysate; IAA-Indole acetic acid; IBA-Indole butyric acid; KIN-Kinetin; NAA-naphthalene acetic acid.

Introduction

The genus *Arnebia* belongs to the family *Boraginaceae* comprising nearly twenty-five different species which includes a variety of herbs, shrubs, and trees, mostly confined to Asia with a few species occurring in the drier parts of North Africa. Seven species are known to occur in India which includes *A. benthami, A. euchroma, A. guttata, A. hispidissima, and A. nobilis*. Plants of the genus *Arnebia* as well as some other species of *Boraginaceae* belonging to genera *Echium, Lithospermum,* and *Onosma* are the source of naphthoquinone-Shikonin known since ancient times as a red-colored dye used for the silk and food industries.

Minakshi Pal, Ashok Chaudhury (✉)
E-mail: ashokchaudhury@hotmail.com
Phone: +91-1662-263306
Fax: +91-1662-276240

Shikonin is known for its tremendous application in the food, health care, and pharmaceuticals industries and possesses antibacterial and antifungal activities and exhibits anti-inflammatory and wound-healing properties. It is predominantly produced in the roots in field-grown plants. Plant cell culture by way of direct plant regeneration offers an attractive source for the production of high-value secondary metabolites. The type and concentration of plant growth regulators are often crucial factors for Shikonin production. Large numbers of herbal, medicinal, ornamental, and tree species serve as excellent sources of a wide variety of secondary metabolite products which offer a number of applications from pharmaceutical and agricultural industries as a food additive. Most plants from which important pharmaceutical compounds have been isolated are growing either in the wild or on large-scale plantations. Nevertheless, it would be of particular interest to the pharmaceutical industry, if

a Factory Type-production was possible in plant bioreactors. An additional problem could be that the places where the plants have been growing are often far from the places where the plants are processed for drug production. Better quality control, higher flexibility to the demand, and more independence are crucial factors. The production of secondary metabolites by the plant cell cultures including direct plant regeneration could, therefore, be an interesting alternative, as many of the deficient problems could be addressed. Thus, cultured plant cell have proven to be a convenient tool for studying basic aspects of plant cell development and metabolism. Wild plant species from the *Boraginaceae* family, accumulating Shikonin fail to provide sufficient raw material for commercial production, owing to which plant tissue culture techniques of direct plant regeneration offer excellent avenues for overcoming this shortfall with respect to increasing demand.

Shikonin is a red dye known to possess antibacterial, antifungal, anti-ulcer activity, anti-allergic, antipyretic, and antihydropic effects, as well as antineoplastic activities and as a medicine for treating a variety of ailments (Hayashi et al. 1969; Khatoon et al. 1994, 2003; Papageorgiou 1978, 1980; Papageorgiou et al. 1999; Terada et al. 1990). Shikonin and its derivatives are predominantly predisposed in the outer surface of mature roots of *Arnebia* and its production depends upon the age of plant and other environmental factors. Middle-aged plants with 6-8 flowering stems, show the highest level of Shikonin induction (Pimenova and Tareeva 1980). Other plant species belonging to the *Boraginaceae* family such as *A. benthamii, A. euchroma, A. guttata, A. nobilis, A. sexatili,s* and *Lithospermum erythrorhizon* also serve as a source of Shikonin (Fukui et al. 1983; Tsukada et al. 1983).

The choice of starting plant material and the media combination employed particularly the type and concentration of plant growth regulators influence the level of secondary metabolite productions. Fujita et al. (1981a, b, 1982, 1983) compared several culture media and reported efficient production of Shikonin by cell suspension culture of *L. erythrorhizon* which resulted in commercial production of Shikonin by Mitsui Petrochemical Company, Japan in 1983. Cell suspension cultures of *L. erythrorhizon* for production of Shikonin was reported by Kim and Chang (1990), Sokha et al. (1996), Yu et al. (1997), and from *A. euchroma* by Davydenkov et al. (1991), Dong et al. (1993), and Sokha et al. (1996). Root-specific production of Shikonin and its regulation by light was reported by Yazaki et al. (2001) in *L. erythrorhizon*. Shikonin production from 0.3-0.4 mm shoot apex-derived, tissue-cultured plants was reported in a Japanese Patent by Toshiro (2001) in *L. erythrorhizon*. Production of Shikonin from seed-derived calli and leaf and root explants obtained from germinated seeds of *A. euchroma* was reported by Ge et al. (2004) and Manjkhola et al. (2005). Estimation of Naphthaquinones from dried roots of mature plants and calli derived from seed in *A. hispidissima* was reported by Singh et al. (2004) but lacks data on plant regeneration. Quite often, the callus and cell suspensions employed for commercial production of secondary metabolites is difficult to establish and maintain, and suffers from Somaclonal variation requiring high degree of *in vitro* culture expertise. Furthermore, due to the fast rate of growth of suspension cells and the callus in the presence of varying concentrations of plant growth regulators, a high level of productivity must be maintained by repeated selection leading to the instability of the cultures which might be due to the presence of many chemotypes within the original cell aggregates.

Therefore, direct plant regeneration without the intervening callus formation by way of direct shoot-bud induction from the inoculated explants through *in vitro* culture offers tremendous applications in circumventing the above problems of callus and cell suspension cultures for the production of secondary metabolites. The protocols help in producing high frequency, direct-plant regeneration from various explants which are true-to-type as well as in maintaining clonal fidelity. A few selective examples of direct plant regeneration of other plant species such as shoot tip cultures of *Oenothera erythrosepala* for the production of Oenothein B (Suzuki et al. 1990); seedling explants of pigeonpea (*Cajanus cajan* L.) (Geetha et al. 1998); zygotic embryos hypocotyls of Tunisian pepper (Arous et al. 2001); a single source lamina at the optimal stage (Martin et al. 2003); immature seeds of *Garcinia indica* popularly known as kokam butter a medicinal tree species (Thengane et al. 2006) in a two-step method; nodal cuttings of *Sarcostemma brevistigma*, a rare medicinal plant popularly known as Somlata (Thomas and Shankar 2008); simple and reproducible method for high frequency direct plant regeneration from leaf, node, internode, and root explants of *Populus deltoides* from our laboratory (Yadav et al. 2009) leading to formation of multiple shoot clusters have been reported.

Even though tissue cultures of *A. euchroma* and *A. hispidissima* were first reported by Davydenkov et al. (1991) and Singh et al. (2004), no detailed studies have so far been reported on high frequency, direct-plant regeneration, callus induction, and Shikonin production in this commercially important medicinal plant of *A. hispidissima*. This is the first report of high frequency, direct-plant regeneration from various explants such as shoot tip, nodal segment, leaf, and internodes, as well as micropropagation and induction of Shikonin production in *A. hispidissima*.

Materials and Methods

Initiation and establishment of in vitro culture

Field-grown plants of A. *hispidissima* obtained from the Northeast location of Teaching Block-1 of Guru Jambheshwar University of Science & Technology, Hisar, Haryana, India during the months November-March (only available during this period) for 2000-2004 were employed as shown in Fig. 1a.

Field-grown plants of *A. hispidissima* washed in running tap water along with a drop of Teepol were rinsed in double distilled water. Explants such as shoot tips (5-8 mm– long) consisting of 10-15 developing leaves surrounding the meristem; young leaves from the tip of the main branch, and nodal segment and internodal segments from third to sixth node from the tip of the branch were excised out and surface sterilized with 0.1% w/v mercuric chloride for 5-7 min and, subsequently washed three times with sterilized, double-distilled water as described by Chaudhury and Pal (2004, 2005) and Pal (2005).

Direct plant regeneration

For direct-plant regeneration, four to five segments of surface sterilized explants of shoot tips, nodal, internodal, and leaf segments were inoculated in 90 mm Petri dishes containing MS medium supplemented with different combinations of auxins and cytokinins either alone or in combination: 0.1-1.0 mg l^{-1} IAA, 0.25-2.0 mg l^{-1} KIN, 0.1-2.0 mg l^{-1} BAP, and 100.0 mg l^{-1} CH coded as $MSAD_0$ to $MSAD_{96}$. All the cultures were incubated in culture room with temperature of 25±2℃, relative humidity of 50-60% and 16 h light/8 h dark cycle at light intensity of 25 µmol m^{-2} s^{-1}. The experiment was conducted in three replicates. Number of explants showing direct-shoot bud initiation and percent explants showing direct plant regeneration were recorded. The regenerated *in vitro*-grown plants were cultured on full-strength or half-strength MS media supplemented with different combinations of auxins and cytokinins either alone or in combination: 0.25-3.0 mg l^{-1} IBA, 0.5-2.0 mg l^{-1} KIN, and 0.5-2.0 mg l^{-1} BAP coded as $MSAR_0$ to $MSAR_{23}$ for induction of rooting.

Acclimatization, hardening and micropropagation

Plantlets with four or more leaves and three to four roots were washed in running tap water to remove the adhering agar and transferred to pots in different potting mixtures of sand and soil in the ratio of 1:1; sand and vermiculite compost in the ratio of 2:1. The plants were acclimatized and hardened in green house with 80-90% RH, 25 ^{0}C and subsequently maintained in earthen pots for micropropagation.

Analysis of shikonin content

Shikonin induced in roots of regenerated plants was quantified as per the method proposed by Fukui et al. (1998), Mizukami et al. (1977), and Yazaki et al. (1998) and with slight modifications. Roots derived from regenerated plants were homogenized in a pestle mortar containing chloroform, allowed to stand for at least 1 day in the dark, and then the homogenate was filtered as shown in Fig. 1i. Filtrate so obtained was dried with magnesium sulphate ($MgSO_4$), filtered, and evaporated to dryness in vacuum. A 5 ml 2.5% KOH solution was added followed by vigorous shaking for 10 min leading to the appearance of blue color as shown in Fig. 1j. Shikonin content was estimated by employing UV-visible spectrophotometer (ECIL, Hyderabad, India) and absorbance was measured at 622 nm and expressed as mg g^{-1} fresh weight.

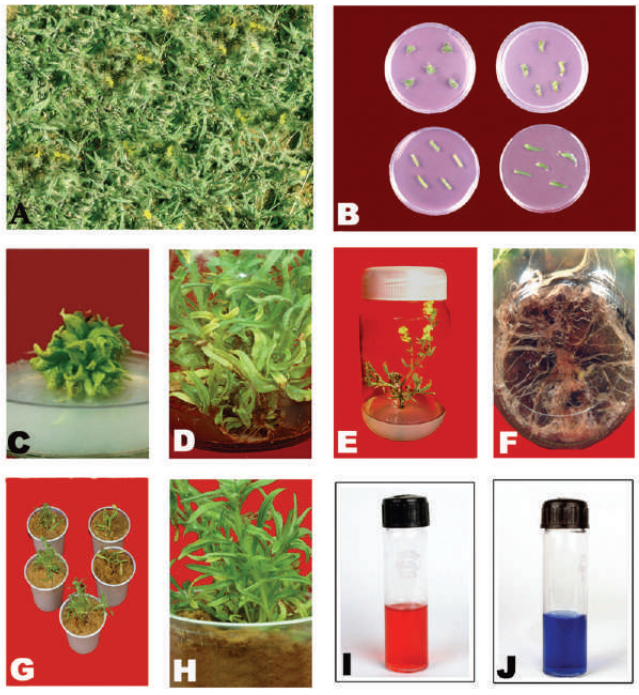

Fig. 1. High frequency direct plant regeneration, shoot bud induction, micropropagation, and induction of Shikonin production from shoot tip, nodal, leaf, and internodal segments in *Arnebia hispidissima*. a *Arnebia hispidissima* plants growing under field conditions which served as a source of explants; b Inoculation of surface sterilized explants viz; shoot tip, nodal, leaf, and internodal segments (clockwise), c Direct plant regeneration and shoot bud induction leading to the formation of cluster from shoot tip explants on MS media ($MSAD_{26}$) supplemented with 0.5 mg l^{-1} KIN, 0.25 mg l^{-1} BAP, 0.1 mg l^{-1} IAA, and 100 mg l^{-1} CH; d *In vitro* regenerated plants with multiple shoot and roots with massive accumulation of red pigment showing induction of Shikonin production; e *In vitro* regenerated plants showing characteristic yellow flower bloom; f Massive root formation on MS medium supplemented with IBA, 2.0 mg l^{-1} showing induction of Shikonin production; g Regenerated plants transferred to pots having sand and vermiculite 2:1 for hardening and acclimatization; h Well established plants after 6-8 weeks of hardening under field conditions; i Solution of extracted Shikonin and its derivatives in Chloroform; Solution of extracted Shikonin and its derivatives in 2.5% KOH.

Results and Discussion

Direct plant regeneration

Various explants of *A. hispidissima*: shoot tip, nodal, leaf, and internodal segments were employed as shown in Fig. 1b. Surface-sterilized explants were cultured on MS media supplemented with various plant growth regulators for direct plant regeneration. Within two weeks of culture, shoot tip and nodal segments began to swell and showed signs of adventitious bud and meristemoid formation. These adventitious shoot buds arose not only from the cut ends but all along the surface of explants.

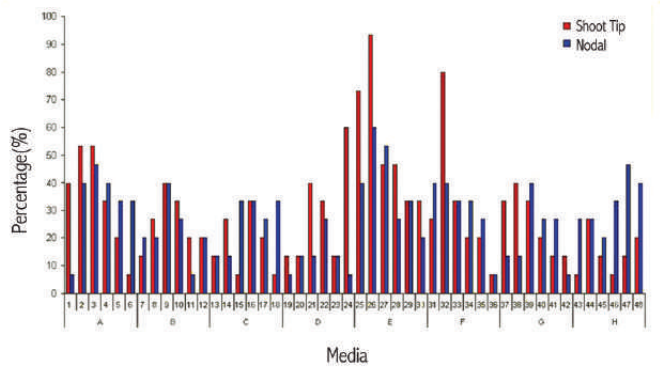

Fig. 2a. Effect of plant growth regulators on *in vitro* induction and direct plant regeneration (%) from shoot tip and nodal segments of *Arnebia hispidissima*.

A. Media $MSAD^{1}$ to $MSAD_{6}$ (MS + KIN 0.25 mgl^{-1} + BAP (0.1, 0.25, 0.5, 1.0, 1.5, and 2.0) mgl^{-1} + IAA 0.1 mg l^{-1} + CH 100.0 mg l-1) (1-6).
B. Media $MSAD_{7}$ to $MSAD_{12}$ (MS + KIN 0.25 mg l^{-1} + BAP (0.1- 2.0) mg l^{-1} + IAA 0.25 mg l^{-1} + CH 100.0 mg l^{-1}) (7-12).
C. Media $MSAD_{13}$ to $MSAD_{18}$ (MS + KIN 0.25 mg l^{-1} + BAP (0.1- 2.0) mg l^{-1} + IAA 0.5 \ mg l^{-1} + CH 100.0 mg l^{-1}) (13-18).
D. Media $MSAD_{19}$ to $MSAD_{24}$ (MS + KIN 0.25 mg l^{-1} + BAP (0.1- 2.0) mg l^{-1} + IAA 1.0 mg l^{-1} + CH 100.0 mg l^{-1}) (19-24).
E. Media $MSAD_{25}$ to $MSAD_{30}$ (MS + KIN 0.5 mg l^{-1} + BAP (0.1- 2.0) mg l^{-1} + IAA 0.1 mg l^{-1} + CH 100.0 mg l^{-1}) (25-30).
F. Media $MSAD_{31}$ to $MSAD_{36}$ (MS + KIN 0.5 mgl^{-1} + BAP (0.1- 2.0) mg l^{-1} + IAA 0.25 mg l^{-1} + CH 100.0 mg l^{-1}) (31-36).
G. Media $MSAD_{37}$ to $MSAD_{42}$ (MS + KIN 0.5 mg l^{-1} + BAP (0.1- 2.0) mg l^{-1} + IAA \0.5 mg l^{-1} + CH 100.0 mg l^{-1}) (37-42).
H. Media $MSAD_{43}$ to $MSAD_{48}$ (MS + KIN 0.5 mg l^{-1} + BAP (0.1- 2.0) mg l^{-1} + IAA 1.0 mg l^{-1} + CH 100.0 mg l^{-1}) (43-48).

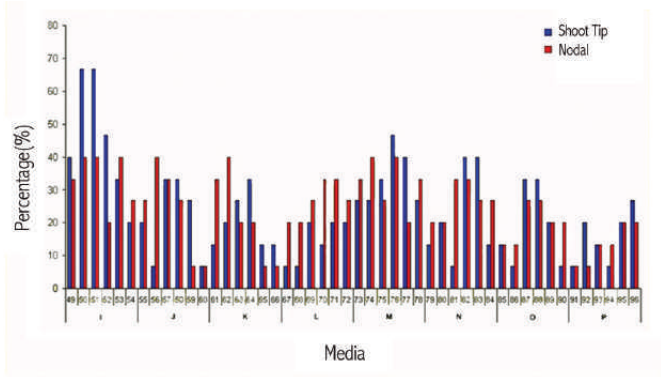

Fig. 2b. Effect of plant growth regulators on *in vitro* induction and direct plant regeneration (%) from shoot tip and nodal segments of *Arnebia hispidissima*.

I. Media $MSAD_{49}$ to $MSAD_{54}$ (MS + KIN 1.0 mg l^{-1} + BAP (0.1, 0.25, 0.5, 1.0, 1.5, and 2.0) mgl^{-1} + IAA 0.1 mg l^{-1} + CH 100.0 mg l^{-1}) (49-54).
J. Media $MSAD_{55}$ to $MSAD_{60}$ (MS + KIN 1.0 mg l^{-1} + BAP (0.1- 2.0) mg l^{-1} + IAA 0.25 mg l^{-1} + CH 100.0 mg l^{-1}) (55-60).
K. Media $MSAD_{61}$ to $MSAD_{66}$ (MS + KIN 1.0 mg l^{-1} + BAP (0.1- 2.0) mg l^{-1} + IAA 0.5 mg l^{-1} + CH 100.0 mg l^{-1}) (61-66).
L. Media $MSAD_{67}$ to $MSAD_{72}$ (MS + KIN 1.0 mg l^{-1} + BAP (0.1- 2.0) mg l^{-1} + IAA 1.0 mg l^{-1} + CH 100.0 mg l^{-1}) (67-72).
M. Media $MSAD_{73}$ to $MSAD_{78}$ (MS + KIN 2.0 mg l^{-1} + BAP (0.1- 2.0) mg l^{-1} + IAA 0.1 mg l^{-1} + CH 100.0 mgl^{-1}) (73-78).
N. Media $MSAD_{79}$ to MSAD84 (MS + KIN 2.0 mg l^{-1} + BAP (0.1- 2.0) mg l^{-1} + IAA 0.25 mg l^{-1} + CH 100.0 mg l^{-1}) (79-84).
O. Media $MSAD_{85}$ to $MSAD_{90}$ (MS + KIN 2.0 mg l^{-1} + BAP (0.1- 2.0) mg l^{-1} + IAA 0.5 mg l^{-1} + CH 100.0 mg l^{-1}) (85-90).
P. Media $MSAD_{91}$ to $MSAD_{96}$ (MS + KIN 2.0 mg l^{-1} + BAP (0.1- 2.0) mg l^{-1} + IAA 1.0 mg l^{-1} + CH 100.0 mg l^{-1}) (91-96).

At the end of 4-6 weeks of culture, proliferated further and resulted in the formation of clusters as shown in Fig. 1c. Adventitious budding and direct plant regeneration was only obtained from shoot tip and nodal explants with varying degrees, while leaf and internodal explants failed to give shoot-bud initiation on all the media combinations ($MSAD_{0}$ to $MSAD_{96}$) employed. The data on direct-plant regeneration from shoot tip and nodal segments of *A. hispidissima* using various media combinations are shown in Fig. 2.

Shoot tips cultured on $MSAD_{26}$ (MS + KIN 0.5 mg l^{-1} + BAP 0.25 mg l^{-1} + IAA 0.1 mg l^{-1} + CH 100 mg l^{-1}) medium produced 93.3% direct shoot regeneration with multiple shoots having short hairs mixed with bristly, bulbous-based hairs (Fig. 2). Leaves of length 1.5-5 cm were linear and lanceolate and developed directly from the explant without callus production (Figs. 1c-d). $MSAD_{26}$ media combination also resulted in 60% direct shoot regeneration from nodal segment (Fig. 2). Thus, the ideal combination of BAP, KIN, and IAA has been identified for effective and high frequency, direct shoot-bud induction and plant regeneration from various explants without the intervening callus. BAP alone was ineffective in inducing shoot proliferation.

Tissue culture response, intensity of shoot development, and quality of shoot (as measured in the case of dividing shoot cultures, shoot elongation, and manifestation of abnormalities such as fasciations, vitrificaton, and etiolation) was recorded weekly up to 8 weeks. The numbers of shoot regenerated from various explants were analyzed after 4 weeks. This experiment was repeated three times. Flowering was also induced in some of the in vitro cultures when plants were maintained for a longer period of time on $MSAD_{26}$ (MS + KIN 0.5 mg l^{-1} + BAP 0.25 mg l^{-1} + IAA 0.1 mg l^{-1} + CH 100 mg l^{-1}) medium (Fig. 1e). The calyx of the *in vitro*-induced flower was 6-8 mm-long divided almost to base, very hispid, and the corolla was yellow, densely pubescent outside at the base and apex. Stamens were inserted at or in the throat and half exerted in the short-style flowers, stigma was small, flattened on the inside and rounded outside. The induced flowering appeared phenotypically similar to the ones in the field-grown plants. The data revealed that shoot tip explants were more responsive than nodal explants for generating direct shoot-bud initiation and plant regeneration. On the other hand, internodal explants and leaf segments hardly showed any direct shoot-bud induction even under all the media combinations and culture room conditions employed in the present investigation.

In vitro rooting and micropropagation

Different media combinations $MSAR_{0}$ to $MSAR_{23}$ were employed for *in vitro* rooting of plantlets regenerated through direct shoot regeneration as shown in Fig. 3. It is evident from the data that maximum rooting (80.0 + 0.11%) was obtained on

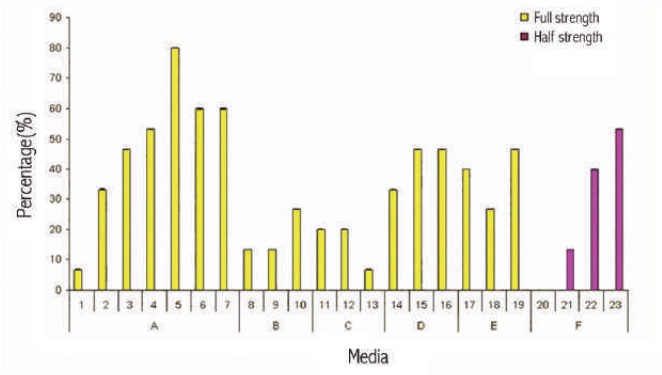

Fig. 3. Effect of plant growth regulators on *in vitro* induction of roots (%) for plants regenerated through direct shoot bud induction in *Arnebia hispidissima*.

A. Media $MSAR_1$ to $MSAR_7$ (MS Full strength + IBA (0.25, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0) mg l^{-1} (1-7).
B. Media $MSAR_8$ to $MSAR_{10}$ (MS Full strength + KIN (0.25, 1.0, and 2.0) mgl^{-1} + IBA 1.0 mg l^{-1} (8-10).
C. Media $MSAR_{11}$ to $MSAR_{13}$ (MS Full strength + BAP (0.25, 1.0, and 2.0) mgl^{-1} + IBA 1.0 mgl^{-1} (11-13).
D. Media $MSAR_{14}$ to $MSAR_{16}$ (MS Full strength + KIN (0.25-2.0) mg l^{-1} + IBA 2.0 mg l^{-1} (14-16).
E. Media $MSAR_{17}$ to $MSAR_{19}$ (MS Full strength + BAP (0.25-2.0) mg l^{-1} + IBA 2.0 mg l^{-1} (17-19).
F. Media $MSAR_{20}$ to $MSAR_{23}$ (MS Half strength + IBA (0.5, 1.0, 1.5, and 2.0) mg l^{-1} (20-23).

MSAR5 (MS + IBA 2.0 mg l^{-1}) medium on which efficient rooting took place after 10-15 days from the culture of plantlets on rooting medium (Fig. 1f). No rooting occurred before 10 days and after 20 days of anchoring the plantlets in rooting media $MSAR_0$ (full strength) and $MSAR_0$ (half strength) without plant growth regulators serving as controls. Very poor rooting (06.7 + 0.07%) was obtained on MSAR1 (MS + IBA 0.25 mg l^{-1}) and $MSAR^{13}$ (MS + BAP 2.0 mg l^{-1} + IBA 1.0 mg l^{-1}) medium. A slightly less efficient rooting (60.0 + 0.13%) was obtained on $MSAR_6$ (MS + IBA 2.5 mg l^{-1}) and $MSAR_7$ (MS + IBA 3.0 mg l^{-1}) media combinations. The number of rooted shoots and quality of roots was recorded after 3-4 weeks of culture. Among the various auxins employed, IBA was the most effective in inducing roots. Thus, the ideal media combination for root induction was found to be $MSAR_5$ medium. Furthermore, Shikonin production was observed in the induced roots. There was a higher amount of Shikonin production in older roots compared to younger ones. Hormones, namely, IAA and NAA, are less effective for root induction. It was also observed that addition of charcoal (0.1%) in both MS media (full strength) and MS media (half strength) with IBA inhibited the Shikonin production (data not shown).

The potting mixtures composed of sand and vermiculite compost mixed in ratio 2:1 was found to be the best compared to soil plus sand (1:1) mixtures (Figs. 1g-h). The percentage survival of plantlets varied from 55-75% in sand and vermiculite mixtures (2:1) as compared to soil plus sand (1:1) mixtures in which a survival of 30-50% was achieved.

In *A. hispidissima* shoot tip explants, MS medium supplemented with BAP (0.25 mg l^{-1}), IAA (0.1 mg l^{-1}), KIN (0.5 mg l^{-1}), and CH (100.0 mg l^{-1}) was found to be optimum for direct plant regeneration. Similarly, shoot tip cultures of *O. erythrosepala* on MS medium supplemented with BAP (1.0 mg l^{-1}), NAA (0.1 mg l^{-1}), or IAA (0.1 mg l^{-1}), proved to be a fast method of propagation in plants with a high content of active Oenothein B (Suzuki et al. 1990). Conversely, tissue culture of *O. paradoxa* showed the highest regeneration from shoot tip explant on MS medium containing BAP (2.0 mg l^{-1}) and IAA (0.1 mg l^{-1}). MS medium supplemented with NAA (2.0 mg l^{-1}) was the best substrate for rooting in *O. erythrosepala*, whereas, *O. paradoxa* showed the best rooting on IAA (2.0 mg l^{-1}). In the present investigation, *A. hispidissima* showed best rooting on IBA (2.0 mg l^{-1}) instead of IAA or NAA. *O.* species micropropagation from both shoot tip and nodal segment follow a similar pattern as reported herein for the *A. hispidissima*, as a result of adventitious budding all along the surface of the explants leading to a massive proliferating cluster of shoots. Eight to fourteen shoots were reported from a single explant in *Oenothera* species within 6-7 weeks.

Plant growth regulators used for direct plant regeneration in flowering plant *Anthurium andraeanum* were similar to that of *A. hispidissima* but the ion concentration was different. In *Anthurium andraeanum*, 300 plantlets were harvested within 200 days using a single-source lamina at the optimal stage (Martin et al. 2003). The lamina explant grown on half-strength MS medium supplemented with BAP (0.25 mg l^{-1}), IAA (0.2 mg l^{-1}), and KIN (0.1 mg l^{-1}) were reported to be optimal for direct shoot regeneration (Martin et al. 2003). Similarly, micropropagation protocol from nodal cuttings of *S. brevistigma*, a rare medicinal plant popularly known as Somlata on MS medium supplemented with 4 µm BAP leading to multiple shoot induction and callus regeneration has been reported by Thomas and Shankar (2008).

Hence, the data presented here for direct plant regeneration and micropropagation in *Arnebia hispidissima* are quite comparable to the other plant species reported earlier. Thus, the simple and reproducible protocol developed here for micropropagation of *Arnebia hispidissima* provides a rapid and efficient method of direct regeneration of plants from various explants throughout the year, as it is difficult to propagate by conventional methods. The rapid multiplication of *Arnebia hispidissima* can help to augment the demand of pharmaceutical industries for the large-scale production of Shikonin.

Quantification of shikonin production *in vitro*

Shikonin content in induced roots of regenerated plants was analyzed. It was observed that when directly regenerated plants were cultured for extended periods of time on MS medium supplemented with 0.5 mg l^{-1} KIN, 0.25 mg l^{-1} BAP, 0.1 mg l^{-1} IAA, and 100 mg l^{-1} CH showed profuse exudation of Shikonin in the medium (Figs. 1d, f). Shikonin extracted from induced roots of directly regenerated plants (Figs. 1i, j) was employed for

Spectrophotometric estimation of Shikonin and its derivatives at 620 nm absorbance by making standard curve using authentic sample of Shikonin. The total content of Shikonin from 1 g of induced root tissue was 0.50 mg at the end of day 50 of in vitro culture.

In conclusion, this is the first report of high-frequency, direct plant regeneration, micropropagation and induction of Shikonin production in *Arnebia hispidissima*. It is envisaged that the protocol developed here for direct plant regeneration without the intervening callus formation will be very useful in meeting the supply of raw products year round irrespective of the growing season necessary for obtaining Shikonin for the cosmetic, dyeing, food, medicinal, and pharmaceutical industries.

Acknowledgements

Thanks are due to Professor V. P. Papageorgiou, Organic Chemistry Laboratory, Chemical Engineering Department, Aristotle University of Thessaloniki, 54006, Thessaloniki (Greece) for kindly providing the authentic sample of Shikonin. Financial assistance for filing the Indian and European Patents by the Department of Biotechnology, Ministry of Science & Technology, Government of India, New Delhi is duly acknowledged. The Transgenic Greenhouse and Tissue culture facility established under FIST program, sanctioned to Prof. A. Chaudhury by the Department of Science & Technology, Ministry of Science & Technology, Government of India, New Delhi is duly acknowledged.

References

Arous S, Boussaïd M, Marrakchi M. 2001. Plant regeneration from zygotic embryo hypocotyls of Tunisian chili (*Capsicum annuum* L.). J. Appl. Hort. 3: 17-22

Chaudhury A, Pal M. 2004. Guru Jambheshwar University of Science & Technology, Hisar, Haryana, India & Department of Biotechnology, Ministry of Science & Technology, Government of India, New Delhi, India. Method of Direct Regeneration and Shikonin Induction in Callus of *Arnebia hispidissima*. Patent Application # 2066/DEL/2004 dated 21-10-2004. Indian Patent. (Patent Pending)

Chaudhury A, Pal M. 2005. Guru Jambheshwar University of Science & Technology, Hisar, Haryana, India & Department of Biotechnology, Ministry of Science & Technology, Government of India, New Delhi, India. Method of Direct Regeneration, Shikonin Induction in Callus and *Agrobacterium*-mediated genetic transformation of *Arnebia hispidissima*. Patent Application # 05256545 dated 21-10-2005. European Patent Granted Published 10th October, 2008

Davydenkov VN, Patudin AV, Popov YuG, Rabinovich SA, Miroshnikov AI. 1991. Cultured *Arnebia euchroma* (Royle) Jonst.- cells a new source of Shikonin production. Khimiko Farmatsevticheskii Zhurnal 25(1): 53-55 (in Russian)

Dong J, Ye H, Wu X, Li G, Wu Z, Chen J. 1993. Studies of sus pension culture and fermentable culture of *Arnebia euchroma*. Acta Bot. Sin. 35: 57-61 (in Chinese)

Fujita Y, Hara Y, Ogino T, Suga C. 1981a. Production of Shikonin derivatives by cell suspension cultures of *Lithospermum erythrorhizon*: I. Effects of nitrogen sources on the production of Shikonin derivatives. Plant Cell Rep. 1: 59-60

Fujita Y, Hara Y, Suga C, Morimoto T. 1981b. Production of Shikonin derivatives by cell suspension cultures of Lithospermum erythrorhizon. II. A new medium for the pro duction of Shikonin derivatives. Plant Cell Rep. 1: 61-63

Fujita Y, Tabata M, Nishi A, Yamada Y. 1982. New medium and production of secondary compounds with the two stage culture method. In A Fujiwara, ed, Plant Tissue Culture, Maruzen, Tokyo, pp 399-400

Fujita Y, Maeda Y, Suga C, Morimoto T. 1983. Production of Shikonin derivatives by cell suspension cultures of *Lithospermum erythrorhizon*. III. Comparison of Shikonin derivatives of cultured cells and Ko-shikon. Plant Cell Rep. 2: 192-193

Fukui H, Yoshikawa N, Tabata M. 1983. Induction of Shikonin formation by agar in *Lithospermum erythrorhizon* cell sus pension cultures. Phytochem. 22: 2451-2453

Ge F, Wang XD, Wang YC. 2004. Studies on highly efficient induction of callus from *Arnebia euchroma* and rapid prolif eration of callus. Chinese Pharma J. 39(10): 735-737

Geetha N, Venkatachalam P, Prakash V, Lakshmi Sita G. 1998. High frequency induction of multiple shoots and plant regen eration from seedling explants of pigeonpea (*Cajanus cajan L.*)

Hayashi M, Tsurumi S, Fujimura H. 1969. Pharmacological effect of Shikon. Folia Pharmacol. Jpn. 65:195-196.

Khatoon S, Mehrotra S, Bajpai VK, Mehrotra BN. 1994. Ultramorphology of some *Boraginaceous* taxa used as Ratanjot. Feddes-Repertorium 105: 61-71

Khatoon S, Mehrotra BN, Mehrotra S. 2003. Pharmacognistic evaluation of Ratanjot of *Arnebia nobilis*. Ref. Natl. Prod. Sci. 9: 286-290

Kim DJ, Chang HN. 1990. Effect of growth hormone modifica tion on Shikonin production from *Lithospermum erythrorhi zon* cell culture with *in situ* extraction. Biotech. Lett. 12(4): 289-294

Manjkhola S, Dhar U, Joshi M. 2005. Organogenesis, embryo genesis and synthetic seed production in *Arnebia euchroma*- A critically endangered medicinal plant of the Himalaya. *In vitro* Cell. Dev. Biol-Plant 41: 244-248

Martin KP, Joseph D, Madassery J, Philip VJ. 2003. Direct shoot regeneration from lamina explants of two commercial cut flower cultivars of *Anthurium andraenanum* Horticulture. *In Vitro* Cell. Dev. Biol-Plant 39: 500-504

Mizukami H, Konoshima M, Tabata M. 1977. Effect of nutri tional factors on Shikonin derivative formation in *Lithospermum* callus culture. Phytochem. 16: 1183-1186

Pal M. 2005. *In vitro* culture and induction of Shikonin produc tion in *Arnebia hispidissima*. Ph. D. Thesis submitted to Department of Biotechnology, Guru Jambheshwar University of Science & Technology, Hisar, Haryana, India

Papageorgiou VP. 1978. Wound healing properties of naph thaquinones pigments from *Alkanna tinctoria*. Experimentia 34: 1499-1501

Papageorgiou VP. 1980. Naturally occurring Isohexenylnaphthazarin pigments: a new class of drugs. Planta Med. 38: 193-203

Papageorgiou VP, Assimopoulou AN, Couladouros EA, Hepworth D, Nicolaou KC. 1999. The chemistry and biology of alkannin, Shikonin, and related naphthazarin natural prod ucts. Angewandte Chemie Int. Ed. 38: 270-301

Pimenova ME, Tareeva NV. 1980. Shikonin content variability in the underground organs of macrotomia dyeing. Rastit Resur. 16: 82-86 (in Russian)

Singh B, Sahu PM, Jain SC, Singh S. 2004. Estimation of Napthaquinones from *Arnebia hispidissima* (Lehm.) DC. *In vivo* and *In vitro*. I. Anti-inflammatory screening. Phytotherapy Res. 18: 154-159

Sokha V, Nikolaeva L, Pank F. 1996. The Shikonin products in plant tissue culture: Selection of cell lines with high produc tivity in *Arnebia euchroma* and *Lithospermum erythrorhizon*. Proc. International Symp. Breed. Res. Medicinal Aromatic Plants, Quedlinburg, Germany. 30(2): 322-326.

Suzuki S, Fujino H, Yamazaki N, Tatsuo Y, Yoshizaki M. 1990. Propagation of *Oenothera erythrosepala* Borbas by shoot apex culture. Jpn. J. Breed. 40: 367-370.

Terada A, Tanoue Y, Taniguchi H. 1990. Chemistry of Shikonin, ancient purple pigment and its derivatives. J. Syn. Org. Chem. Jpn. 48: 866-875 (in Japanese).

Thengane SR, Deodhar SR, Bhosle SV, Rawal SK. 2006. Direct somatic embryogenesis and plant regeneration in *Garcinia indica* Choiss Current Sci. 91: 1074-1078.

Thomas TD, Shankar S. 2008. Multiple shoot induction and cal lus regeneration in *Sarcostemma brevistigma* Wight & Arnott, a rare medicinal plant. Plant Biotechnol. Rep. 3: 67-74

Toshiro O. 2001. Massive growth of *Boraginaceae* plant by tis sue culture and massive production of Shikonin derivative thereby, Patent Japan JP2000004702

Tsukada M, Fukui H, Habara C, Tabata M. 1983. Comparative studies on naphthoquinone derivatives in various crude drugs of "Zicao" (Shikon). Shoyakugaku Zasshi 37: 299-305

Yadav R, Arora P, Kumar D, Katyal D, Dilbaghi N, Chaudhury A. 2009. High frequency direct plant regeneration from leaf, internode, and root segments of Eastern Cottonwood (*Populus deltoides*). Plant Biotechnol. Rep. 3: 175-182

Yazaki K, Tanaka S, Matsuoka H, Sato F. 1998. Stable transfor mation of *Lithospermum erythrorhizon* by *Agrobacterium rhizogenes* and Shikonin production of transformants. Plant Cell Rep. 18: 214-219

Yazaki K, Matsuoka H, Shimomura K, Bechthold A, Sato F. 2001. A novel dark inducible protein, LeDI-2 and its involve ment in root-specific secondary metabolism in *Lithospermum erythrorhizon*. Plant Physiol. 125: 1831-1841

Yu HJ, Oh SK, Oh MH, Choi DW, Kwon YM, Kim SG. 1997. Plant regeneration from callus cultures of *Lithospermum ery throrhizon*. Plant Cell Rep. 16(5): 261-266