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In vitro plant regeneration system for *Cassia siamea* Lam., a leguminous tree of economic importance

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Abstract A method for rapid in vitro propagation of *Cassia siamea* Lam. using cotyledonary node explants, excised from 14-day old aseptic seedlings, has been established. Murashige and Skoog (MS) medium supplemented with different concentrations of 6-benzyladenine (BA), kinetin (Kn) and thidiazuron (TDZ) singly or in combination with auxins was used for regeneration studies. Among the single treatment of three cytokinins BA at 1.0 μM was found to be optimum for direct shoot regeneration as it induced an average of 8.20 ± 0.66 shoots per explant. The regeneration frequency further enhanced with the application of auxin along with optimal BA concentration. The highest frequency for shoot regeneration (90%), the maximum number of shoots per explant (12.20 ± 0.73) and the maximum shoot length (6.40 ± 0.07) cm were obtained on the medium consisted of MS + 1.0 μM BA + 0.5 μM NAA. Successful in vitro rooting was induced from cut end of the microshoots when placed on half-strength MS + IBA (2.5 μM). The regenerated shoots with well developed root system were successfully acclimatized and established in pots containing sterilized garden soil and garden manure (1:1) and grown under greenhouse conditions with 85% survival rate.

Keywords *Cassia siamea* · Fabaceae · Cotyledonary node · In vitro shoot regeneration

Abbreviations

BA	6-Benzyladenine
Kn	Kinetin
TDZ	Thidiazuron
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
NAA	α-Naphthalene acetic acid
GA_3	Gibberellic acid
MS	Murashige and Skoog medium
PGR	Plant growth regulator

Introduction

Cassia siamea Lam. (Siamese senna) is a fast growing, multipurpose nitrogen fixing tree species belonging to family Fabaceae (sub family Caesalpinaceae). The plant is native to South East Asia and is very popular in arid regions, particularly West Africa, it has been extensively planted as an avenue and shade tree in tea estates and found useful for afforestation of degraded and wastelands where organic manure is deficient. It decreases soil erosion, while improving soil fertility in the plantation site, well adapted to a variety of climatic conditions within the tropics, and highly resistant to

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drought. The plant also provides excellent fuel wood, premium quality timber, good fodder and green manure and compost (Anonymous 1992). Therefore, the tree species possess a great potential to be employed as a tool for traditional agroforestry systems and social and community forestry (Ouinsavi and Sokpon 2008) by conservation of germplasm through in vitro techniques.

Medicinally, it is reported to exhibit laxative and purgative properties and has been traditionally used for the treatment of liver problems, urticaria, loss of appetite from gastrointestinal trouble and rhinitis. Young leaves and flower extracts of the plant contain a type of chromone called barakol, two novel alkaloids cassiarins A(1) and B(2), anthraquinone glycosides and other alkaloids which are effective against insomnia, anxiety, periodic fever, malaria and showed a potent antiplasmodic activity (Morita et al. 2007).

C. siamea is commonly propagated through seeds. However, several fungi, sap suckers and wood borers are reported to attack the plant and a considerable damage is caused by *Dendrophthoe falcata* (a stem parasite). Thus, reducing the survival percentage of the seedlings and increasing its mortality rate. Besides, wild-stand seedlings are highly variable in terms of growth and biomass production. More uniform, healthy and vigorously growing planting stock of *C. siamea* that can benefit agroforestry systems can be obtained through micropropagated superior trees. Micropropagation techniques can be used as an alternative tool for rapid establishment and conservation of control pollinated seeds. Although, the regeneration of forest trees in general and legumes in particular have been a difficult task (Lakshmisita et al. 1992), the strategies to regenerate legumes by exploiting tissue culture techniques have been evolved steadily during the past few years (Rhagavaswamy et al. 1992; Gulati and Jaiswal 1996; Shahzad et al. 2006; Husain et al. 2008). Thus, to ensure an availability of large number of planting propagule for afforestation programmes, biomass production and germplasm conservation an alternative and feasible method (micropropagation) is of great value. As far as literature is available there has been no report of any attempt for micropropagation of *C. siamea*. In the present study the cotyledonary node explant (CN) was selected for the development of efficient regeneration protocol. CN explants have already been recommended, as an excellent tissue, in several earlier studies conducted in other forest tree species (Pradhan et al. 1998; Sinha et al. 2000; Anis et al. 2005).

Materials and methods

Explant collection and establishment of aseptic seedlings

The certified seeds of *C. siamea* obtained from Prem Nursery and Seed Store, Dehradun, India were used for raising aseptic seedlings. Seeds were first thoroughly washed under running tap water for 30 min to remove adherent particles. Seeds were kept in 1% (w/v) Bavastin (Carbendenzim Powder), a broad spectrum fungicide, for 25–30 min, followed by thorough washing with 5% (v/v) Teepol, a liquid detergent, by continuous shaking for 15 min. Seeds were washed with sterile double distilled water (DDW) for 3–4 times under the laminar flow hood followed by a short treatment with 70% (v/v) ethanol for 30–60 s, rapidly washed with sterile DDW and then surface sterilized using 0.1% (w/v) freshly prepared mercuric chloride ($HgCl_2$) for 4 min. Finally, the seeds were washed (5–6 times) with sterile DDW to remove the traces of sterilant. The sterilized seeds were inoculated in the culture flask (100 ml, Borosil) containing 0.8% (w/v) agar solidified germination medium composed of MS (Murashige and Skoog 1962) basal medium or half-strength MS medium with 3% (w/v) sucrose and Gibberellic acid (GA_3) or without GA_3. Ten culture flasks were taken for each treatment and 10 seeds were inoculated in each flask.

CN measuring 1–1.5 cm excised from 14-day-old aseptic seedlings raised on half-strength MS supplemented with GA_3 (1.0 μM) were used as explants.

Culture media and conditions

The MS medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar (Hi-media, India) were used throughout the experiments. The pH of the medium was adjusted to 5.8 using 1 N NaOH or 1 N HCl prior to autoclaving at 121°C at 1.06 kg cm^{-2} pressure for 20 min. All the cultures were maintained at 24 ± 2°C under 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 50 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes (40W; Phillips, India) and with 50–60% relative humidity.

Shoot induction, multiplication and maintenance

The MS medium supplemented with various cytokinins 6-Benzyladenine (BA), Kinetin (Kn), Thidiazuron (TDZ) at different concentrations (0.1, 0.5, 1.0, 2.0 and 5.0 μM) individually or in combination with different auxins—Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA) or α-Naphthalene acetic acid (NAA) (0.1, 0.5 and 1.0 μM) were used for multiple shoot induction through CN, excised from aseptic seedlings. For shoot multiplication and long-term establishment the regenerating tissues were subcultured onto the fresh medium comprised of MS plus BA (1.0 μM) after every 4 weeks. The percentage of explants producing shoots, number of shoots per explant and shoot length were recorded after 8 weeks of culture.

In vitro rooting in microshoots

Regenerated shoots of about 3–4 cm length were excised and transferred to the rooting media composed of MS basal and half-strength MS supplemented with different auxins like IAA, IBA and NAA (1.0, 2.5, 5.0 and 10.0 μM). Data were recorded for rooting percentage, mean number of roots and root length after 4 weeks of transplantation onto the rooting medium.

Hardening and acclimatization

Plantlets with well developed root and shoot system were removed from the culture medium and washed gently under running tap water to remove any adherent gel from the roots and transferred to thrermocol cups containing sterile soilrite. These were kept under diffuse light conditions (16:8 h photoperiod) covered with transparent polythene bags to ensure high humidity. These were irrigated after every 3 days with ¼ strength MS salt solution (without vitamins) for 2 weeks. Polythene bags were removed gradually after 2 weeks in order to acclimatize the plantlets and after 4 weeks they were transferred onto earthen pots containing sterilized garden soil and garden manure (1:1) and maintained in green house under normal day length conditions.

Data collection and statistical analysis

The data for percentage regeneration, number of shoots per explant and shoot length were recorded after 8 weeks and for rooting experiment after 4 weeks. All the experiments were conducted with ten replicates per treatment and repeated thrice. The data were analyzed statistically using SPSS ver.12 (SPSS Inc., Chicago, USA). The significance of differences among means was carried out using Tukey's multiple range test at $P = 0.05$ and the results are expressed as a mean ± SE of three repeated experiments.

Results and discussion

Establishment of aseptic seedlings and explant collection

To raise aseptic seedlings, the seeds were germinated under controlled conditions on MS basal and half-strength MS media with or without GA_3. A maximum of 90% seed germination along with healthy growth was achieved on half strength MS + 1.0 μM GA_3 (Table 1; Fig. 1a). The media lacking GA_3 showed poor response (40–50%) for seed germination. On increasing the concentration of GA_3 from 1.0 to 5.0 μM the germination percentage was reduced to 60%. GA_3 is known to break dormancy of several types of seeds at a critical concentration. It stimulates seed germination via synthesis of α-amylase and other hydrolases (Shepley et al. 1972). Thus, the supplementation of GA_3 has been found to enhance the rate of seed germination in *C. siamea* in consonance with earlier findings in *Clitoria ternatea* (Shahzad et al. 2007). CN explants excised from 14-day-old aseptic seedlings of *C. siamea* were used for regeneration studies throughout the experiment.

Table 1 In vitro seed germination of *C. siamea*

Medium	% Germination
MS basal	40
½ MS	50
½ MS + GA_3 (0.1 μM)	70
½ MS + GA_3 (0.5 μM)	80
½ MS + GA_3 (1.0 μM)	90
½ MS + GA_3 (5.0 μM)	60

Data recorded after 4 weeks

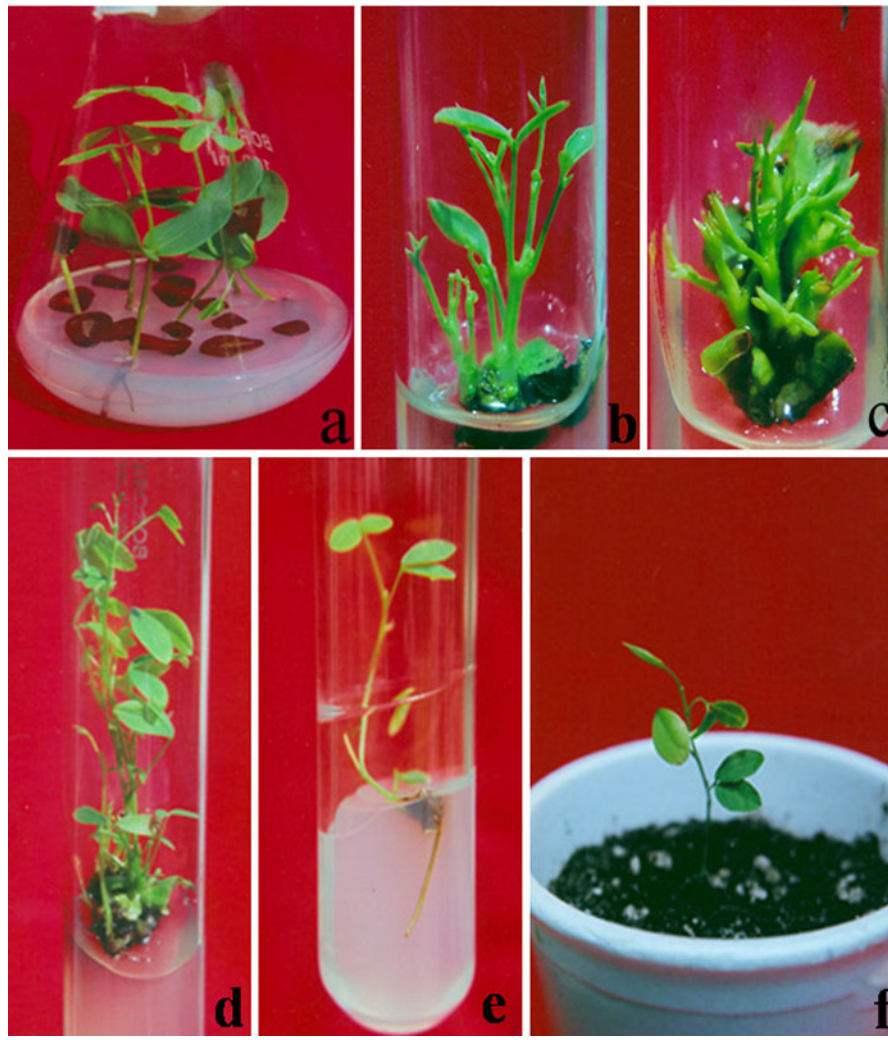

Fig. 1 In vitro plant regeneration in *C. siamea* through CN explant. **a** Aseptic seedlings; **b** development of multiple shoots on MS + BA (1.0 μM) after 4 weeks; **c** multiplication and proliferation of shoots on MS + BA (1.0 μM) + NAA (0.5 μM) after 4 weeks; **d** fully developed, elongated micro-shoots after 8 weeks; **e** rooted plantlet on half-strength MS + IBA (2.5 μM); **f** an acclimatized plantlet

Induction and multiplication of shoots

The response of various cytokinins (BA, Kn and TDZ) for shoot regeneration from CN explants is depicted in Table 2. The explants cultured on MS basal medium without growth regulators (control) did not show any regeneration response. However, the addition of plant growth regulators enhanced the multiplication rate and the number of shoots per explant. The percentage response varied with the type of growth regulator used and its concentration. All concentrations of BA, Kn and TDZ (0.1, 0.5, 1.0, 2.0 and 5.0 μM) alone resulted in direct shoot bud differentiation from the explant within 3 weeks of incubation. The initial response was noticed in the form of swelling of the explants within 8–10 days of incubation and then differentiation of green protuberances occurred which transferred into direct shoot buds in successive weeks. Among the three cytokinins tested, BA was found to be more efficient than others with respect to initiation and subsequent proliferation of shoots (Table 2). Of the various concentrations of BA tested, 1.0 μM proved to be most effective as in this medium an average of 8.20 ± 0.66 shoots per explant were developed in 80% of cultures (Fig. 1b). Our results are in

Table 2 Effect of various cytokinins on direct shoot regeneration from CN explants of *C. siamea* on MS medium

PGR (μM)			Regeneration (%)	Mean number of shoots/explant	Mean shoot length (cm)
BA	Kn	TDZ			
0.1	–	–	60	3.60 ± 0.51^{abcde}	3.42 ± 0.14^{bcd}
0.5	–	–	75	4.80 ± 0.37^{def}	3.78 ± 0.12^{de}
1.0	–	–	80	8.20 ± 0.66^{g}	4.14 ± 0.13^{e}
2.0	–	–	68	6.20 ± 0.37^{fg}	3.60 ± 0.13^{cde}
5.0	–	–	64	5.20 ± 0.37^{ef}	3.10 ± 0.07^{abc}
–	0.1	–	50	2.80 ± 0.37^{abcd}	2.82 ± 0.18^{ab}
–	0.5	–	68	4.20 ± 0.40^{bcdef}	3.38 ± 0.07^{bcd}
–	1.0	–	72	4.80 ± 0.37^{def}	3.68 ± 0.10^{cde}
–	2.0	–	65	3.80 ± 0.32^{abcde}	3.08 ± 0.17^{abc}
–	5.0	–	55	2.40 ± 0.37^{abc}	2.86 ± 0.11^{ab}
–	–	0.1	45	2.20 ± 0.37^{ab}	1.82 ± 0.12^{g}
–	–	0.5	60	4.40 ± 0.37^{cdef}	2.50 ± 0.16^{ef}
–	–	1.0	50	3.80 ± 0.32^{abcde}	1.96 ± 0.09^{fg}
–	–	2.0	40	3.20 ± 0.24^{abcde}	1.56 ± 0.07^{g}
–	–	5.0	32	2.00 ± 0.21^{a}	1.50 ± 0.14^{g}

Data recorded after 8 weeks

Value represents Mean ± SE of three repeated experiments with 10 replicates each. Means followed by the same letter within columns are not significantly different ($P = 0.05$) using Tukey's test

consistence with earlier findings in woody tree species like *Sterculia urens* (Purohit and Dave 1996), *Dalbergia sissoo* (Pradhan et al. 1998) and *Pterocarpus marsupium* (Husain et al. 2008) where BA was found to be more effective than other cytokinins for mass multiplication.

Increasing the concentration of BA (5.0 μM) resulted in decrease in regeneration potential (64%) and the number of shoots per explant (5.20 ± 0.37). On this medium induction of callus from basal cut end was very much pronounced, which might be one of the reason for reduction of multiple shoot regeneration. Inhibitory effect of higher concentrations of BA on shoot multiplication has also been reported in other plant species such as *Withania somnifera* (Sen and Sharma 1991), *Albizia chinensis* (Sinha et al. 2000) and *Simmondsia chinensis* (Agrawal et al. 2002). There are several reports available in which the production of callus was considered inhibitory for direct shoot regeneration (Lakshmanan et al. 1997; Pattnaik and Chand 1997). Shoot induction was also observed in the explants cultured on MS medium fortified with Kn and TDZ. Kn was found to be less effective than BA as it induced an average of 4.80 ± 0.37 shoots per explant with 72% regeneration frequency at an optimal concentration of 1.0 μM. However, the regenerated shoots were healthy and showed good growth as compared to TDZ supplemented medium. Thus, among three cytokinins tested, TDZ was proved to be least effective (BA > Kn > TDZ). The MS medium supplemented with 0.5 μM TDZ produced only 4.40 ± 0.37 shoots per explant with 60% regeneration potential, furthermore the regenerated shoots were stunted with poor internodal elongation (Table 2). When the concentration of TDZ was increased beyond optimum level (0.5 μM) a gradual decrease was observed in regeneration frequency, number of shoots per explant and average shoot length. Similar inhibitory effects of TDZ on growth and elongation at higher concentration have also been observed in other woody trees *Pyrus malus* (Van Nieuwekerk et al. 1986), *Rhododendron* (Preece and Imel 1991), *Albizia julibrissin* (Sankhla et al. 1994), *Albizia chinensis* (Sinha et al. 2000) and *Vitex negundo* (Ahmad and Anis 2007). The formation of stunted shoots or inhibition of internodes elongation may be due to the high cytokinin activity of TDZ since cytokinins are known to inhibit stem elongation (Mok et al. 1982; Huettman and Preece 1993). Unlike adenine-type cytokinins (BA and Kn), TDZ was more

Table 3 Effect of various auxins with optimal concentration of BA (1.0 μM) on direct shoot regeneration from CN explants of *C. siamea* on MS medium

PGR (μM)			Regeneration (%)	Mean number of shoots/explant	Mean shoot length (cm)
IAA	IBA	NAA			
0.1	–	–	64	9.80 ± 0.86^{bcd}	5.74 ± 0.12^{de}
0.5	–	–	74	10.20 ± 0.58^{cd}	5.54 ± 0.07^{bcd}
1.0	–	–	60	8.00 ± 0.55^{abc}	4.90 ± 0.10^{ab}
–	0.1	–	60	7.20 ± 0.37^{ab}	5.32 ± 0.06^{bcd}
–	0.5	–	72	8.40 ± 0.51^{bc}	6.06 ± 0.15^{ef}
–	1.0	–	76	5.20 ± 0.37^{a}	4.74 ± 0.13
–	–	0.1	74	8.40 ± 0.51^{bc}	5.10 ± 0.20^{abc}
–	–	0.5	90	12.20 ± 0.73^{d}	6.40 ± 0.07^{f}
–	–	1.0	80	10.60 ± 0.75^{cd}	4.98 ± 0.14^{abc}

Data recorded after 8 weeks

Value represents Mean ± SE of three repeated experiments with 10 replicates each. Means followed by the same letter within columns are not significantly different ($P = 0.05$) using Tukey's test

effective at lower concentrations and therefore, a comparison of those cytokinins with TDZ at equimolar concentrations is not possible.

The synergistic effect of auxins such as IAA, IBA and NAA (0.1, 0.5 and1.0 μM) with optimal concentration of BA (1.0 μM) was also evaluated. Among various combinations used, BA (1.0 μM) + NAA (0.5 μM) was found to be most effective (Table 3) (Fig. 1c). MS medium supplemented with BA (1.0 μM) + NAA (0.5 μM) exhibited 90% shoot regeneration and induced maximum (12.20 ± 0.73) shoots per explant with an average shoot length of 6.40 ± 0.07 cm (Fig. 1d). Upon increasing the concentration of NAA to 1.0 μM, regeneration frequency (80%) as well as the number of shoots per explant was reduced (10.60 ± 0.75) moderately. Higher concentration of NAA reduced the regeneration frequency and resulted in increased basal callusing, which is not desirable for direct shoot regeneration. Our results substantiate with earlier findings in *Psoralia corylifolia* (Saxena et al. 1997), *Acacia catechu* (Kaur et al. 1998), *Mucuna pruriens* (Faisal et al. 2006) and *Balanites aegyptiaca* (Anis et al. 2010) where the addition of lower concentration of auxin with cytokinin promoted shoot regeneration. Among the different combinations of BA - IAA used, the highest shoot regeneration frequency (74%), maximum number of shoots per explant along with the maximum shoot length were recorded on MS medium augmented with BA (1.0 μM) + IAA (0.5 μM) (Table 3) followed by BA - IBA combinations, wherein the highest shoot regeneration frequency (72%), maximum number of shoots (8.40 ± 0.51) per explant and the maximum shoot length (6.06 ± 0.15 cm) were recorded on MS medium supplemented with BA (1.0 μM) + IBA (0.5 μM).

In vitro rooting in microshoots

In vitro raised microshoots (3–4 cm) were transferred to rooting media comprised of full strength MS and half-strength MS along with various concentrations of auxins; IAA, IBA and NAA (1.0, 2.5 and 5.0 μM). Full strength MS medium failed to induce rooting in all the treatments whereas half-strength MS with different concentrations of auxins induced rooting from basal cut end of microshoots within 2 weeks of transferring (Fig. 1e). The best rooting in terms of rooting percentage and root length was achieved in the medium fortified with IBA (2.5 μM) (Table 4), giving a maximum of 3.60 ± 0.24 roots per shoot with a root length of 7.88 ± 0.28 cm. The roots produced were thick, long and well developed with secondary branching. Comparatively lesser number of roots were produced when IBA was replaced with other auxins, NAA and IAA. However, among all the three auxins tested the lower concentrations of auxins were more satisfactory than the higher concentrations because the higher concentration favored basal callusing before root induction, which resulted into poor vascular

Table 4 Effect of various auxins on rooting of excised microshoots of *C. siamea* cultured on half strength MS medium

PGR (μM)			Rooting (%)	Mean number of roots/shoot	Mean root length (cm)
IAA	IBA	NAA			
1.0	–	–	30	1.40 ± 0.24^{b}	3.92 ± 0.33^{def}
2.5	–	–	40	1.60 ± 0.24^{b}	4.26 ± 0.12^{bc}
5.0	–	–	36	2.20 ± 0.20^{ab}	5.18 ± 0.13^{bc}
10.0	–	–	20	1.80 ± 0.37^{b}	$4.42 \pm .14^{cdef}$
–	1.0	–	60	2.20 ± 0.20^{ab}	4.60 ± 0.40^{cde}
–	2.5	–	84	3.60 ± 0.24^{a}	7.88 ± 0.28^{a}
–	5.0	–	76	2.80 ± 0.37^{ab}	6.20 ± 0.17^{b}
–	10.0	–	62	2.00 ± 0.32^{b}	4.76 ± 0.22^{cde}
–	–	1.0	40	2.40 ± 0.24^{ab}	3.74 ± 0.28^{de}
–	–	2.5	50	2.60 ± 0.24^{ab}	4.94 ± 0.30^{c}
–	–	5.0	46	1.80 ± 0.37^{b}	3.68 ± 0.33^{ef}
–	–	10.0	30	11.60 ± 0.40^{b}	3.34 ± 0.19^{f}

Data recorded after 4 weeks

Value represents Mean ± SE of three repeated experiments with 10 replicates each. Means followed by the same letter within columns are not significantly different ($P = 0.05$) using Tukey's test

connection between the shoot and induced roots, consequently affecting the survival percentage of plants (Data not shown). Our results are similar with earlier findings in other leguminous plants such as *Pterocarpus marsupium* (Chand and Singh 2004), *Cassia angustifolia* (Agrawal and Sardar 2006), *Pterocarpus santalinus* (Prakash et al. 2006) and *Clitoria ternatea* (Shahzad et al. 2007) where lower concentration of IBA was proved to be the best for rhizogenesis.

Hardening and acclimatization

The most critical and important step of micropropagation studies is the transfer of regenerants from artificial to natural environment to ensure the maximum application of the technique. Plantlets with well-developed root and shoot system were successfully hardened off inside the growth room in sterile planting substrate (soilrite) for 4 weeks and irrigated with ¼ strength of MS in the first two weeks (Fig. 1f). The pots were covered with polythene bags to ensure high relative humidity for the first two weeks. Thereafter exposed gradually to controlled environment followed by their transfer to earthen pots containing sterilized garden soil and garden manure (1:1) and maintained in green house under normal day light condition. About 85% of regenerated plants survived after transferring from soilrite to soil-garden manure mixture. All the plants were exhibiting normal morphology and growth when compared with naturally grown plants.

Conclusions

In conclusion, an efficient and effective protocol was developed for micropropagation of *C. siamea*, a leguminous tree of great importance. This protocol provides a successful and rapid technique that can be used for the propagation and ex situ conservation of this important species.

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