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Indirect shoot organogenesis from leaf explants of threatened medicinal plant *Cleistanthus collinus*

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Abstract

In this study an effective plant regeneration protocol for multiple shoot bud induction was optimized for the micropropagation of plantlets through indirect organogenesis from leaf explants of *C. collinus.* The young leaves were cultured on Murashige and Skoog (MS) medium with Thidiazuron (TDZ), 6- Benzylaminopurine (BAP), Kinetin (KIN), 2,4-Dichlorophenoxyacetic acid (2,4-D) and 1-Naphthaleneacetic acid (NAA) at 0.5-3.0 mg/l for callus induction. The induced calli was excised and subcultured on MS medium supplemented with BAP, KIN and TDZ (0.5-4.0 mg/l) in combination with NAA (0.5 mg/l) to find out the optimum hormone concentration for shoot bud induction. For rooting, half strength MS medium with various concentrations (0.5-3.0 mg/l) of Indole 3-acetic acid (IAA), Indole 3- butyric acid (IBA) and NAA was used. Initially, a creamy white callus was formed from the cut ends of leaf explants and gradually these mass of unorganised cells transformed to light green regenerable callus. The maximum callus induction (100%) was observed in MS medium with TDZ at 1.0 mg/l concentration. The highest frequency of organogenesis was observed in the combination of BAP (1.0 mg/l) and NAA (0.5 mg/l) with a mean number of 12.1±1.1 shoots followed by TDZ (1.0 mg/l) and NAA (0.5 mg/l) with 10.2±1.0 shoots per explants. The healthy shoots were rooted best on half strength MS medium supplemented with 0.5mg/l IAA. The well developed plantlets with healthy roots were successfully acclimatized. The development of thriving protocol for the callus induction and regeneration would make possible for the mass multiplication and *in vitro* production of plantlets with somaclonal variations.

Keywords: Callus, *Cleistanthus collinus*, Euphorbiaceae, Leaf explants, Regeneration.

Introduction

Medicinal plants are used in the production of pharmaceutical compounds, as most of the drug industry partly depends upon these medicinal plants. The overexploitation leads to the loss of these medicinal plants. Plant tissue culture is considered as the most powerful tools in modern plant biotechnology to save these plants and conserve its germplasm. The callus induction and successful plant regeneration with the help of plant growth regulators speed up the multiplication of young and strong plantlets.

The plant regeneration system is mainly based on two types, namely direct and indirect regeneration (Mukherjee et al., 2011). Indirect methods are based on callus mediated shoot regeneration (Kumar et al., 2011; Varshney and Johnson, 2010). The shoots are produced either through adventitious shoot formation

or axillary shoot formation. The former is the differentiation of non-meristamatic tissues and the latter is derived through preexisting meristematic tissues. The plant regeneration depends on the suitable choice of the explants, specific media formulation, appropriate growth regulators, gelling agent and physical factors such as temperature, light, humidity etc (Sujatha et al., 2005; Deore and Johnson, 2008).
Callus induction from the leaf was performed to study the culture conditions necessary to the explants for the survival, growth, primary and secondary metabolite production and to produce cell suspension in large scale production. It is also helpful in isolating economically important phytochemicals and also stays away collecting plants from the natural resources (Ogita et al., 2009; Berkov et al., 2009). The most important source of pharmaceuticals is derived from the phytochemicals which serve as a major source in medicinal plants.

Cleistanthus collinus (Roxb.) Benth and Hook (Euphorbiaceae) is a tree habit plant which grows in the dry forests of southern parts of India (Subramanian and Krishnamurthy, 1975; Annapoorani et al., 1984). It is traditionally known as Garari in Hindi, Nilapala in Malayalam and Oduvanthalai in Tamil. All parts of the plants are reported as highly toxic (Subrahmanyam, 2003). The toxic nature of the plant is mainly due to the accumulation of a large amount of specific secondary metabolites. However, those compounds were used as drugs to treat various illnesses. The active principles in the leaves are arylnaphthalene lignin lactones- Diphyllin and its glycoside derivatives such as cleistanthin A and B. These two glycosides are collectively termed as 'Oduvin' (Rajagopal, 1944). Recent studies also suggest that these contain Cleistanthin C, D and Cleistanone (Ramesh et al., 2003).

Especially in rural South India these leaves are used as fish and cattle poison and also used as abortifacient (Devaprabhu et al., 2007). The whole parts of this plant, especially leaves, roots, and fruits were used to treat gastrointestinal disorders (Anugral, 2012). This plant also possesses anticancer activity. Alcoholic extracts of this plant shows high antiproliferative properties of the nasopharynx against human epidermal carcinoma during tissue culture (Bhakuni et al., 1969). The tissue culture cell lines show high cytotoxic effects of the glycosides. The glycoside derivatives such as cleistanthin A causes DNA strand breaks through arresting the growth of the cells by inhibiting the synthesis of DNA and cell division. This causes the DNA damage and moreover, it induces cell apoptosis (Pradheepkumar et al., 2000; Meenakshi et al., 2000). These cleistanthins also cause neutrophilic granulocytosis in monkeys, cats etc (Rao and Nair, 1970). The leaf extracts contains larvicidal property against vector mosquitoes (Arivoli and Samuel, 2011). These plants also has insecticidal activity and used in rice fields as insecticides against the red flour beetle, *Tribolium castaneum* (Harwansh et al., 2010; Gupta et al., 2010).

In the present study both cytokinins and auxins were deliberated for their ability of callus induction. The optimum concentration for callus induction was combined with different concentration of auxin (NAA) to identify the best combination of growth regulators for the regeneration of plant and also that triggered the greatest callusing capacity of the leaf explants of *C .collinus* in the basal nutrient medium. In addition, the effect of thidiazuron (TDZ) at varying concentration was also examined for the callus induction and regeneration. To the best of our knowledge, there is no study on callus induction and regeneration from leaf explants of this plant. Hence the present study was focused to find out the proficient regeneration protocol using leaf derived calli of *C. collinus.*

Materials and Methods

Cleistanthus collinus plant was collected from Ariyalur (11.1401° N, 79.0786° E), Tamilnadu, India and maintained in the institute herbal garden. Young leaves were excised from the healthy mother plant and rinsed in running tap water for 10 minutes, followed by liquid detergent Teepol and rinsed thoroughly with sterilized water. Then 70% ethanol was used to treat the explants for 30 seconds and sterile water wash was done for two times and consequent immersing in 0.1% (w/v) mercuric chloride for 3 minutes and washed by vigorous rinsing in sterile double distilled water for 3-5 times to remove the traces of chemicals. This process was done in aseptic conditions under laminar air flow chamber. After three to four washings with sterile water, the explants were inoculated in culture tubes containing plant growth regulators. Explants were treated with various concentrations of ascorbic acid (10-50 mg/l) for almost five minutes before inoculation and the same was additionally supplemented with the culture medium.

Callus induction

For callus induction, the excised leaves from healthy plants were cut into appropriate size (0.5-1.0 cm) and inoculated in Murashige and Skoog medium

(Murashige and Skoog, 1962) supplemented with ascorbic acid (10-50 mg/l) and different growth regulators viz., TDZ, BAP, KIN, 2,4-D and NAA at varying concentrations ranging from 0.5 to 3.0 mg/l to find out the effect of callus induction. Optimum concentration of the growth regulator for callus induction was combined with NAA to find out the regeneration efficiency and embryogenic callus induction frequency. The pH of the medium was adjusted to 5.8 with 1 N NaOH and 1 N HCl solutions prior to add agar and autoclaving. The medium was supplemented with 3% (w/v) sucrose and was autoclaved at 121°C for 15 min. The cultures were incubated at 25°C ± 1°C with a 16 h photoperiod. A light intensity of 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ was provided by cool white fluorescent lamps. The percentage of callus induction was calculated after 3 weeks. The explants produce large amount of callus and these were maintained by sub culturing at 3 weeks intervals.

Plant regeneration

After 4 weeks the induced callus from the leaf explants were transferred to regeneration medium (MS medium with BAP, KIN, TDZ at the range of 0.5-4.0 mg/l in the combination of 0.5 mg/l NAA) for the induction of shoots buds and shootlets. The cultures were incubated at 25±1ºC in light with an intensity of 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux density for about 4 weeks to stimulate shoot bud development. The proliferated callus was regularly sub cultured in the fresh medium to avoid the phenolic exudation on the medium which results in the browning of callus. During subculture the unhealthy callus were removed from being transferred. The percentage of the explants forming callus, proliferation and multiple shootlets production was recorded after 7 weeks. All the treatments were repeated three times with ten replicates for each experiment. Statistical analysis was performed utilizing SPSS 13 (SPSS Inc., Chicago, IL, USA) and Excel 2007 (Microsoft, Redmond, WA, USA) software. Data were analyzed using one and two-way ANOVA to consider for significant and Least Significant Differences test was utilized to compare means at $p<0.05$.

Rooting and Hardening

The microshoots regenerated from the callus (30-40 mm in length) was excised and transferred to root induction medium containing half strength MS with IAA, IBA, NAA (0.5-3.0 mg/l) alone or in combination with activated charcoal. The number of induced roots with response of each explants and the length of the roots per shoots were recorded after 4 weeks. The well developed plantlets with healthy roots were removed from the culture tubes and washed carefully in running tap water to remove the agar debrices. Then the plant was transplanted into plastic cups containing sand:soil:vermiculite (1:1:1) and covered with polythene covers to maintain humidity under aseptic conditions inside the culture room. After 2 weeks the bag was carefully removed and transferred to earthen pots with garden soil.

Results

The leaf explants of *C. collinus* was cultured on MS medium supplemented with various concentrations of TDZ, BAP, KIN, 2, 4-D and NAA alone or in combination for callus induction and regeneration. The results point out that the lethal browning of the explants was reduced by the addition of ascorbic acid (50 mg/l) and the culture was maintained for long period of time.

Callus induction

After 4 -5 days of explants inoculation, swelling and expansion was observed in leaf explants and callus induction was initiated at the cut ends. Two types of calli were initiated on leaf explants. One consists of white and soft calli; other has nodular, greenish yellow calli. The frequency of callus formation was higher in TDZ compared to other hormones. The maximum percentage (100%) of leaf callus was achieved with TDZ (1.0 mg/l) followed by BAP (70%) and NAA (60%). Callus induction was declined when higher concentrations of growth regulators were used. TDZ (1.0 mg/l) showed good response for the initiation of callus compared to KIN and 2,4-D. The explant cultured on basal medium fails to produce callus. The callus produced from the induction medium was frequently subcultured on fresh medium with the same hormone concentration. The friable callus thus formed was transferred to regeneration medium.

Table 1: *In vitro* responses of different Plant Growth Regulators (Cytokinin and Auxin) on Callus induction of *C.collinus.*

Concentration of Plant Growth Regulators (mg/l)					Percentage of Callus Induction	Intensity of callus formation	Texture and color of callus
BAP	KIN	TDZ	2,4-D	NAA			
0.0	0.0	0.0	0.0	0.0	0.0	-	-
0.5	-	-	-	-	65	++	Hard,white, slow growth
1.0	-	-	-	-	70	+++	Soft, huge growth
2.0	-	-	-	-	60	++	Hard,yellowish brown
3.0	-	-	-	-	55	+	Very hard,brown
	0.5	-	-	-	50	+	Hard,slow growth
-	1.0	-	-	-	55	+	Very hard, brown
-	2.0	-	-	-	45	+	Hard, yellowish brown
-	3.0	-	-	-	30	+	Very hard
-	-	0.5	-	-	100	+++	Greenish, white, nodular
-	-	1.0	-	-	100	+++	Greenish white, soft, nodular
-	-	2.0	-	-	95	++	Hard, Green, Profuse growth
-	-	3.0	-	-	80	++	Very Hard, Dark green
-	-	-	0.5	-	40	+	Hard,white
-	-	-	1.0	-	50	+	Very Hard, white
-	-	-	2.0	-	45	+	Hard, Brown
-	-	-	3.0	-	40	+	brown
-	-	-	-	0.5	50	+	Soft, brown
-	-	-	-	1.0	60	+	Slightly hard,brown
-	-	-	-	2.0	55	+	Very hard, brown
-	-	-	-	3.0	50	+	Hard, brown

'+' indicates the degree of response; '-' indicates no response

Shoot regeneration

Subculturing of these large masses of soft, green, friable organogenic callus on MS medium with BAP, TDZ and KIN at 0.5-4.0 mg/l in combination with NAA (0.5 mg/l) produced embryogenic callus and shoots were induced after few days. BAP (1.0 mg/l) and TDZ (1.0 mg/l) along with NAA (0.5 mg/l) was comparatively found better for shoot regeneration of the three cytokinins used (BAP, TDZ and KIN). Highest response of shoot proliferation and regeneration was observed from leaf callus on MS medium along with 1.0 mg/l BAP and 0.5mg/l NAA (Table 2 and Fig. 1B). In addition, there is also shoot regeneration in leaf callus when cultured on MS medium with 1.0 mg/l TDZ and 0.5 mg/l NAA. The optimum response was observed in 1.0 mg/l BAP and 0.5 mg/l NAA. On this medium 85% of cultures produced shoots with an average number of 12.1±1.1 shoots per callus clumps. The shoots elongated well on MS medium supplemented with 0.5 mg/l BAP.

Table 2: Responses of different concentration of Plant Growth Regulators (Cytokinin and Auxin) on shoot bud regeneration from callus cultures of *C.collinus*.

Concentration of Plant Growth Regulators (mg/l)				Mean Shooting response (%)	Mean No. of shoot buds /explant ± SD*	Mean height of Shootlet (cm) ± SD
BAP	TDZ	KIN	NAA			
0.0	0.0	0.0	0.0	0.0	0.0^{g}	0.0
0.5	-		0.5	70	8.2 ± 0.7^{b}	2.4±0.7
1.0	-	-	0.5	85	12.1 ± 1.1^{a}	3.8±1.2
2.0	-	-	0.5	60	6.1 ± 0.8^{c}	2.7±0.6
3.0	-	-	0.5	55	1.8 ± 0.7^{e}	1.8±0.7
4.0			0.5	30	1.0 ± 0.0^{f}	1.0±0.0
	0.5	-	0.5	60	7.7 ± 1.0^{b}	2.2±1.2
-	1.0		0.5	80	10.2 ± 1.0^{a}	3.4±1.1
-	2.0	-	0.5	75	8.1 ± 1.1^{b}	2.4±0.8
-	3.0	-	0.5	55	2.9 ± 0.7^{d}	1.8±0.7
-	4.0	-	0.5	45	1.0 ± 0.0^{f}	1.0±0.0
-	-	0.5	0.5	40	2.1 ± 0.9^{d}	2.4±1.1
-	-	1.0	0.5	50	2.2 ± 0.7^{d}	2.8±0.9
-	-	2.0	0.5	30	1.9 ± 0.7^{de}	1.5±0.7
-	-	3.0	0.5	20	1.0 ± 0.0^{f}	1.0±0.0
-	-	4.0	0.5	20	1.0 ± 0.0^{f}	1.0±0.0

*Values are mean ± SD (n=10) dissimilar letters indicated significant difference between means within the treatment at α=0.05 level based on LSD mean separation.

Shoot multiplication and rooting

A single shoot was excised from the cluster of regenerated shoots and cultured on low concentration of BAP (0.3 mg/l) for further multiplication. The shoots multiplied and elongated well on this medium. The elongated shoots measuring a length of above 2.0 cm were rooted best on half strength MS medium supplemented with 0.5 mg/l IAA and activated charcoal (500 mg/l). Induction of roots in shootlets inoculated in IAA was found better and quick while compared to IBA and NAA. The maximum response (75%) was observed with an average number of 3.7 roots per shootlet with a mean root length of 2.8 cm. Thick, long white roots were formed from the *in vitro* derived shoots on application of auxin individually (Table 3 and Fig. 1H).

The well developed plantlets with healthy roots were carefully removed from the culture tubes and washed with sterile water to remove the agar attached to the shoots. Then the plantlets were transferred to the polyethylene cups containing sand:soil:vermiculite (1:1:1) mixture and maintained in tissue culture room for a week. The plant was covered by polyethylene cover and Humidity was maintained by continuous spraying of water. Then it was moved to the greenhouse conditions. After one week well growing plants were transferred to earthen pots containing 3:2:1 (v/v/v) mixture of garden soil, vermiculite and sand for hardening and acclimatization.

Table 3: Response of different concentration of Plant Growth Regulators (Auxins) on the induction of rooting on half strength MS medium.

Concentration of Plant Growth Regulators (mg/l)		Mean rooting response (%)	Mean number of rootlets /explant ± SD*	Mean root length (cm) ± SD
Control	0.0	0.0	0.0^{g}	0.0
IBA	0.5	70	3.5 ± 0.2^{b}	2.3 ± 0.1
	1.0	35	2.0 ± 0.7^{c}	2.0 ± 0.1
	2.0	30	1.4 ± 0.5^{e}	1.9 ± 0.3
	3.0	20	1.5 ± 0.6^{e}	1.8 ± 0.4
IAA	0.5	75	3.7 ± 0.9^{a}	2.8 ± 0.3
	1.0	30	2.0 ± 0.8^{c}	2.0 ± 0.3
	2.0	25	1.5 ± 0.7^{e}	1.7 ± 0.4
	3.0	20	1.0 ± 0.0^{f}	1.0 ± 0.0
NAA	0.5	20	1.8 ± 0.4^{d}	2.2 ± 0.1
	1.0	25	1.5 ± 0.5^{e}	1.7 ± 0.5
	2.0	25	1.0 ± 0.0^{f}	1.0 ± 0.0
	3.0	20	1.0 ± 0.0^{f}	1.0 ± 0.0

*Values are mean ± SD (n=10) dissimilar letters indicated significant difference between means within the treatment at α=0.05 level based on LSD mean separation.

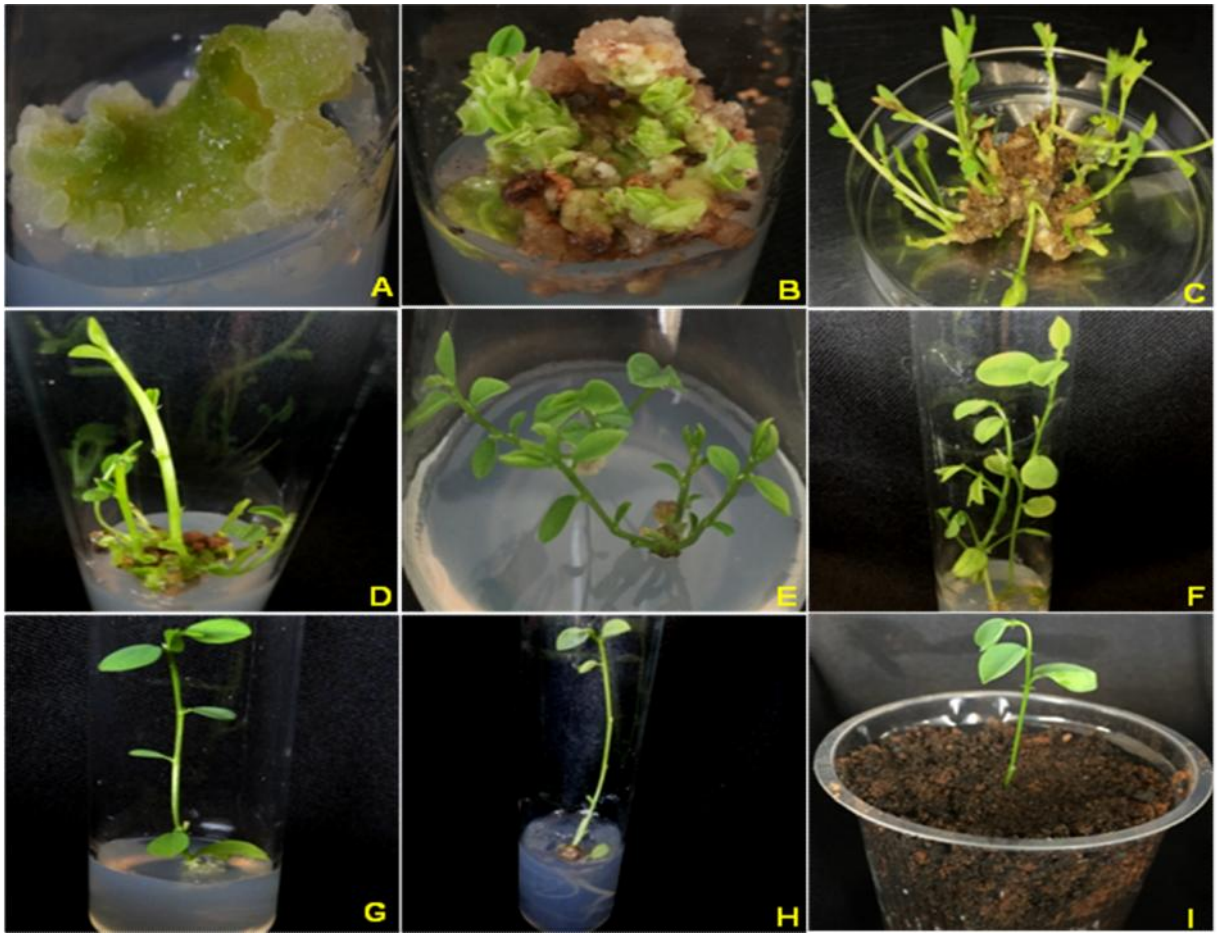

Fig. 1: Callus morphogenesis and shoot bud regeneration of *Cleistanthus collinus*.(A) The induced leaf callus tissue on MS medium supplemented with TDZ (1.0 mg/l); (B) Adventitious shoot bud regeneration with primary callus; (C, D, E & F) Emerging of multiple shoots; (G) Elongation of shoots in MS medium with BAP (0.3 mg/l); (H) Rooting of the regenerated shootlet; (I) Healthy rooted plantlets were transferred into poly cups containing bio-compost nutrients.

Discussion

Leaching of phenolic compounds was found to be a major problem in regeneration of this plant. Ascorbic acid is an antioxidant used to control oxidation of phenols, and so it was added along with MS medium at low concentration to avoid the exudation of phenolic compounds. Cosmos *et al*. (2014), proposed an approach to overcome the leaching of phenolic compounds during the growth of *Brahylaena huillensis* through the addition of ascorbic acid in the medium. Similar symptom was observed in the plant *Cavendish banana c.v.* Formosana (Ko et al., 2009) local Musa spp. (Banana) cv. Mzuzu (Munguatosha et al., 2014; Soniya and Sujitha, 2006) and it was reduced by adding ascorbic acid. But there is an inverse effect when high concentration of ascorbic acid is added. Frequent subculturing also reduces the problem significantly (Wang et al., 2005; Hoque and Arima, 2002).

After 4-5 days of inoculation swelling and expansion was observed in the explants. There is a fast rate of cell division, growth and development and the formation of plant organs when cytokinin in combination with auxin was used. This was already reported in studies on the plant *Jatroba curcus* (Purkayastha, 2010). Recently, TDZ was found to be a cytokinin like activity which is one of the substituted ureas. It was found to be the best hormone for the induction of callus. It took only a short period for the production of callus. It has the highest activity when low concentration was added to the culture medium (Kim et al., 2001; Kumar et al., 2011). In the present study, TDZ plays an important role in the callus induction as well as regeneration. These results were in accordance with Sharma *et al.*, 2011 (Sharma et al., 2011). The callus induction was also promoted by the combination of cytokinin and auxins (Capuana et al., 2007). TDZ is also considered to be one of the most active cytokinins for the shoot induction in plant tissue culture (Pelah et al., 2002; Mithila et al., 2003).

The regeneration frequency of the plant depends on the various types of callus such as embroyogenic and non-embryogenic callus. All the tested concentrations generate both types of callus. The embryogenic callus was formed in 1.0 mg/l TDZ whereas 2,4-D and NAA produced non-friable callus that was not able to proliferate further resulting in the browning and necrosis. The watery callus which was white and soft eventually turned to brown and dried up. But the nodular, creamy yellow calli was further proliferated and turned to compact in structure. The embryogenic callus formed in TDZ was soft and friable and further subcultured on the regeneration medium such as combination of cytokinin with auxin. The presence of both auxin and cytokinin in appropriate concentration is necessary for optimum shoot regeneration from callus. MS medium supplemented with BAP (1.0 mg/l) and NAA (0.5 mg/l) in combination produce a higher percentage of shoot regeneration. Previous reports have also shown that the regeneration frequency was higher in combination of BAP and NAA in *Aristolochia indica* and *Abelmoschus moschatus* (Remashree et al., 1997; Ashish et al., 2011). The proliferation of callus was also higher in BAP and NAA compared to other hormone combinations. This was also reported in other plants such as *Orthosiphon aristatus and Solanum nigrum* (Reshi et al., 2013; Sridhar et al., 2011). Morphogenesis was controlled by several factors such as physical, chemical and hence a definite concentration of plant growth regulators at appropriate value and optimum culture conditions has been used for the successful plant regeneration in culture. The adventitious roots were observed on the surface of calli when subcultured on IBA (results not shown). This was also similar to the results of previous reports in *S. franchetiana* and *S. davidii* (Xiang et al., 1999; Huang et al., 2002). The organogenic calli which were most friable remained light creamy white and proliferate rapidly during monthly subcultures without any significant changes in its regeneration potential.

On the other hand, the increased concentration of NAA results in the reduction of callus proliferation and regeneration while BAP concentration was kept constant. However, NAA concentration was kept stable and the BAP concentration was increased, there is a better percentage of regeneration. The choice of explants and the concentration of plant growth regulators play an important role in the callus induction and regeneration. On the whole, callus induced from plant growth regulator TDZ was tremendous in the regeneration of shoots than the combination of other PGRs. The medium supplemented with BAP (1.0 mg/l) and NAA (0.5 mg/l) shows maximum regeneration percentage. The combination of KIN and BAP failed to produce shoots from the induced calli. BAP was the most effective cytokinin for the multiplication of shoots in *C. collinus.* This result was in accordance with the findings of *Portulaca grandiflora* (Jain and Bashir, 2010), *Thymus satureioides* (Aicha et al., 2013) and *Salvia guaranitica* (Echeverrigaray et al., 2010). On the other hand when the cytokinin level was increased there was a reduction and stunted shoots. Previous

reports in the plants such as *Majorana hortensis* (Tejavathi and Padma, 2012) and *Vitex agnus-castus* (Balaraju et al., 2008) also shows the same results. BAP also promotes the cell division that is essential for the growth and development and also it was essential for the shoot regeneration (Mok and Mok, 2001). Leaf is one of the desirable explants for the *in vitro* culture because the regeneration of plants from these explants would conserve the genetic characteristics of the parent genotype to some extent (Monokesh et al., 2014). Callus induction is mainly required for the large scale production of the secondary metabolites and for genetic engineering (Jose et al., 2012).

The regeneration response of one plant varies from the other. It has been obvious that MS medium supplemented with NAA and BAP helps in highest percentage of shoot development in short duration of time (Yasmin et al., 2003). IAA was found to be the best hormone for the induction of roots. This was already reported in many other medicinal plants such as Carnation (Aamir et al., 2008), *Abelmoschus moschatus* (Ashish et al., 2011). The advantage of IAA over other auxins for root initiation was also reported in *C. cajon*, *Murraya konini* and *Quillaja brasiliensis* (Rout, 2005; Fleck et al., 2009). Activated charcoal was also essential for the development of rooting (Kassim et al., 2012; Thomas, 2008). The results were in accordance with the *Swertia chirayita* micropropagation where activated charcoal in half strength MS medium supplemented with NAA produced good number and healthy rooting (Joshi and Dhawan, 2007).

Conclusion

In the present study, the successful protocol for callus induction and shoot regeneration from leaf derived callus of *C. collinus* was optimized. The ability to regenerate complete plants from the immature leaf derived callus of *C. collinus* has been established. This optimized protocol helps in providing a promising method for the large scale propagation of this species. The plant growth regulators and the culture conditions strongly affect the callus induction and its regeneration. This protocol could be much useful in the mass propagation of the important plant and also useful for various biotechnological studies like modified cell and callus line for the production of specific secondary metabolites at *in vitro* level and helps in the study of plant transformation also.

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