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RESEARCH ARTICLE

In vitro Plant Regeneration, Flowering and Fruiting from Nodal Explants of *Andrographis lineata* Nees (Acanthaceae)

Arifullah Mohammed[a,b,*], Kishore K. Chiruvella[b], Rama Gopal Ghanta[b]

[a] *Faculty of Agrobased Industry, Universiti Malaysia Kelantan, Jeli Campus, Locked bag-100, 17600, Jeli, Kelantan, Malaysia*
[*] *Corresponding author.*
[b] *Division of Plant Tissue Culture, Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India*

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Abstract

In the present study, *in vitro* propagation of tribal endemic medicinal plant, *Andrographis lineata* has been established using mature nodal explants. High frequency of regeneration (91.4%) was achieved on MS medium containing BA (3.0 mg L^{-1}) along with IAA (0.2 mg L^{-1}). Strikingly, irrespective of the season and collection period, we observed axillary flower induction and fruit formation from the above *in vitro* cultures supplemented with BA and NAA. Transition from the vegetative to the reproductive phase occurred within 2 months of culture, and importantly was influenced by factors such as sucrose and cytokinins. *In vitro*-regenerated flowers were morphologically identical to *in vivo* flowers. Furthermore, our scanning electron microscopic studies revealed that pollen external morphology of both *in vitro* and *in vivo* flowers were similar. The flowers self-fertilized and produced fruits *in vitro*. Elongated shoots rooted well on half-strength MS basal medium, and were successfully acclimatized to the garden conditions. Altogether, our established protocol can be utilized in plant breeding for the purpose of *ex situ* conservation, quick flowering, and fruit set.

Key words : *Andrographis*, axillary proliferation, flowering, fruiting, pollen, multiple shoots

Key words : NAA, *α*-naphthalene acetic acid; IAA, indole-3-acetic acid; BA, 6-benzylaminopurine; TDZ, thidiazuron; PGR, plant growth regulator; MS, Murashige and Skoog; WPM, woody plant medium; B5, Gamborg medium; SEM, scanning electron microscope; PVP, polyvinyl pyrolidine

Introduction

Medicinal plants are unscrupulously harvested from the wild populations resulting in the depletion of species, facing the threat of extinction, and finally causing loss of biodiversity. Hence, application of plant tissue culture techniques would be useful for large-scale propagation of medicinal plants and the production of phytochemicals (Briskin 2000). Micropropagation offers a rapid mass multiplication, a proven method for efficient *in vitro* propagation and for commercial exploitation of valuable plant-derived pharmaceuticals. Flowering is a complex process regulated by a combination of environmental and genetic factors. Season independent *in vitro* flowering in tissue culture has been reported in many medicinal plants (Jeyachandran and Bastin 2013; Lin et al. 2003 ; Saritha and Naidu 2007; Sharma et al. 2014; Taylor et al. 2005).

Transition from the vegetative state to reproductive stage during plant development is poorly understood. Under natural growth conditions, flower formation usually commences when a plant attains maturity. The age of the plant is genetically controlled and is species specific. The plant flowers only when genetic factors including photoperiod response are congenial and these conditions can often be altered so that the plant can be induced to undergo an early reproductive phase. Such an attempt to induce flowering *in vitro* from juvenile explants has been made in many plant systems. *In vitro* culture provides an ideal experimental system for studying the molecular biological mechanism of flowering. *In vitro* flowering has been reported in many plant species as described above. Reduction

Arifullah Mohammed(✉)
E-mail: arifullahmd@gmail.com, drkishorekumaar@gmail.com
ghantargopal@yahoo.co.in

in the duration of flowering *in vitro* and seed settings is an advantage and in turn reduces the duration required for the completion of life cycle.

Andrographis lineata Nees is a tribal endemic herbal medicinal plant that grows wildly throughout the plains and hills of India and Ceylon (Rani and Matthew 1991). This plant is used by traditional practitioners as stomachic, anti-helminthic, anti-inflammatory, antipyretic, antiperiodic and in intermittent and remittent fevers (Anonymous 1948; Balu et al. 1993; Balu and Alagesaboopathi 1993; Chopra et al. 1956; Kirtikar and Basu 1975). It is also used for snake bite, diabetes, skin diseases, fever, dog bite, constipation, bronchitis, cancer and inflammation (Alagesaboopathi 2012; Ayyanar 2008; Balu and Alagesaboopathi 1993, 1995). For all respiratory infections, the leaves are made into a paste with hot water and then administered with a glass full of warm water, three times a day. A decoction of the root is astringent and cooling and is a powerful antidote for poisonous stings of all insects. It is a good substitute for *A. paniculata*. Three flavonoids are isolated from leaf extracts of *A. lineata* along with andrographolide (Kishore et al. 2003). Recent pharmacological studies suggest that *A. lineata* leaves are used for various ailments. It possess hepatoprotective, antidiabetic, antibacterial and diuretic properties (Perumalsamy and Ignacimuthu 2000; Sangameswaran et al. 2007, 2008; Santhi 2006). Overall *A. lineata* is attributed to have several medicinal properties in traditional Indian medicine (Alagesaboopathi 2012).

Conventional propagation of *A. lineata* through seed germination is very limited (20-30%), due to a very short span of seed viability. Seedlings of this plant are browsed by cattle which makes plant availability and existence in small patches. Nonetheless, vegetative propagation through stem cuttings is with a low frequency (< 50%) of rooting (Alagesaboopathi and Ramachandran 2000). No other alternative method is available till today to propagate this endemic ethnomedicinal plant effectively. Overexploitation, scanty availability, and poor regeneration efficiency by conventional methods urged to develop efficient methods for rapid propagation and *ex situ* conservation of *A. lineata*.

Here, we established *in vitro* regeneration of *A. lineata* through direct organogenesis using mature nodal explants. We observed *in vitro* flowering and fruit formation on MS medium containing BA and NAA. Importantly the time period of flowering and fruiting under *in vitro* conditions was much shorter compared to natural flowering. Shoots rooted well on half-strength MSB medium and were successfully acclimatized.

Materials and Methods

Plant Material

Nodal explants collected from mature plants were initially washed with running tap water for 15 min then in 5% teepol detergent for 15 min followed by repeated washing in running tap water until all traces of detergent were removed and then they were rinsed 4-five times in distilled water. Surface sterilization of these explants was then made by immersing them in 70% alcohol for 15s followed by rinsing three times in sterile distilled water. Finally the explants were treated with 0.1% $HgCl_2$ (w/v) for 2 min. The surface sterilization was followed by 5-6 rinses in sterile distilled water. The cut ends of the explants were further trimmed with sharp edge sterile surgical blades. Then the explants were blotted on sterile filter paper discs before planting them vertically on agar gelled MS medium taken in 25 × 150 mm culture tubes or 150 ml conical flasks.

Culture Initiation and Shoot Multiplication

Initial experiments were designed for the selection of most appropriate suitable medium. MS media were supplemented with different concentrations of BA (0.5-3.5 mg l^{-1}) alone and nodal explants were inoculated. Nodal segments which showed a good response for shoot multiplication were then used in the further experiments to enhance multiple shoot production. After assessing the effect of BA on shoot induction and multiplication, its effect in combination with other basal media like WPM and B5 were tried, and MS medium was found to be the best medium for shoot induction and multiplication. Hence, MS medium supplemented with various cytokinins (BA, TDZ, and Kn) alone and along with auxins (NAA, IAA, and IBA) was used to study their effects on shoot induction and multiplication. In addition to this, growth adjuvants like tender coconut milk (TCM), casein hydrolysate (CH), and antioxidants like ascorbic acid, polyvinyl pyrrolidone (PVP), and activated charcoal (AC) were added to the media to improve the shoot initiation and multiplication efficiency (Fig. 1G). Subculture was done by transferring the microshoots to fresh shoot induction medium. The stumps with remnant meristem were also subcultured in fresh medium.

All cultures were maintained at 25 ± 2°C under a 16 h photoperiod using cool, white fluorescent bulbs (80 m E m^{-2} S^{-1}). Ten replicates were maintained for each treatment and the experiments were repeated three times. Shoots and flowers were examined and counted at 2-week intervals. The number, size, and type of inflorescence/distribution of flower buds were determined after 7 weeks.

Root Initiation and Field Establishment

The microshoots of 4-5 cm length were dissected from proliferated shoot cultures and inoculated on agar gelled medium containing full strength, half strength, and quarter strength MS salts with/without different auxins. After 6-8 weeks of inoculation, rooting frequency and the number of roots with other characters like callusing were recorded. The rooted microshoots were planted in plastic trays containing vermiculite, covered with plastic sheets, and kept in a mist chamber. The plastic trays were kept open during night and covered with plastic sheets during the day. After one week, the plantlets were directly exposed to light during morning hours. Then they were transferred to root trainers containing vermicompost and sand (1:1) for 10 days, and 10 ml of autoclaved/MS salts were directly supplied to substratum in

Table 1. Shoot morphogenic response of mature nodal explants of *A. lineata* on MS medium with various PGRs. Observations are after 6 weeks. Values represented above are the means of 20 replicates. Mean values having the same letter in each column do not differ significantly at $P \leq 0.05$ (Duncan's multiple range test); * indicates flowering.

PGR (mg l^{-1})					Mature Node			
BA	Kinetin	TDZ	IAA	NAA	Frequency of Regeneration (%)	Mean no. of shoots/explant	Mean length of the Shoot (cm)	Mean no. of propagules/ explant
2.0	-	-	0.2	-	73.6 ^{k}	47.7^{j}	4.4^{d}	4.8^{kj}
2.0	-	-	-	0.2	60.6 ^{j}	*25.8^{f}	5.0^{g}	4.5^{j}
3.0	-	-	0.2	-	91.4^{l}	55.5^{k}	4.0^{c}	3.0^{g}
3.0	-	-	-	0.2	72.4^{k}	*44.6^{i}	4.8^{f}	2.5^{e}
-	1.0	-	-	-	42.8^{f}	15.4^{e}	5.2^{gh}	4.4^{j}
-	2.0	-	-	-	50.4^{h}	30.6^{h}	5.4^{h}	3.4^{h}
-	3.0	-	-	-	30.6^{e}	26.8^{f}	4.7^{f}	4.0^{i}
-	2.0	-	-	0.2	48.5^{g}	28.5^{g}	4.6^{ef}	4.2^{ij}
-	2.0	-	0.2	-	54.7^{i}	42.3^{i}	6.4^{i}	1.0^{a}
-	-	0.01	-	-	26.5^{d}	7.5^{a}	1.2^{b}	1.0^{a}
-	-	0.02	-	-	30.4^{e}	11.0^{c}	0.8^{a}	1.0^{a}
-	-	0.03	-	-	18.8^{c}	9.1^{b}	0.6^{a}	2.0^{d}
-	-	0.10	-	-	15.4^{b}	callus	-	-
-	-	0.02	0.2	-	16.7^{b}	12.3^{d}	1.2^{b}	2.7^{fg}
-	-	0.02	-	0.2	10.8^{a}	12.7^{d}	1.3^{b}	1.3^{b}

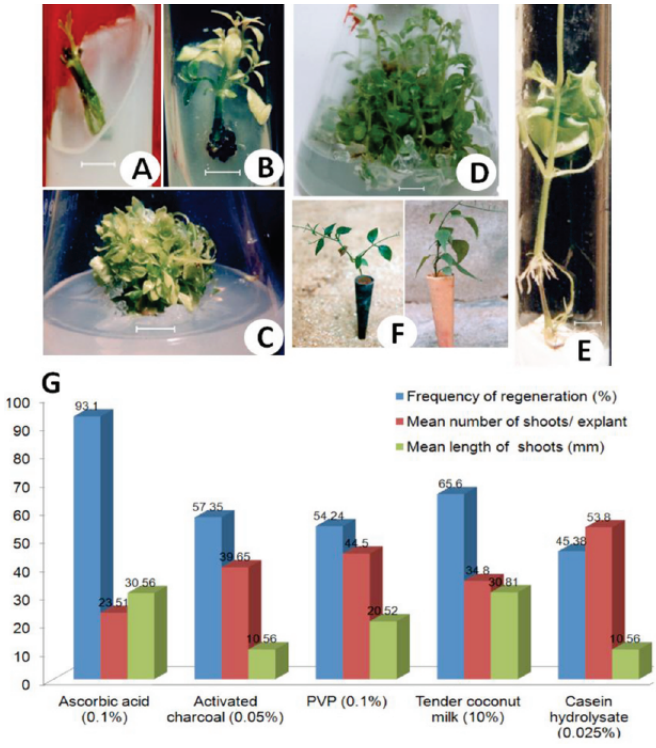

Fig. 1. *In vitro* multiple shoot production, shoot elongation, and rooting from mature nodal explants of *A. lineata on* MS medium. A. Single shoot initiation after one week, B. Shoot proliferation after 3 weeks on 1.0 mg l^{-1} BA (Bar = 3.5 mm, 4.8 mm). C. Induction of multiple shoots after 5 weeks, D. Shoot elongation after 6 weeks on 3.0 mg l^{-1} BA + 0.2 mg l^{-1} IAA (Bar = 6.1 mm, 5.1 mm). E. *In vitro* rooting on half-strength MS medium after 2 weeks (Bar = 5.9 mm). F. Hardening of plantlet growing in plastic root trainers after three months. G. Effect of different growth adjuvants and antioxidants on shoot multiplication of mature nodal explants of *Andrographis lineata.*

root trainers. After one week, the plantlets were transferred to plastic bags containing a mixture of soil, organic manure and sand in 2:1:1 ratio. The plants in plastic bags were removed from the mist chamber and kept in open shade and watered twice a day. After one week, they are transferred to the field.

Pollen Morphology

The anthers were dried to allow pollen release. Pollen was collected in small vials and stored in desiccator at 3-5°C until used. Pollen external morphology was studied by scanning electron microscopy (SEM). For SEM evaluation, dehydrated pollen grain samples were mounted on aluminium stubs with double-sided cellophane tape, and air dried at room temperature under an inverted flask. Specimens were coated with gold using a fine coater ion sputter SC–1100E and then examined under a Scanning electron microscope (ESEM–XL–30 at Central Instrumentation Laboratory, University of Hyderabad, Andhra Pradesh, India).

Experimental design and Statistical analysis

Each culture tube with one shoot explant was considered as one replicate. Each treatment in each set of experiments consists of 20 replicates and each experiment was repeated three times. Standard error of means was calculated in each experiment. The data was statistically analyzed using SAS software package by one way analysis of variance (ANOVA) and means were compared using the Duncan's multiple range test (DMRT). The *p*-values were considered statistically significant at $P < 0.05$ and $P < 0.01$.

Results

Shoot Initiation and Multiplication

Direct shoot multiplication was carried out using mature,

Table 2. *In vitro* flowering and multiple shoot production from mature nodal explants of *A. lineata* on MS medium supplemented with BA and NAA. Observation is after 4 weeks. Values represented above are the mean of 20 replicates; ± represents Standard error.

Concentration of PGR (mg L^{-1})		Nodes		
BA	NAA	Initiation of flowering (%)	Mean number of shoots/ explant	Mean number of flowers/ Explants
2.0	0.1	45.50 ± 0.16	6.10 ± 0.54	2.85 ± 0.17
2.0	0.3	48.40 ± 0.08	6.30 ± 0.33	4.18 ± 0.04
2.0	0.5	49.37 ± 0.08	4.04 ± 0.66	3.59 ± 0.11
2.0	0.7	52.50 ± 0.05	3.52 ± 0.48	4.68 ± 0.06
2.0	0.9	54.45 ± 0.13	2.85 ± 0.53	2.13 ± 0.06
2.0	1.0	57.40 ± 0.52	4.30 ± 0.32	5.42 ± 0.03
2.0	1.5	70.00 ± 0.11	3.25 ± 0.30	5.51 ± 0.03
2.0	2.0	43.45 ± 0.11	2.45 ± 0.47	1.6 ± 0.03
2.0	2.5	-	2.10 ± 0.21	-
2.0	3.0	-	4.15 ± 0.02	-
1.0	2.0	-	4.78 ± 0.02	-
1.5	2.0	-	4.24 ± 0.02	-
2.5	2.0	50.2 ± 0.08	3.10 ± 0.54	2.85 ± 0.17
3.0	2.0	52.40 ± 0.07	4.30 ± 0.33	4.18 ± 0.04
3.5	2.0	55.37 ± 0.08	5.40 ± 0.66	3.59 ± 0.11
4.0	2.0	22.50 ± 0.05	8.20 ± 0.48	1.68 ± 0.06
5.0	2.0	19.50 ± 0.05	7.85 ± 0.53	3.13 ± 0.06

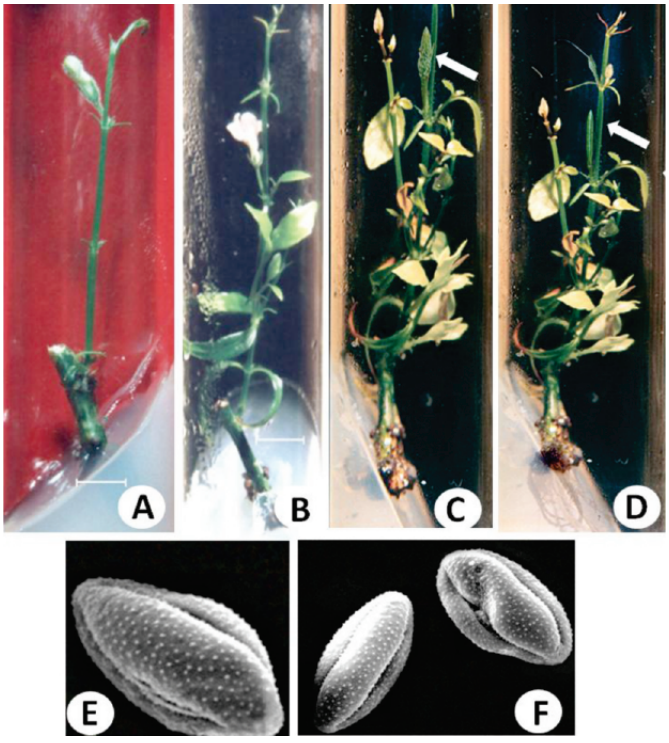

Fig. 2. *In vitro* flowering and fruiting from mature nodal explants of *A. lineata on* MS medium containing 2.0 mg L^{-1} BA + 0.1 mg L^{-1} NAA + 3% sucrose + 0.01% PVP. A. Mature flower bud formation after 3 weeks (Bar = 6.4 mm). B. *In vitro* flowering on after 2 weeks (Bar = 6.7 mm). C, D. *In vitro* fruiting after 3 weeks (C), 5 weeks (D) (Bar = 3 mm). Fruit formation is indicated by arrows. E, F. Scanning electron microscopy of pollen grains obtained from *in vitro* flowers (E) and *in vivo* flowers (F) (Bar = 20 mm).

field-grown nodal explants. Initial experiments on culture initiation and establishment were carried out on MS basal medium without growth regulators formed only two shoots. Nodal explants produced the maximum number (43.1) of multiple shoots (Fig. 1A-C) on MS medium with 2.5 mg L^{-1} BA with high frequency (80.2%) of regeneration (Table 1). However, they formed basal callus at all concentrations of BA tested, but this decreased at higher concentrations of BA. Further experiments were carried out to examine the effect of various cytokinins (BA, Kn, and TDZ) in combination with auxins (NAA and IAA) on shoot multiplication. High frequency (91.4%) of regeneration was achieved on MS medium supplemented with 3.0 mg L^{-1} BA along with 0.2 mg L^{-1} IAA (Fig. 1C, Table 1). BA in combination with IAA was effective in shoot multiplication, when compared to BA alone or in combination with NAA and other treatments. BA at 2.0 mg L^{-1} along with 0.2 mg L^{-1} NAA did not increase the shoot number but did increase in shoot length (5.0 cm) (Fig. 1, Table 1).

Kinetin at 2.0 mg L^{-1} in combination with 0.2 mg L^{-1} IAA produced the highest mean shoot length (6.4 cm), but it decreased the shoot number compared to other combinations of PGR. Kinetin alone was also less effective when compared to BA. Kinetin at 2.0 mg L^{-1} produced maximum (30.6) shoots with mean shoot length of 5.4 cm with 50.4% regeneration (Table 1). A very low number of shoots with lower frequency of regeneration (26.5%) was observed on MS medium supplemented with 0.01 mg L^{-1} of TDZ. TDZ at 0.02 mg L^{-1} produced a maximum number (11.0) of shoots with shoot length of 0.8 cm. TDZ alone and in combination with NAA or IAA was not effective in inducing multiple shoots and shoot length (Table 1).

In vitro Flowering

A single nodal explant was planted upright on MS medium (Murashige and Skoog 1962) supplemented with various plant growth regulators (BA, IAA, NAA, and IBA). We observed axillary flowers formation nodal explants cultured on MS

Table 3. Effect of different concentrations of sucrose on *in vitro* flowering of *A. lineata* nodal explants on MS medium fortified with 2.0 mg L^{-1} BA and 1.5 mg L^{-1} NAA along 0.01% PVP.

Sucrose concentration(%)	Frequency of Flowering(%)	Mean number of flowers/explant
0	00.0	0.00 ± 0.00
1	55.0	04.0 ± 0.11
2	65.0	07.5 ± 0.20
3	82.1	14.0 ± 0.14
4	60.0	08.5 ± 0.13
5	45.0	2.50 ± 0.11
6	32.0	01.0 ± 0.12

medium with BA and NAA combinations (Fig. 2) irrespective of the collection period/season. *De novo* flower frequency and number were effected by concentrations of both the PGR (Table 2). The maximum number (5.5 flowers/explant) and frequency (70%) of flowers were noticed from the nodes cultured on a medium containing 2.0 mg L^{-1} BA and 1.5 mg L^{-1} NAA (Fig.2 A, B; Table 2). Most of the flowers initiated on non-leafy shoots.

Next, we analyzed the influence of sucrose on flowering. Various sucrose concentrations (1.0, 2.0, 3.0, 4.0, 5.0, and 6.0% w/v) were used in the MS medium supplemented with 2.0 mg L^{-1} BA plus 2.0 mg L^{-1} NAA (Table 3). Altogether, high frequency of flowering (82.1%) and maximum number of flowers (14 flowers/shoot) was obtained on MS medium (pH 5.7) fortified with 2.0 mg L^{-1} BA and 1.5 mg L^{-1} NAA along with 3.0% sucrose and 0.01% PVP. Addition of growth adjuvants to MS medium did not markedly increase flowering response (data not shown).

The *in vitro* developed flowers were identical to *in vivo* flowers having two bracteoles, a 6.5 mm 5 lobed calyx with glandular hispid, acute (Fig. 2B). Corolla was white with dark purple streaks on the limb or white with purple blotches (1 ´ 0.5 cm across). Corolla tube cylindric and 7.4 mm in length with five lobes, glandular hispid. Upper lip oblong, 7.4 mm in length and shortly bifid. Lower lip lengthens 7.5 mm. Corolla ventricose. Two stamens (8.6 mm) with glabrous anthers (3.4 mm). Filaments were glabrous. Ovary was oblong (2.3 mm, puberulous) with a sparsely hairy style (1.5 cm).

In vitro Pollen Morphology

Pollen grains obtained from *in vitro* flowers germinated on Brewbaker and Kwack's medium with 15% sucrose only when kept under low temperatures. Further, the external morphology of the pollen grain obtained from *in vitro* flowers was observed by SEM. The pollen grains from were found to be trizonosyncolpate, prolate spheroidal–subprolate (polar axis 51.3 µm; equatorial axis 42.7 µm) and subprolate–euprolate (polar axis 53.4 µm; equatorial axis 42.0 µm), respectively. The exine surface was relatively smooth with regular perforations (conical and dome shaped) (Fig. 2E). Altogether, the germinated pollen grains were found to be similar to those from field-grown plants (Fig. 2F).

In vitro fruiting

Flower anthesis occurred after 10 days of flower bud induction. Flower shedding was observed after 5 days of anthesis. Shedding of leaves were observed with the onset of flowering. *In vitro* flowers self-fertilized leading to fruit set, resembled the natural fruits. Fruits were formed on same media after 3-4 weeks (Fig. 2C, D). Fruit is green linear oblong capsule. *In vitro* floral parts and fruits were comparatively similar to *in vivo*. Altogether shoot induction, elongation was followed by *in vitro* flowering and fruiting (Figs. 2A-D). The latter two events were observed before rooting of shoots.

Effect of Growth Adjuvants, Antioxidants, and Sucrose on Shoot Multiplication

Shoot regeneration efficiency of *in vitro* shoots and nodal stumps during subculture was studied by inoculating them on MS medium supplemented with BA (0.5-5.0 mg L^{-1}) alone, and along with IAA (0.1-0.3 mg L^{-1}). Among all the treatments, 2.0 mg L^{-1} BA + 0.1 mg L^{-1} IAA showed maximum response in all explants. Addition of IAA resulted in good shoot elongation in all the explants that were subcultured. Increasing the concentration of BA (3.0-5.0 mg L^{-1}) hindered the shoot multiplication as well as elongation with the interference of undesirable basal callus.

BA at 3.0 mg L^{-1} along with 0.2 mg L^{-1} IAA on other basal media viz., WPM and B5 did not influence shoot multiplication and frequency of regeneration. Nodal explants on MS medium responded more than on other media (data not shown). Although less effective than MS medium, WPM was better than B5 medium. Among all adjuvants tested in the study, CH (0.025%) was effective in inducing multiple shoots. Addition of ascorbic acid (0.1%) increased frequency of regeneration (93.1%) with least number of shoots per explant (23.51) (Fig. 1G).

Optimum sucrose concentration for maximum shoot bud proliferation was found to be 3%. Below the optimum concentration, percentage of response, number of shoots, and shoot length drastically decreased and at higher concentrations profuse basal callusing was observed (Table 3). Response of nodal segments cultured on MS medium supplemented with 3.0 mg L^{-1} BA + 0.2 mg L^{-1} IAA fortified with four sugars viz., cane sugar, fructose, glucose and sucrose, at 3%. Among the four sugars tested, sucrose was found to be the best followed by cane sugar and glucose.

In vitro Rooting and Field Establishment

Micro shoots (measuring about 4-6 cm long) were isolated from proliferating bud cultures growing on MS medium and used for *in vitro* rooting. Root initiation was observed only on auxin-free MS basal medium (MSB). When auxins were added to the medium, callus was found developing from the base of the shoot, which hindered the root initiation. MSB medium was modified by reducing the concentration of nutrients to half and

quarter strength to study the effect of nutrients on root induction. MS half-strength basal medium showed the maximum frequency (74.2%) of root formation with the maximum number (28.5) and the length (5.6 cm) of the roots (Fig. 1E). Sturdy and healthy roots have developed from the micro shoots, 8 weeks after implantation onto this medium (Fig. 1E).

Roots regenerated on MS full strength and quarter strength medium were very thin, fragile, and short. Shoots rooted on MS half-strength medium showed the maximum percentage of survival (96%). Eighty-six percent of the plants planted on both the substrates (sand:garden soil 1:1 and autoclaved vermiculite) were successfully acclimatized to the garden conditions (Fig. 1F). However, loss occurred at the time of exposing the plants to the environment by removing bags.

Discussion

Micropropagation is indispensable for *ex situ* conservation of medicinal plants and also serves as vehicles for in-depth investigation of physiological and biochemical processes (Smith et al. 2002). Firstly we demonstrate mature nodal explants showing high frequency of regeneration on MS medium containing BA along with auxin NAA or IAA. A low concentration of auxin along with cytokinin increased the rate of shoot multiplication (Rout 2005; Rout et al. 2000). The beneficial aspects of BA and auxin for bud induction followed by shoot multiplication has been demonstrated in other plants (Nguyen et al. 2005; Rout 2005; Serkan et al. 2005). Altogether, auxins are essential along with cytokinins for shoot elongation and their balance during culture initiation, establishment for initiating the growth of axillary buds and types of cytokinins are crucial for organogenesis (Kohlenbach 1977). Usage of nodal explants obtained from *in vitro*-raised plantlets ensures contamination free compared to *ex vitro* explants. Importantly, nodal explants from *in vitro*-raised shoots can be utilized as propagules depending on field material. Strikingly, these cultures can be used for production of plant specific phytochemicals such as andrographolide, echoidin, echoidinin, and methyl wogonin (Arifullah et al. 2015).

Secondly, we show a transition from vegetative to reproductive phase with flower and fruit formation on MS medium fortified with BA and NAA. Flowering can be induced in explants that are either in the vegetative or reproductive states. The ability of explants to form flowers *in vitro* depends on numerous internal and external, chemical, and physical factors and virtually all of these factors interact in various complex and unpredictable ways (Compton and Vielleux 1992). Especially important are carbohydrates, growth regulators, light, and pH of the culture medium (Heylen and Vendrig 1988). In the present study, irrespective of the collection period/season, axillary flowers were observed on nodal explants cultured on MS medium fortified with BA and IAA/NAA combinations. Flower bud formation was also noticed from cotyledonary nodes of the aseptic seedlings of *A. lineata* on MS medium supplemented with 2.0 mg L^{-1} BA + 0.5 mg L^{-1} NAA. The stimulation of *in vitro* flowering by cytokinins has been reported for a number of diverse plant species (Jana and Shekhawat 2011; Kishore et al. 2003; Lin et al. 2003; Naor et al. 2004; Sharma et al. 2014; Taylor et al. 2005). Synergistic effect of auxin and cytokinins on flowering *in vitro* indicate a species-specific role of the endogenous level of hormones in flowering plants (Jeyachandran and Bastin 2013; Lin et al. 2005; Saritha and Naidu 2007; Sudhakaran and Sivasankari 2002; Taylor et al. 2005; Thiruvengadam and Jayabalan 2001).

Sugars are necessary carbon sources in culture media for reliable induction and development of flowers (Nguyen et al. 2006). The interaction of sucrose and light to promote *in vitro* flowering has been reported in a number of species (Jumin and Nito 1996). In the present study, organogenesis did not occur in medium without sugar or without exposure to light even with sugar. Explants kept under light (16 h photoperiod) with 2% sucrose produced 30% shoots and did not flower, and 3% sucrose produced 60% shoots without flowers and 45% shoots with flowers; whereas those kept under light with 4% sucrose were prolific in both shoot (85%) and flower (75%) production. Shoot and flower formation decreased with 5% sucrose. Similar results were reported in (Jumin and Nito 1996; Kachonpadungkitti et al. 2001). Cultures grown in complete darkness or in continuous light, after flower bud initiation, were found to grow poorly compared to the cultures grown in the other photoperiod treatments in the current study. Impairment of anther development and degeneration of anthers was also observed in this treatment. Photoperiod is critical for sterile flower induction (Kerbauy 1984). It was observed in the present study that *in vitro* developed flowers were typical to *in vivo* flowers and were large and healthy. Most of the flowers developed on leafy shoots. However undersized and malformed flowers have been observed previously in other species (Ramanayake et al. 2001).

Thirdly, we reveal similarity of pollen external morphology of both *in vitro* and *in vivo* flowers. Flowers that possess anthers and stigmas simultaneously are more likely to experience intrafloral self-pollination (Lloyd 1979) as observed in the current study. This is evidenced in large flora, displaying increased opportunities for geitonogamous self-pollination (Harder and Barrett 1995). Because self-pollination can result in inbreeding depression (Charlesworth and Charlesworth 1987) and pollen discounting (Harder and Wilson 1994), it is not unexpected that many floral adaptations have evolved to reduce these mating costs. It is well known that successful fruit setting depends largely on viable pollen grains. Pollen viability could be measured by *in vitro* pollen germination techniques using organic and inorganic substances like sucrose, H_3BO_3, $Ca(NO_3)_2,4H_2O$, KNO_3, and $MgSO_4.7H_2O$ which would exert effect on *in vitro* pollen germination. The occurrence of flowering in tissue culture has become widespread; the

systematic study of fruiting *in vitro* becomes plausible. Our study provides a useful tool to study the reproductive aspects associated with plant differentiation *in vitro*. *In vitro* floral parts were similar to *in vivo*. Importantly, we observed flowering in shoots without roots, suggesting rooting is not essential for *in vitro* flowering and fruiting of *A. lineata*. Similar results have been reported by bamboo *in vitro* flowering, which induced flowers only in absence of roots when shoots were cultured on media containing BA (Nadgauda et al. 1997).

Conclusion

Our results provide nutritional conditions for efficient *in vitro* plant regeneration, for the purpose of quick *in vitro* flowering and fruiting of *A. lineata* any time in a year for commercial propagation. Further it can be used to understand the snapshots of physiological, molecular and hormonal regulation of flowering.

Competing interests

The authors declare that there is no competing interest.

Author contributions

AM, KKC, RG designed experiments; AM, KKC performed experiments; AM KKC, RG interpreted the data; AM, KKC wrote the manuscript.

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