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In Vitro Micropropagation of *Janakia arayalpathra* Joseph & Chandras.- a RET Medicinal Plant

K. Thangavel[1*], P. Ravichandran[2], R. Mahesh[3] and K. Muthuchelian[3]

[1] Department of Botany and Biotechnology, Thiagarajar College (Autonomous), Madurai – 625 009. Tamil Nadu.

[2] Department of Plant Sciences, Manonmaniam Sundaranar University, Tirunelveli – 627 012.

[3] Centre for Biodiversity and Forest Studies, School of Energy, Environment and Natural Resources, Madurai Kamaraj University, Madurai – 625 021. Tamil Nadu. India.
*E.mail: thangamspkces@gmail.com

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Abstract

An *in vitro* clonal propagation protocol was evolved for *Janakia arayalpathra* Joseph & Chandras. - a rare endemic and threatened medicinal plant. Axillary bud explants cultured in Murashig and Skoog medium supplemented with 8µM BAP + 4µM IAA evinced maximum shoot multiplication response than the other combinations. The survival of shoots in root induction medium necessitated the addition of BAP along with IBA. Maximum root induction was observed in medium supplemented with 8µM BAP + 6µM IBA. An average of 45 shoots could be produced by three sub cultures within 5 months' period. Plants were successfully acclimated and established in field conditions. About 60 % of in vitro derived plantlets survived in field conditions.

Key Words: Clonal propagation, Axillary bud culture, *Janakia arayalpathra.*

Introduction

Plants have been the traditional source for raw materials and finished medicines since the dawn of civilization. Plant derived medicines constitute a substantial component of present day human healthcare systems in industrialized as well as developing countries. In India, herbal medicines have been the basis of treatment for various diseases, physiological conditions in traditional systems such as Ayurveda, Unani and Siddha. Indian folk medicine comprises numerous herbal prescriptions for therapeutic purposes which may be as varied as healing wounds, treating inflammation due to infection, skin lesions, leprosy, diarrhea, scabies, venereal diseases, snake bite and ulcers. In recent years there has been a gradual revival of interest in the use of medicinal plants in developed as well as in developing countries because herbal medicines have been reported to be safe without much adverse side effects. Thus a research for new drugs with better substitutes and plant resources are a natural choice (Sushil *et al.,* 1999).

Genetic diversity of traditional medicinal species is continuously under threat of extinction as a result of habitat destruction and unmonitored trade of medicinal plants and their products. Therefore, the conservation and sustainable utilization of medicinal plants are more desirable need of the hour (Bahadur *et al.,* 2007). In recent years, tissue culture has emerged as a promising technique to obtain genetically pure elite populations under *in vitro* conditions rather than have in different populations. Tissue culture has become a well established technique for culturing and studying the physiological behavior of isolated plant organs, tissues, cells, protoplasts and even cell organelles under precisely controlled physical and chemical conditions. Most of the medicinal plants either do not produce seeds or seeds are too small and do not germinate in soil. Thus mass multiplication of disease free planting material is a general problem. In this regard micropropagation holds significant promise for true to type, rapid and mass multiplication under disease free conditions.

In Vitro Conservation of Medicinal Plants

In vitro propagation of plants holds tremendous potential for the production of high quality plant based medicines (Murch *et al.,* 2000). This can be achieved through different methods including micropropagation. With micropropagation, the multiplication rate is greatly increased. It also permits the production of pathogen free material. Plant regeneration from shoot and stem meristems has yielded encouraging results in medicinal plants like *Catharanthus roseus, Cinchona ledgeriana and Digitalis spp, Rehmannia glutinosa, Rauvolfia serpentina, Isoplexis canariensis, Citrullus colocynthis, Zephyranthes bulbous, Plectranthus vetiveroids* and *Glossocardia bosvallea* (Roy *et al.,* 1994; Maridass *et al.,* 2010; 2012; Paek *et al.,* 1995; Perez-Bermudez *et al.,* 2002; Sivasubramanian *et al.,* 2002; Geetha and Gopal, 2007; Gayathri and Ramagopal, 2007; Meena and Patni, 2007; John De Britto *et al.,* 2009; Mahesh *et al.,* 2010; 2011; 2012; Rafael *et al.,* 2008; Mishra *et al.,* 2008; Supriya Das *et al.,* 2013; Ondo Ovono *et al.,* 2013; Mubo *et al.,* 2014; Gill *et al.,* 2014; Bekim Gashi *et al.,* 2015; Tomasz Pniewski *et al.,* 2016; Jian Ma *et al.,* 2016).

Most of the available micropropagation protocols were generally based on restricted genotypes and were adapted to achieve the best response from specific materials which were used for commercial purposes. Germplasm conservation programs on the other hand demand a protocol that ensures a sufficiently effective response over the whole range of genotypes of crop species (Mandal and Chandel 1996). Using *in vitro* propagation technology many rare and endangered plant species can now be quickly and successfully propagated and preserved from a minimum plant material and with low impact on wild population (Branka *et al.*, 1997; Cuenca *et al.*, 1999). Micropropagation of various medicinal plants has been reported (Withers, 1986; Marina *et al.*, 1994; Augustine and D'souza, 1997; Benny *et al.*, 1999; Komalavalli and Rao, 2000; Anu *et al.*, 2001; Ahuja *et al.*, 2002; Ashok Kumar *et al.*, 2002). In the present study an attempt was made to develop a rapid micropropagation protocol for clonal multiplication of the endangered medicinal plants – *Janakia arayalpathra* through axillary bud culture.

Janakia arayalpathra (Periplocaceae) as a new type genus and species was described by Joseph and Chandrasekharan (1978). *Janakia arayalpathra* is a medium sized shrub growing in a peculiar habitat of rock crevices and on sedimentary rocky slopes at an altitude of above 800 meters. The stem is slender and reaches a girth not beyond 3 cm. The stem tubers are aromatic and highly valued for medicinal properties. The tubers are black in colour and grow up to 60 cm in length and 18 mm width with beaded appearance. Single plant produces approximately 1 – 2.5 kg of tubers. It is a monotypic endemic genus with the species having narrow and restricted distribution with a threat status of CR- Critically Endangered (B1 & 2c, d) - Globally (Ravikumar and Ved, 2000). The specimen was first collected near Bonaccord estate at Kursumalai – 875 m (77º 10' – 77º 11'). Trivandrum District, Kerala (Holotype - Joseph 46503); (Joseph and Chandrasekaran, 1978). After which several collections were made for botanical investigations from the same habitat as well as from many new localities. This species is an endemic plant to Southern Western Ghats - Kerala and Tamil Nadu states. In Tamil Nadu, it has been collected from the exposed rocky slopes of Kanniyakumari and Thirunelveli districts (Ravikumar and Ved, 2000; K. Thangavel, Field No. 143, SPKCESH, 2000, 2002, and 2005) which includes Thirukurangudi, Narai kadu, Kodaiyar, Kuthiraivetti, Utthumalai, these are also the new locations and besides that so far not collected from any other part of India. The tubers are being ruthlessly collected from its natural habitat by the local people for trade. This has led to the acute scarcity of this plant. Consequently, it has been enlisted as critically endangered (B1 & 2c, d) - Globally (Ravikumar and Ved, 2000). The moniliform tuberous roots of this plant are highly aromatic and the native Kani tribes use it as an effective remedy for peptic ulcer, cancer-like afflictions and as a rejuvenating tonic (Nayar, 1996). Recent pharmacological investigations of the root extract of the plant have revealed immunomodulatory and anticancer properties (Subramoniam *et al.*, 1996). The active principle (2-hydroxy - 4-methoxy benzaldehyde) of the fleshy tuberous roots of this plant was extracted and characterized by Sudha and Seeni (2001).

Very few tissue cultures studies have been known in *Janakia. In vitro* establishment of normal root cultures and the hormonal supplementations were analyzed towards extraction of the aromatic compounds (Sudha and Seeni, 2001). Regeneration of whole plants through micropropagation has been reported with single shoot development from shoot tip cultures (Sudha *et al.*, 2005; Gangaprasad *et al.*, 2005). However, a lack of comprehensive understanding of the morphogenetic potential for large scale multiplication and the restoration of the species is necessary. Hence, the present study focused on development of economically viable and rapid tissue culture protocol for shoot multiplication.

Materials and Methods

Fresh plant materials of *Janakia arayalpathra* was collected from its natural habitat Naraikadu forest - Kottangathatti Hills – at Thirukurangudi range, Kalakad Mundanthurai Tiger Reserve (Southern Western Ghats. Few plants from each species were potted and maintained in the green house for ready reference and use. Herbarium specimens (in triplicate) were deposited at the SPKCES herbarium (F.No.143). The identity of the specimens was confirmed and authenticated by K. Ravikumar, Angiosperm Taxonomist, FRLHT, Bangalore.

Murashige and Skoog (1962) medium (MS) was used to culture axillary bud, shoot tip explants of *Janakia arayalpathra*. The Basal Medium was supplemented with different concentration and combinations of plant growth regulators. Sucrose as a carbon source was added at a concentration of 3% and 0.8% agar. pH was adjusted 5.6 – 5.8 using 0.1N HCl and 0.1N NaOH solutions prior to adding agar. Molten media were dispensed into culture vials sterilized in an autoclave at 15 lbs/inch^2 for 15 minutes. Liquid endosperm from fresh tender coconuts was carefully collected, boiled to precipitate complex proteins and cooled. The cooled endosperm was filtered using several layers of cheese cloth and used as natural plant growth regulator was at

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a concentration of 5-15 % (added before adjusting the pH). Plant growth regulators such as naphthalene acetic acid (NAA), indole 3-butyric acid (IBA), indole 3-acetic acid (IAA), 6-benzyl-aminopurine (BAP) and Kinetin (KIN) were also used as supplements with MS medium in the range of 0.5µM to 20µM. Silver nitrate was used at concentrations ranging from 10 – 40 µM to assess its potential in adventitious root induction. Polyvinylpyrollidone was used at a concentration of 0.05% to overcome the problems associated with the phenolic exudation.

Explants such as axillary bud and shoot tip were surface sterilized stepwise as follows. The explants were initially washed with tap water and then soaked in liquid commercial bleach (0.5-1 % v/v NaOCl) containing a few drops of "Teepol" as surfactant for 5 minutes. This was followed by a thorough wash in running tap water. Explants were transferred to sterile laminar air flow cabinet, under such sterile conditions they were immersed in 0.05 % mercuric chloride ($HgCl_2$) solution for 5 min and rinsed thoroughly in sterile distilled water. Surface sterilized explants were placed in a sterile glass plate and trimmed into optimum size (shoot tip 0.5-1.0 cm, axillary bud 0.5 – 1.0 cm). One or two explants were aseptically placed per culture vial and at least 10 replicates were maintained for each treatment. Cultures were incubated at16/8-hour light/dark regime under 4000 lux light intensity provided by Philips (Cool While fluorescent tube). The temperature of culture room was maintained at 25 ± 2ºC. Sub-culture was done in 15 days' interval. Subculture was periodically done up to a period of 6 months. The clump of shoots were separated into single shoots or minor clumps and transferred into fresh medium for further multiplication. After 2-3 subcultures the regenerated shoots were subcultured on different shoot elongation medium. Regenerated shoots with 3-5 leaves were selected for inducing roots. Shoots were cultured on rooting medium supplemented with various concentrations of IBA, NAA and 1.5-2.5 % sucrose and 0.8 - 1.4 % agar. Plantlets were transferred to 10cm high plastic cups filled with soil mix (sterile garden soil and vermiculite soil mix in 1:1 ratio) and half strength MS liquid medium. The open top portion of plastic cups were covered with a transparent plastic sheet and kept under high light intensity (4800 lux) of 16 h photoperiod at 25 ± 2ºC. High humidity was provided for a minimum period of 2 weeks. After 15 days' small openings were made in the plastic cover to lower the humidity. In such a condition plantlets were maintained for an additional period of 10 days. Further, establishment of root and other morphological changes were observed during this stage.

Results

0.5 - 1.0 cm long shoot apices and axillary buds cultured on MS basal medium produced only 2 shoots after 10 days of culture. Among these two explants, axillary buds responded quickly. The growth of shoots in basal medium was not affected by basal callus interference. Supplementation of cytokinin (2 – 10 µM BAP) and auxin (2- 4 µM IAA) in the medium enhanced the shoot multiplication. The cytokinins, BAP and KIN supplemented individually and in combination with auxin effected shoot multiplication activity. Among the various combinations tested 8 µM BAP + 4 µM IAA evinced better response than the other combinations in induction of shoot multiplication (Table 1). The adventitious shoots arose possibly from the preexisted shoot primordia near the axillary bud. The multiplied shoots commonly depicted apical dominance where one shoot dominated the other adventitious shoots. Though shoot multiplication resulted in 10 days of culture initiation subsequent multiplication needed very long time (3 months) with regular subcultures. Addition of NAA along with BAP in shoot multiplication medium induced basal callus and suppressed the growth of adventitious shoot. KIN did help multiply shoots and the number of shoots produced was 3 - 4. Increasing the level of cytokinins did not have any dramatic effect on multiplication of shoots.

Three month old shoots in culture (5 cm long) were individually separated and transferred to half MS root induction medium. The survival of shoots in root induction medium necessitated the addition of BAP along with IBA. Besides the PGR supplement the medium was added with AC and $AgNO_3$ to induce adventitious roots. Table 2 summarizes the effect of various media adjuvants in stimulation of roots *in vitro*. The shoots cultured on hormone free medium, did not induce any root growth. Maximum root induction capacity was observed in medium supplemented with 8 µM BAP + 6 µM IBA (after 40 days). The roots were initially fragile but over period of time they turned sturdy and an average of 3 roots was produced per shoot. Amending $AgNo_3$ in root induction medium helped in production of sturdy roots. The inclusion of PVP in the entire root induction medium was essential to contain the phenolic exudation. 1.4 % of agar in the medium matrix also seemed to be essential for normal growth of roots and shoots. While shoots in the root induction medium grew longer (more than 10 cm) and exhibited climbing habit and also produced basal adventitious shoots (Figs. 4, 5). The internodes elongated unusually. Based on the growth responses evoked by PGRs and medium adjuvants in shoot multiplication and root induction a protocol for clonal propagation of *Janakia* was standardized to raise a large number of plantlets using axillary buds as explants (Fig. 3, 5). An average of 45 shoots could be produced by three cycles of cultures after shoot multiplication within 5 months. Rooted plantlets were acclimated in poly cups containing sterile soil mix soaked in half strength MS

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medium. Acclimation process took about 45 days for the plantlets to adapt and survive in the green house conditions. About 60 % survival was observed after one month of transfer. The plantlets established well under green house conditions and there were no morphological differences between the stock and *in vitro* derived plants.

Table -1: Effects of PGRs and adjuvants on clonal propagation of *Janakia arayalpathra* from axillary bud explants

S. No.	Medium Composition	Avg. No. of shoots produced / explant[a] (10 replicates)	No. of shoots survived [b] after subculture (%)	Health of shoots
1	MS BM	-	-	-
2	MS+ 2 µM BAP + 3 % S + 0.8 % A + 0.05 % PVP	3±0.81	2 (66.6)	+
3	MS + 4 µM BAP + 2.5 % S + 0.8 % A + 0.05 %P VP	3±0.76	2 (66.6	+
4	MS + 6 µM BAP + 2.5 % S + 1.0 % A + 0.05 % PVP	3±0.72	2 (66.6)	+
5	MS + 8 µM BAP + 2 % S + 1.0 % A + 0.05 % PVP	4±0.13	2 (50)	++
6	MS + 2 µM BAP + 2 % S + 1.2 % A + 0.05 % PVP	3±0.68	2 (66.6)	++
7	MS + 8 µM BAP + 2 % S + 1.2 % A + 5 % CM + 0.05 % PVP	4±0.64	3 (75)	++
8	MS+ 10 µM BAP + 2 % S + 1.2 % A + 10 % CM + 0.05 % PVP	4±0.58	3 (75)	++
9	MS + 10 µM BAP + 2 % S + 1.2 % A + 15 % CM + 0.05 % PVP	5±0.56	3 (60)	++
10	MS + 8 µM BAP + 2 µM IAA 2 % S + 1.2 % A + 15 % CM + 0.05 % PVP	4±0.72	3 (75)	++
11	MS + 8 µM BAP + 4 µM IAA 2 % S + 1.2 % A + 15 % CM + 0.05 % PVP	6±0.74	5 (83.3)	++
12	MS + 2 µM KIN + 2 % S+ 1.2 % A + 0.05 % PVP	4±0.78	3 (75)	++
13	MS + 4 µM KIN + 2 % S + 1.2 % A	5±0.82	3 (60)	++
14	MS + 6 µM KIN + 2 % S + 1.2 % A + 0.05 % PVP	4±0.80	3 (75)	++
15	MS + 8 µM KIN + 2 % S + 1.2 % A	3±0.91	2 (66.6)	++
16	MS + 10 µM KIN + 2 % S + 1.2 % A + 0.05 % PVP	3±0.84	2 (66.6)	++
17	MS + 10 µM KIN + 2 µM IAA 2 % S + 0.8 % A+0.05 %PVP	4±0.81	2 (50)	++
18	MS + 10µM KIN + 4 µM IAA 2 % S+ 1.2 % A + 0.05 % PVP	4±0.74	3 (75)	++
19	MS+ 10µM KIN+ 4µM IAA 2% S+ 1.2% A+ 5% CM+0.05%PVP	3±0.72	2 (66.6)	++
20	MS+ 10µM KIN+ 4µM IAA 2% S+ 0.8 % A+ 10% CM+0.05%PVP	3±0.64	2 (66.6)	++
21	MS+ 10µM KIN+ 4µM IAA 2% S+ 1.2 % A+ 15% CM+0.05%PVP	4±0.68	3 (75)	++

P^H 5.6

+ Thin shoots (pale green); ++ Healthy shoots (dark green)

A - All values are average of 10 replicates ± SD; B – In parenthesis values represent Percentage

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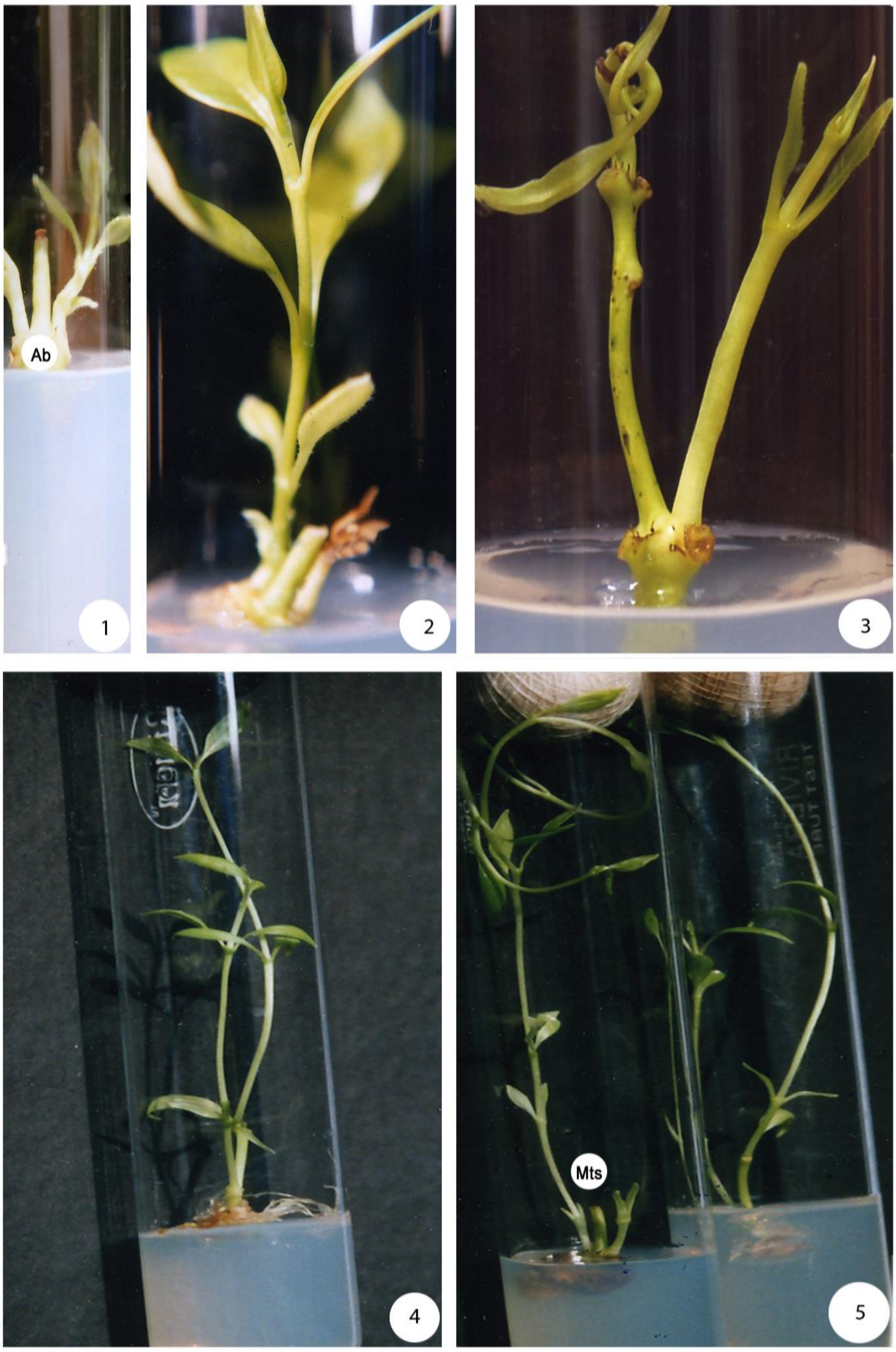

1-2. Axillary bud (Ab) cultured on MS medium containing 8 µM BAP + 4 µM IAA + 15 % CM + 0.05 % PVP. New shoots developed after 10 days. Sub-cultured at 15days interval for further multiplication.

4&5. Multiple shoots (Mts) were transferred to rooting medium (MS medium supplemented with 8 µM BAP + 6 µM IBA 30µM Ag NO_3). Fragile roots induced after 20 days of transfer.

Figs. 1 – 5: Axillary bud culture of *Janakia arayalpathra*

Table -2: Effects of PGRs and adjuvants on root induction in regenerated shoots of *Janakia arayalpathra* developed from axillary bud explants

S. No.	Media Composition	No. of shoots transferred to rooting medium	% of root induction	Nature of Established shoots
1	MS BM	5	-	-
2	MS + 8 µM BAP + 2 µM IBA + 2.5 % S + 0.8 % A + 15 % CM + 0.05 % PVP	5	2 (40)	+
3	MS + 8 µM BAP + 4 µM IBA + 2.5 % S + 0.8 % A + 15 % CM + 0.05 % PVP	5	2 (40)	+
4	MS + 8 µM BAP + 6 µM IBA + 2.5 % S + 1.0 % A + 15 % CM + 0.05 % PVP	5	3 (60)	+
5	MS + 8 µM BAP + 8 µM IBA + 2 % S + 1.0 % A + 15 % CM + 0.05 % PVP	5	3 (60)	++
6	MS + 8 µM BAP + 6 µM IBA + 2 % S + 1.2 % A + 10 µM Ag NO_3 + 0.05 % PVP	5	3 (60)	++
7	MS + 8 µM BAP + 6 µM IBA + 2 % S + 1.2 % A + 20 µM Ag NO_3 + 0.05 % PVP	5	4 (80)	++
8	MS + 8 µM BAP + 6 µM IBA + 2 % S + 1.4 % A + 30 µM Ag NO_3 + 0.05 % PVP	5	5 (100)	++
9	MS + 8 µM BAP + 6 µM IBA + 2% S+ 1.4 % A + 40 µM Ag NO_3 + 0.05 % PVP	5	4 (80)	++
10	MS + 8 µM BAP + 6 µM IBA + 2 % S + 1.4 % A + 0.05 % AC	5	4 (80)	++
11	MS + 8 µM BAP + 6 µM IBA + 2 % S + 1.4 % A + 0.075 % AC	5	5 (100)	++
12	MS + 8 µM BAP + 6 µM IBA + 2 % S + 1.4 % A + 0.1 % AC	5	4 (80)	++

P 5.6
+ healthy shoots with fragile roots
+ + healthy shoots with sturdy roots

Discussion

Janakia arayalpathra require a protocol for rapid regeneration and establishment in field conditions as it is categorized as critically endangered. Hence, this study was aimed at understanding the morphogenetic potential of explants such as shoot tip and axillary buds. MS medium supplemented with auxins such as IAA, IBA and cytokinins including BAP and KIN have been evaluated. Plant growth regulators were amended at different concentrations as per the requisite of culture and response either individually or in combinations.

Clonal propagation has many advantages over conventional methods of vegetative propagation which are advantages for producing true to type plants and virus and pathogen free material. Numerous factors are reported to influence the success of *in vitro* clonal propagation of different medicinal plants. Clonal propagation of various plant species including medicinal and rare and endangered species has been reported (Tsay *et al.*, 1989; Anu *et al.*, 2001; Ahuja *et al.*, 2002; Rai, 2002; Srivastava and Srivaarva, 2004; Koul *et al.*, 2005; Geetha and Gopal, 2007; Meena and Patni, 2007). Propagation from existing meristems (shoot tips and axillary buds) produces plants that are genetically identical with the donor plants (Hu *et al.*, 1983; Gupta *et al.*, 1996; Saha *et al.*, 2003; Nikam and Savant, 2007; Sharma *et al.*, 2007). Plant regeneration from shoot and stem meristems yielded encouraging results in medicinal plants like *Catharanthus roseus, Cinchona ledgeriana, Digitalis spp., Rehmannia glutinosa, Ravulfia serpentine, Isoplexis canariensis* (Roy *et al.*, 1994; Perz- Bermudez *et al.*, 2002; Tripathi and Tripathi, 2003).

A successful protocol for rapid regeneration of *Janakia arayalpathra* has been developed using existing meristems

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from axillary buds (Figs. 1-5). During the initial attempts, the initiation and shoot multiplication was inhibited due to the development of callus from the cut ends of the axillary bud explants. To overcome the accompanied callus growth in both the species low concentrations of IAA (2 - 4 µM) was supplemented in combination with cytokinins (BAP, KIN). Callus interference could be completely prevented and shoot multiplication was enhanced. Emergence of new shoots from the cultured axillary buds was higher in MS medium supplemented with IAA along with cytokinins than the medium augmented only with cytokinins (Tables 1 & 2). The degree of growth and differentiation of multiple shoots varied considerably with the media combination. An average of 45 shoots could be produced by three cycles of cultures after shoot multiplication within five months.

Media adjuvants like coconut milk, activated charcoal, silver nitrate and polyvinyl pyrollidone have support shoot multiplication and control of phenolic substances in the medium. The role of these supplements in tissue culture has been widely reported (Antis and Northcote, 1973; Weatherhead *et al.,* 1979; Hossain *et al.,* 1993; Chi *et al.,* 1990, 1994; Teng, 1997; Obel Reddy *et al.,* 2002). Further establishment of the newly multiplied shoots was facilitated by the supplementation of enhanced level of coconut milk. The effect of CM on shoot multiplication was due its innate contents of cytokinins such as zeatin, zeatin ribosides and kinetin (Shirgurkar *et al.,* 2001). Comparing the different shoot multiplication media used in this study, MS medium supplemented with 8 µM BAP and 4 µM IAA was superior to other media combinations. The effect of auxin and cytokinin combination in shoot multiplication has been well understood and employed in large to rapidly increase the multiple shoot (Bhagyalakshmi, 1988; Rahman *et al.,* 2004; Gayathri and Ramagopal, 2007; Meena and Patni, 2007).

It has been observed that cytokinin is required in an optimal quantity, for shoot proliferation in many genotypes but inclusion of low concentration of auxins along with cytokinin triggers the rate of shoot proliferation (Rout *et al.,* 1992; Rout and Das, 1993; Tsay *et al.,* 1989). Cut ends of axillary bud have released minute milky latex. As the latex inhibited the uptake of nutrients during *in vitro* culture, the medium was supplemented with 0.05% of PVP (Reeth and Shivamurthy, 2005). Activated charcoal was also reported to have the similar kind of activity in suppressing the action of milky latex and other polyphenols in culture conditions.

The effects of activated charcoal in establishment of phenolics free culture and plant regeneration has been reported in various species. Activated charcoal improves the establishment of protoplast culture (Kunitake *et al.,* 1995), prevents the development of abnormal plantlets (Ziv an Gadasi, 1986) and enhances somatic embryogenesis, shoot formation, plant recovery and rooting (Buter *et al.,* 1993; Dumas and Monteuuis, 1995; Fuchs, 1991; Mathews *et al.,* 1993; Patel and Thorpe, 1984; Sinha and Mallick, 1991). Moreover, activated charcoal restores the abilities for somatic embryogenesis and consequently plant regeneration from long-term sub-cultured *Festuca rubra* callus and shows a decline in regeneration potential (Zaghmount and Torello, 1988). Various aspects of the effects of activated charcoal on *in vitro* culture have been investigated in higher plants, including the quantity (Teixeira *et al.,* 1995), the application method (Ziv and Gadasi, 1986), the timing of application (Kunitake *et al.,* 1995), and the treatment duration (Zaghmout and Torello, 1988).

Root induction was accomplished by adding IBA, NAA and other adjuvants like activated charcoal and silver nitrate individually and in combination at different concentrations. Roots were developed after 10 days from the date of transfer of shoots into rooting medium. Maximum root induction capacity was observed in medium supplemented with 8 µM BAP + 6 µM IBA along with silver nitrate. The role of silver nitrate in root induction has been well reported (Chi *et al.,* 1990, 1994; Obul Reddy *et al.,* 2001).

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