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Cytokinin-Induced High Frequency Shoot Multiplication in *Celastrus paniculatus* Willd., a Red Listed Medicinal Plant

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ABSTRACT

A protocol for rapid propagation of *Celastrus paniculatus* Willd. has been developed. A range of cytokinins and gibberelic acid were tested on Murashige and Skoog (MS) medium for multiple shoot induction and high frequency shoot multiplication with axillary nodes of seedlings. While the seeds were subjected to three different treatments (room temp, cold (4°C) and water soaking treatments), cold treatment of seeds when cultured for 3-4 weeks on MS medium with a combination of 6-benzylaminopurine (BA) and kinetin (KN) was sufficient to induce maximal shoot response (81.2%) and gave the highest number of shoots per explant (4.4). All shoots regenerated directly without a callus phase and individual hormones BA, KN and GA_3 were less effective. The excision of the node and shoot tip from *in vitro* grown seedlings and their subsequent culture on MS medium supplemented with 2.2 µM BA and a combination of MS+ 4.4 µM BA + 1.1 µM KN facilitated enhanced axillary bud proliferation (66.6%). Best rooting was observed on MS medium fortified with 10.7 µM α-naphthaleneacetic acid. The new plantlets were weaned and subsequently acclimatized in a glasshouse and a 80-90% survival rate was achieved in the field. This study has significant implications in achieving considerable progress in *ex situ* conservation initiatives for *C. paniculatus*.

Keywords: cold treatment, combination of cytokinins, *ex situ* conservation, multiple shoot induction, nearly threatened
Abbreviations: BA, N^6-benzylaminopurine; **CT**, cold-treated; **GA_3**, gibberelic acid; **$HgCl_2$**, mercuric chloride; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **KN**, kinetin; **MS**, Murashige and Skoog medium; **NAA**, α-naphthaleneacetic acid; **RT**, room temperature; **TDZ**, thidiazuron; **WS**, water-soaked

INTRODUCTION

Celastrus paniculatus Willd. (Celastraceae) is a perennial medicinal plant, known for centuries as the "elixir of life", and occurs from South Asia eastwards through Indochina to Australia and New Caledonia (Godkar *et al*. 2006). It is found throughout most parts of India, chiefly in deciduous forests (Parrotta 2001). It has a great medicinal value and the seeds are used to increase intelligence and memory power (Nadkarni 1976). Pharmacological studies suggest that the seed oil obtained from the seeds possesses sedative and anticonvulsant properties (Gaitonde *et al*. 1957) along with a number of additional pharmacological actions such as analgesic (Ahmad *et al*. 1994), anti-malarial (Ayudhaya *et al*. 1987), anti-inflammatory (Dabral and Sharma 1983), bactericidal (Patel and Trivedi 1962), insecticidal (Atal *et al*. 1978), hypolipidemic (Khanna *et al*. 1991) and anti-spermatogenic (Wangoo and Bidwai 1988). Seed oil is also shown to have beneficial effects in treating psychiatric patients and improving certain psychological attributes, including IQ in mentally retarded children (Hakim 1964; Nalini *et al*. 1986). Seed oil consists of noval β-dihydro agaroform sesquiterpene polyesters "celapanin and celapanigin" (Zhang *et al*. 1998). The seeds are the major source of regeneration in *C. paniculatus*. However poor seed germination and over-exploitation are the major constraints in the sustainable availability of *C. paniculatus* in the wild and resulted in a marked decline in the populations globally and the plant is now categorized as "nearly threatened" (Anonymous 2000).

In vitro propagation methods are essential components of plant genetic resources management and they are becoming increasingly important for conservation, rapid large-scale propagation of rare and endangered plant species (Fay 1992; Pradhan *et al*. 1998; Benson *et al*. 2000; Bhatia *et al*. 2002). Similarly, these techniques facilitate long-term storage, to comply with the need of the herbal and pharmaceutical industry (Hawkes *et al*. 2000). Conservation of *C. paniculatus* via micropropagation (*ex situ*) is an effecttive alternative for meeting the need for plants, within a reasonable timeframe without affecting the wild bioresources.

Micropropagation of *C. paniculatus* via nodes, shoot tips, internodes and leaf bases derived from mature plants has been reported by Nair and Seeni (2001) and regeneration of *C. paniculatus* plantlets via callus cultures was reported by Sharada *et al*. (2003). However, the shoot proliferation rate achieved in both the cases was very low. The present article deals with the comparative performance of cytokinins on multiple shoot induction and high frequency shoot proliferation from the explants of *in vitro* grown seedlings and subsequent acclimatization of plantlets in natural habitats. This *in vitro* micropropagation protocol using seedling explants is novel, and hence reported. Many medicinal plants such as *Sterculia urens* (Purohit and Dave 1996), *Gymnema sylvestre* (Komalavalli and Rao 2000), *Anoectochilus formosanus* (Shiau *et al*. 2002) have been propagated through seedling-derived explants.

MATERIALS AND METHODS

Mature pods were collected from a 10-15 years old plant at the Srisailam Forest Reserve, Andhra Pradesh, India. Seeds were washed thoroughly with running tap water for 15 minutes and soaked in 0.5% (w/v) fungicide bavistin (Carbondazim) for 30 minutes. Further surface sterilization was done using 70% (v/v) ethanol for 2 minutes, followed by 5 minutes in 0.1% (w/v) $HgCl_2$ and rinsed 2-3 times with sterile double distilled water. Seeds were subjected to

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three different treatments. In the first, treatment seeds were maintained at room temperature (RT) throughout the experiment. Seeds were subjected to 4°C cold (CT) and soaked in water (WS) for 24 hours during the course of second and third treatments, respectively. Initially the seed explants were transferred to sterile Whatman No. 1 filter paper bridges on semi solid Murashige and Skoog (1962) (MS) medium fortified with different concentrations (µM, w/v) of hormones 6-benzylaminopurine (BA) at 0.4-13.3 µM, kinetin (KN) at 0.4-13.9 µM and gibberelic acid (GA_3) at 0.2-8.6 µM] individually and in the following combinations, all in MS: 4.4 µM BA + 1.1 µM KN; 4.4 µM BA + 2.3 µM KN; 2.2 µM BA + 1.1 µM KN; 2.2 µM BA + 2.3 µM KN. These treatments were investigated to optimize the hormonal requirements for multiple shoot induction in addition to MS (control), ½MS and ¼MS medium with 3% (w/v) sucrose and 0.3% (w/v) agar. The pH of the medium was adjusted between 5.6-5.8 before autoclaving at 121°C for 20 minutes.

After 3-4 weeks, germination of seeds on filter paper was noticed in all the three above-mentioned methods i.e RT, CT and WS. Shoot tips and axillary node explants (0.5-1 cm) were dissected and cultured on MS shoot proliferation medium supplemented with various concentrations of BA (0.4-8.8 µM) and combinations of cytokinins (BA+KN) as described earlier, in addition to phytohormone-free MS, the control. The cultures were maintained in a culture room at 22±3°C under a 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 80 $\mu Em^{-2} s^{-1}$ provided by white fluorescent tubes with 70% relative humidity. All cultures were transferred every 2-3 weeks onto fresh medium with the same composition.

Shoots (4-5 cm long) regenerated from different explants were excised and individually transferred to MS medium fortified with various concentrations of auxins IAA (0.5-17.1 µM), IBA (0.4-14.7 µM) and NAA (0.5-16.1 µM) in addition to ½MS and ¼MS for root induction. The culture conditions were same as those of the multiplication phase. Six micro shoots were used for each treatment and the experiment was repeated twice. Plantlets with well developed roots were removed from culture tubes and washed in sterile distilled water to remove traces of agar and then transplanted into plastic containers (7.5 cm diameter) filled with a sterile mixture of soil, sand and farmyard manure (1:1:1) covered with transparent polythene bags to maintain high humidity under controlled growth chamber conditions (22±3°C, 16 h photoperiod, 70-80% relative humidity and 80 $\mu Em^{-2} s^{-1}$ light intensity) and irrigated daily with a few ml of tap water. After 10-15 days in culture room conditions, the polythene bags were removed and the plantlets were shifted to green house for further acclimatization. Established plantlets were transferred to the field for hardening and the percentage survival was recorded at this stage.

The data were scored in terms of percentage of seed explants that responded and produced shoots after 4 weeks, the number of shoots produced per explant in the multiple shoot induction phase, the percentage of shoots that rooted and the number of roots formed in the rooting phase. The experiment had four replicates each of which consisted of four culture tubes repeating twice. Data were subjected to analysis of variance (ANOVA) to assess treatment differences. Significance between means was tested by Duncan multiple range test (DMRT) at a 5% probability level using MSTAT-C computer programme (Michigan State University). Data given in percentages were subjected to arcsin ($\sqrt{X}$) transformation (Snedecor and Cochran 1967) before statistical analysis.

Fig. 1 Micropropagation of *C. paniculatus*. (**A, B**) Multiple shoot induction from seed explants on MS semi-solid medium supplemented with 4.4 µM BA + 1.1 µM KN after 4 weeks. (**C**) Shoot multiplication from node explants on MS + 2.2 µM BA + 1.1 µM KN after 2nd subculture. (**D**) A clump of rapidly proliferating shoots developed from node explants on 2.2 µM BA after 2nd subculture. (**E**) Rooting of *in vitro* regenerated shoots on 10.7 µM NAA. (**F**) Potted plantlets covered with polythene covers maintained in culture room before transfer to glass house. (**G**) Well grown plants established in soil after 4-5 weeks of hardening.

RESULTS AND DISCUSSION

Influence of treatments

The results showed that all three (i.e. RT, CT and WS) pretreated seeds responded well to cytokinin treatments within 2-3 weeks. Significant differences ($P \leq 0.05$) were noticed in the response and multiple shoot induction when these pretreated seeds were subjected to different cytokinins individually and in combination. Seeds germinated within two weeks and multiple shoots were visible after approximately three to four weeks of culture (**Fig. 1A, 1B**). Seeds subjected to overnight cold treatment (CT) (at 4°C) responded well to hormonal treatments. Whereas in room temp (RT) and watersoaked (WS) seeds, the response was suppressed during the same culture period. This experiment suggested that seeds subjected to cold treatment enhanced seed germination due to breakage of dormancy. Using this cold treatment the highest germination percentage was noticed in the four combinations of cytokinins (BA+KN) than in individual hormone BA, KN and GA_3 application. Multiple shoot induction from seed explants was highly effective in all combinations of cytokinins, with all the three pretreated seeds (**Table 1**). However in the control, ½MS and ¼MS, the seed response was poor (**Table 1**). Among the different treatments of seeds tested with hormones CT was found to be the best treatment for shoot response (52.57%), and number (2.1) followed by RT and WS seeds (**Table 1**). In both RT and WS seeds the mean response and sprouting was more or less similar. Thus the degree of response and sprouting varied considerably with various pretreatments. This shows the need of CT, which stimulates the embryo making it active for regeneration of seeds of *C. paniculatus* to overcome the dormancy. Our findings are similar to those for the medicinal plant *Echinacea* where cold treatment of seeds enhanced the germination percentage (Romero *et al.* 2005).

Table 1 Influence of room temperature, cold and water soaking treated seeds to different hormones, on *in vitro* seed response and number of shoots of *Celastrus paniculatus* after four weeks of culture. Values are means of two replicates with 16 seeds in each replication.

Pretreatment of seeds	Percentage of seeds responding	Mean number of shoots/ seed
Room temp.	47.91^{b}	1.54^{c}
Cold treated	52.57^{a}	2.12^{a}
Water soaked	46.40^{c}	1.65^{b}

In each column means followed by different letters are significantly different ($P \leq 0.05$) as indicated by one-way ANOVA followed by Duncan's multiple range test.

Table 2 Influence of BA, KN, GA_3 and BA+KN treated seeds subjected with pretreatments (RT, CT, WS), on *in vitro* seed response and number of shoots of *Celastrus paniculatus* after four weeks of culture. Values are means of two replicates with 16 seeds in each replication.

Media treatments	Percentage of Seeds responding	Mean number of shoots/ seed
MS	48.60^{de}	1.45^{ghij}
½ MS	42.59^{g}	1.23^{lm}
¼ MS	36.49^{h}	1.11^{m}
MS + 0.4 µM BA	45.59^{fg}	1.36^{ijkl}
MS + 2.2 µM BA	50.43^{bcd}	1.41^{hijk}
MS + 4.4 µM BA	51.05^{bcd}	1.56^{efgh}
MS + 8.8 µM BA	46.80^{ef}	1.48^{fghi}
MS + 13.3 µM BA	43.78^{fg}	1.31^{jkl}
MS + 0.4 µM KN	44.30^{fg}	1.46^{fghij}
MS + 2.3 µM KN	53.13^{b}	1.66^{de}
MS + 4.6 µM KN	52.94^{bc}	1.80^{d}
MS + 9.2 µM KN	44.38^{fg}	1.61^{ef}
MS + 13.9 µM KN	42.59^{g}	1.28^{kl}
MS + 0.2 µM GA_3	46.78^{ef}	1.33^{ijkl}
MS + 1.4 µM GA_3	51.02^{bcd}	1.45^{ghij}
MS + 2.8 µM GA_3	58.15^{a}	1.68^{de}
MS + 5.7 µM GA_3	51.04^{bcd}	1.58^{efg}
MS + 8.6 µM GA_3	49.79^{cd}	1.41^{hijk}
MS + 2.2 µM BA + 1.1 µM KN	51.02^{bcd}	2.78^{c}
MS + 2.2 µM BA + 2.3 µM KN	57.37^{a}	3.40^{a}
MS + 4.4 µM BA + 1.1 µM KN	58.23^{a}	3.46^{a}
MS + 4.4 µM BA + 2.3 µM KN	51.04^{bcd}	3.08^{b}

RT- room temperature; **CT -** cold treated; **WS -** water soaked
In each column means followed by different letters are significantly different ($P \leq 0.05$) as indicated by one-way ANOVA followed by Duncan's multiple range test.

Influence of hormones

Experimental results of the effect of different cytokinins (BA, KN, BA+KN) and GA_3 combined with three treatments were investigated on enhancing the percentage response and shoot induction and are summarized in **Table 2**. Multiple shoots were visible after approximately two to three weeks of culture and the maximum number of shoot initials was observed in 4-5 week old cultures in BA, KN and BA+KN. In the case of GA_3 multiple shoot induction could be observed only after 4 weeks. ANOVA revealed that the treatments had a significant effect ($P \leq 0.05$) on percentage of seed germination response and multiple shoot induction. A comparison of different cytokinin concentration treatments showed that maximum response and shoot bud induction per seed explant was observed in BA+KN, followed by KN, GA_3 and BA. In BA+KN the response and sprouting was huge in all three parameters. A total four combinations of BA+KN were used to test the seed germination response and multiple shoot induction. This combination worked well for *C. paniculatus* seeds with a huge response and multiple shoot induction. BA+KN hormone combinations produced almost three times the response as compared to individual hormones BA, KN and GA_3. The highest response (81.2%) with as many as 4.4 multiple shoots were obtained after 4-5 weeks culture in MS medium supplemented with 4.4 µM BA + 1.1 µM KN, thus indicating the synergistic effect of hormones on multiple shoot induction (**Table 2; Fig. 1B**). With an increase in concentration of BA+KN (4.4 µM BA + 2.3 µM KN) the percentage response and multiple shoot induction decreased. This regeneration of multiple shoots from seeds might be due to a phenomenon such as polyembryony or apomixis. However no anatomical studies were done to understand the type of regeneration pattern from seed explants in combination of hormones. Generally cytokinins are known to promote cytokinesis (cell division) in certain plant tissues, and it is not unlikely that they have this role in the developing seed too (Sawan *et al*. 2000). The period of active cell division and enlargement in the seed (in both the embryo and endosperm) is longer when the cytokinins are at their highest level. This may indicate recognition of the cells, or mechanisms of action of the cytokinin compounds (Huetteman and Preece 1993). They also reported that shoot proliferation can be significantly enhanced by the addition of a low concentration of cytokinin to the cytokinin proliferation medium. Similar findings observed by the addition of TDZ to a BA containing medium that enhanced axillary shoot proliferation in *Pyrus communis* (Singha and Bhatia 1988) and *Vitis rotundifolia* (Sudarsono and Goldy 1991). In the other three combinations of BA and KN the response resulted in a reduced number of shoots (**Table 2**).

In the case of KN at 4.6 µM, the highest average number of multiple shoots were 2.3, 1.5 and 1.5 in CT, RT and WS, respectively. The use of GA_3 was also investigated for sprouting as it is a dormancy-breaking hormone. When GA_3 was used alone (0.2-8.6 µM) in the media, at 2.8 µM, the highest average number of multiple shoots were 2.0, 1.5 and 1.5 in CT, RT and WS, respectively. This is in sharp contrast to the reports of Arora and Bhojwani (1989) where increasing concentration of GA_3 (0.3-10.0 mg/L) was shown to have a promotive effect on differentiation of shoot buds in *Saussurea lappa*. GA_3 at a high concentration resulted in the suppression of seed germination response and shoot induction (**Table 2**). According to earlier reports on micropropagation (for example, Mao *et al.* 2000) BA was more effective than the other cytokinins, but in the current study the response and induction rate was lesser than those obtained in individual hormones GA_3, KN and combined BA+KN treatments. No callus regeneration was noticed with any of the above treatments. Retention of the explants with proliferated shoots in the initiation medium for more than 4 weeks resulted in a decline as evidenced from the formation of smaller new leaves and premature leaf fall. The results of different hormonal treatments clearly demonstrate that seeds of *C. paniculatus* have stringent hormonal requirements for initiation of germination. In this experiment all hormones (with respect to treatments), the percentage of response and multiple shoot induction were high in CT. Overall the percentage response and number of shoots were influenced by pretreatments and by different hormone concentrations ($P \leq 0.05$; **Table 2**).

Explants obtained from four week-old seedlings showed maximum proliferation and this was found to be the optimum seedling age for multiple shoot induction. Among the various explants tested, only axillary node and shoot tip explants showed a high response for multiple shoots. The excision of axillary node and shoot tip segments and their culture on MS medium supplemented with the same growth regulators BA (0.4-8.8 µM) and combinations of BA+KN (as described earlier for seed regeneration) enhanced the shoot multiplication rate with a resulting increase in the number of shoots (12±2) which developed. This response of shoot bud proliferation was enormous and no decline in shoot number was observed, even up to the third culture without any basal callus. The control in this experiment showed normal growth of explants with minimum number of shoots (**Table 3**). Initially, small protuberances emerged on the epidermal cell layer at the basal

Table 3 Evaluation of *in vitro* shoot proliferation of node and shoot tip explants derived from initial multiple shoot induction phase of *Celastrus paniculatus*, cultured on BA and BA+KN medium after 2nd subculture.

Media treatments	Node segment	Shoot tips
Control	7.4^{g}	5.4^{c}
MS + 0.4 µM BA	10.0^{ef}	9.3^{ab}
MS + 2.2 µM BA	15.4^{a}	10.8^{ab}
MS + 4.4 µM BA	11.3^{cde}	12.1^{a}
MS + 8.8 µM BA	9.1^{fg}	9.3^{ab}
MS + 2.2 µM BA + 1.1 µM KN	12.5^{bcd}	8.4^{bc}
MS + 2.2 µM BA + 2.3 µM KN	14.3^{ab}	10.6^{ab}
MS + 4.4 µM BA + 1.1 µM KN	13.1^{bc}	10.6^{ab}
MS + 4.4 µM BA + 2.3 µM KN	10.8^{def}	9.6^{ab}

In each column means followed by different letters are significantly different ($P \leq 0.05$) as indicated by one-way ANOVA followed by Duncan's multiple range test.

portion of the existing shoot, which later developed into a bulge and subsequently differentiated into a shoot buds. Once the shoot buds attained 1-1.5 cm in length, the cycle of initiation and development of new shoot buds was repeated. These new adventitious shoots arose from peripheral epidermal cells but not from the pre-existing buds. The extensive branching pattern observed supports the suggestion that BA inhibits shoot growth and also promotes adventitious bud initiation. Maximum number of shoots (14±2) were obtained after the 2nd sub-culture in the medium containing BA (2.2 µM) with node explants (**Fig. 1D**). While reporting shoot multiplication in *Gymnema sylvestre* using BA, similar observations were also made by Martin (2003), Komalavalli and Rao (2000). The present shoot proliferation rate from nodes is superior to the results of Nair and Seeni (2001) who observed 8±1 shoots at 4.4 µM BA and Sharada *et al*. (2003) who reported 25.6±0.8 shoots at 10 µM BA. According to our study, lower concentrations of BA (2.2 µM) resulted in the regeneration of the highest number of shoots (14±2). Repeated subculturing of node and shoot tip explants in BA (2.2 µM) enabled continuous production of callus-free shoots. Compared to node explants, shoot tips produced fewer multiple shoots in further subcultures. The BA+KN combination resulted in a number of shoots and percentage response that was on par with that observed with BA alone (**Fig. 1C**). This is similar to reports in which shoot multiplication was enhanced in *Eucalyptus impensa*, when a BA+KN combination was used (Bunn 2005). With increase (2.2 µM) in BA levels reduced the number of shoots and shoot length per explants with characteristic symptoms of browning, thin, weak shoots and shoot tip decay. BA at higher concentrations (>2.2 µM) not only reduced the number of shoots but also resulted in stunted growth of shoots, according to Martin (2003). Prolonged delay in subculture resulted in accumulation of phenolics, resulting in the shoots turning brown with little callus at the base. The elongated, subcultured shoots were longer (4.2 cm) than the previous seed regenerated shoots (3.5 cm). This is similar to the results reported in *Withania somnifera* (Sen and Sharma 1991). Because of the superior *in vitro* response of the node and shoot tip explants in both BA and combinations of BA+KN, only these explant derived shoots were used in subsequent cultures, rooting and field acclimatization experiments.

On the basis of the results obtained in the present study, we estimate that a single juvenile shoot from seed explants can produce an average of 70-80 shoots after 11-12 weeks in culture. This method of multiple shoot induction using mature seeds is simple and can result in a high frequency of multiple shoot formation.

The regenerated shoots were excised and transferred to different rooting treatments with IAA, IBA and NAA. In most cases root initiation started within two weeks of culture. The shoots that failed to form roots in this period were unable to produce roots even after 5 weeks of culture. In control, ½MS and ¼MS, a low frequency of rooting was noted, showing thin, short and slow growing shoots. However, other hormones improved the rooting percentage, root quality and the number of roots per cutting (**Table 4**). Among the three types of auxins, NAA (90%) was found to be comparatively superior followed by IAA (63%) and IBA (81.5%) with respect to percentage response during induction of roots. The best rooting percentage (100%) and the highest number (6.1) of roots per shoot was obtained using 10.7 µM NAA with minimum callus formation within 5-6 weeks (**Fig. 1E; Table 4**). IAA and IBA treated shoots produced callus along with root formation at the basal end of the *in vitro* shoots. Effectiveness of NAA at lower concentrations in *in vitro* rooting has been reported in various medicinal plants, for example *Verbascum thapsus* (Turker *et al*. 2001), *Santolina canescens* (Casado *et al*. 2002) and *Rotula aquatica* (Martin 2003). Our study, however, shows an improvement in rooting over previous reports. For hardening, the rooted plantlets obtained from the culture tubes were washed thoroughly to remove medium and were planted in small plastic containers containing sterilized soil. For the initial period these plantlets were kept in culture room conditions. Humidity was maintained by covering the plantlets with transparent plastic bags (**Fig. 1F**). Within 10-15 days the plantlets started to develop new leaves and resumed growth. Successful acclimatization was achieved under greenhouse conditions in two weeks and plants were adapted to field conditions within 4-5 weeks of hardening. Plantlets were subsequently transferred to larger pots and were allowed to grow normally (**Fig. 1G**). The plantlets exhibited 80-90% survival in field conditions.

Table 4 Effects of auxins IAA, IBA and NAA on rooting *in vitro* regenerated shoots of *C. paniculatus* cultured on MS medium after 5-6 weeks.

Treatment	Percentage of response	Mean number of roots/explant
Control	52.24^{fgh}	2.0^{i}
1/2 MS	49.78^{gh}	1.8^{i}
1/4 MS	45.00^{h}	1.6^{i}
MS + 0.5 µM IAA	54.70^{efg}	3.4^{h}
MS + 2.8 µM IAA	60.00^{def}	4.2^{efg}
MS + 5.7 µM IAA	62.94^{cde}	4.4^{de}
MS + 11.4 µM IAA	62.94^{cde}	4.7^{cd}
MS + 17.1 µM IAA	57.35^{defg}	4.3^{def}
MS + 0.4 µM IBA	60.00^{def}	3.1^{h}
MS + 2.4 µM IBA	62.94^{cde}	3.4^{h}
MS + 4.9 µM IBA	65.88^{bcd}	3.9^{fg}
MS + 9.8 µM IBA	81.57^{a}	4.3^{def}
MS + 14.7 µM IBA	62.94^{cde}	3.8^{g}
MS + 0.5 µM NAA	62.94^{cde}	4.0^{efg}
MS + 2.6 µM NAA	69.51^{bc}	5.1^{bc}
MS + 5.3 µM NAA	90.00^{a}	5.4^{b}
MS + 10.7 µM NAA	90.00^{a}	6.1^{a}
MS+ 16.1 µM NAA	73.15^{b}	4.9^{c}

In each column means followed by different letters are significantly different ($P \leq 0.05$) as indicated by one-way ANOVA followed by Duncan's multiple range test.

CONCLUDING REMARKS

The main objective of this study was to establish an alternative method of micropropagation system using seed regenerated multiple shoots for conservation and mass propagation of *C. paniculatus*. MS medium containing 4.4 µM BA + 1.1 µM KN is the best for seed regeneration. MS basal medium supplemented with 10.7 µM NAA is the best for root induction. The use of axillary nodes for multiplication is more beneficial than shoot tips as explants would help in germplasm conservation for commercial cultivation and also alleviate the pressure on natural populations. Even though *in situ* conservation has high priority in conservation strategy programmes, there are some limitations, such as the risk of material being lost due to environmental hazards while the cost of maintaining a large number of plants in the field is extremely high. In *ex situ* conservation, conventional methods of propagation using different modes is limited and delayed. So the remaining viable strategy to meet the demand is *in vitro* propagation. *In vitro* propagation using tissue culture has clearly proved to be a useful technique for multiplication of a number of medicinal plants (Afolayan 2004). Direct regeneration of shoots from seed explants seems to be a desirable method since there is no callus phase involved in the shoots obtained and thus chance for increasing variation. Such an efficient regeneration is a prerequisite for large-scale multiplication and production within a short time span.

Though regeneration of plantlets via cotyledonary leaf-derived callus cultures was established by Sharada *et al*. (2003), our protocol on the study of regeneration of plantlets using seed explants directly, makes the large scale multiplication of *C. paniculatus* plants within a short timespan simple, effective and reproducible. Seed explants are frequently used for woody species because researchers have had the greatest success with their regeneration (Huetteman and Preece 1993). Moreover seed regenerated shoots exhibit a relatively high amount of heterozygosity, which is helpful in further studies on seed quality and quantity. In the present study the rate and percentage of shoot production per

explant was high when compared to previous reports. We could obtain 70-80 shoots after 4 subcultures from a single node explant. Thus, this protocol proved to be very useful in breaking seed dormancy and enhancing germination to build up seedling stocks. Moreover this seems to be the desirable method since there is no callus phase involved in the shoots obtained.

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