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RESEARCH ARTICLE

SHOOT BUD DIFFERENTIATION AND PLANTLET REGENERATION FROM *IN VITRO* DERIVED LEAF EXPLANTS OF *Celastrus paniculatus* Willd

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Abbreviations:

MS - Murashige and Skoog's medium, BAP - 6-Benzyl aminopurine, NAA- Naphthalene-3-acetic acid, IAA - Indole-3-acetic acid, 2,4-D - 2,4-dichlorophenoxy acetic acid.

ABSTRACT

Leaf explants of *Celastrus paniculatus* initially inoculated on MS medium devoid of plant growth regulators evoked no response in terms of adventitious shoot bud differentiation. Incorporation of cytokinins like Kinetin (Kn) and BAP induced shoot buds at their various concentrations. Shoot buds were visible after 3 weeks on petiolated leaf explants (PL) while non- petiolated leaf explants (DL) took 6 weeks to have similar response. Shoot bud differentiation was much better on BAP media as compared to Kn. BAP alone at its 2.0 mgl^{-1} concentration produced a maximum of 11 shoots buds on PL explants while more than 13 shoot buds per explants could be obtained when both BAP and Kn at 0.5 mgl^{-1} concentration each were incorporated in the medium. Although shoot buds could be differentiated from both PL and DL explants, there was a great degree of difference in their response to PGR treatments. Leaves derived from *in vitro* multiplying shoots responded equally, irrespective of their size and position on shoots. However, leaf explants with petioles were more responsive in terms of adventitious shoot bud differentiation. Most of the shoot buds originated from petiolar region while occasional development of shoot buds was also observed from leaf apices and margins of leaf lamina. In case of DL explants buds originated from shoot apices and margins of leaf lamina though most regeneration events occurred from midrib and depetiolated leaf base. PL explants showed cent percent regeneration while 66% response was observed with DL explants. Shoot buds differentiated from various explants could be elongated during their sub-culture for 2 passages of three weeks each and the shoots were subsequently multiplied at a rate of 3.0 - fold. Elongated shoots could be easily rooted and plantlets successfully hardened using previously described procedure.

INTRODUCTION

Celastrus paniculatus Willd. (Celastraceae) commonly known as Malkangani, Jyotishmati, Bitter sweet, is an important Indian medicinal deciduous, forest climber seen growing mostly in the hilly regions of northern India at an altitude of 1250 meters (Sharada *et al.,* 2003). The plant is valued for its immense medicinal properties. The plant parts are constituents of numerous indigenous medicines. Leaves are emmenagogue and the leaf sap is used as an antidote for opium poisoning. The oil obtained from the seeds has been recommended for the treatment of beri-beri, rheumatism, paralysis, gout, cough, asthma, leprosy, headache and leucoderma (Chopra *et al.,* 1986). The chief phytoconstituents of medicinal value reported in *C. paniculatus* include malkangunin (sesquiterpene polyster), celapanin celapanigin, celapagin (sesquiterpene alkaloids) and celastrol, pristimerin, zeylasterone and zeylastral (quinine-methide and phenolic triterpenoids) (Anonymous, 2002). Over-exploitation and poor natural regeneration either by seed or other methods have resulted into depleting population of *Celastrus paniculatus* in natural habitats and therefore, it is currently listed as threatened species (Kirtikar and Basu, 1987). In recent years, induction of multiple shoots and recovery of complete plantlets from tissue culture of many rare, endangered, aromatic and medicinal plants have been reported (Bhojwani and Razan, 1996; Komalavalli and Rao, 2000; Sebastian *et al.,* 2002; Sridhar and Naidu, 2011). *In vitro* shoot proliferation and plantlet regeneration in *C. paniculatus* have been attempted using different pathways and variety of explant sources (Nair and Seeni, 2001; Sharada *et al.,* 2003; Martin *et al.,* 2006). However, there are no reports on direct regeneration from leaf explants without any intervening callus phase.

Adventitious shoot bud regeneration is considered to be a better option for gene delivery through a variety of explants (James and Barbara, 1990; Swartz *et al.,* 1990; Petri and Scorza, 2010) and success has been achieved to obtain transgenic plants in number of species (Uematsu *et al.,* 1991; Moore, 1992). There are several reports which suggest that leaves are better source of explants for induction of multiple shoots *in vitro* (Shrivatava and Rajani, 1999; Babu *et al.,* 2000; Temjensangba and Deb, 2005). The present investigation was aimed to demonstrate the potential of tissue culture derived leaf explants for shoot differentiation and plantlet regeneration in *C. paniculatus.*

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MATERIALS AND METHODS

In vitro shoot cultures of *C. paniculatus* were established using nodal explants as described by Bilochi (2001). *In vitro* multiplying shoot cultures were used as the source of leaf explants in the present investigation. Whole leaf explants with (PL) or without (DL) petioles were harvested aseptically from *in vitro* multiplying shoot cultures of *C. paniculatus.* The petiole was removed in order to avoid its influence on regeneration capacity of leaf explants. Both type of leaf explants (PL and DL) were placed firmly with their adaxial surface uppermost on the medium in culture tubes and stoppered with non-absorbent cotton plugs. The medium for all the experiments consisted of MS salts and vitamins plus 30 gl^{-1} sucrose solidified with 0.8% agar and supplemented with different concentrations of cytokinins (0.0 - 5.0 mgl^{-1}) viz. BAP and Kn. Range of auxins (0.0 - 1.0 mgl^{-1}) viz. IAA, NAA, 2,4-D either individually or in combination with cytokinins was also attempted. The pH of the medium was adjusted to 5.8 prior to autoclaving at 1.06 kg cm^{-2} for 15 min and was solidified to provide slanted surface. All the cultures were maintained under the standard culture room conditions as have been described earlier. Explants were sub-cultured on to the same fresh medium after an interval of every 21 days and were maintained for 63 days and observations were recorded. The response has been expressed in terms of average number of shoot buds produced per explants and per cent response. Each treatment consisted of six replicates with one explants per culture tube and each experiment was repeated thrice. The experiments were conducted in a completely randomized design (CRD) and the data were analyzed using one way ANOVA to identify best treatment combination.

After 63 days of culture, differentiated shoot buds along with mother explants were sub-cultured on to fresh MS medium supplemented with different concentrations of BAP (0.0 - 2.0 mg l^{-1}) for further elongation and multiplication. The cultures were kept under culture room conditions and scored for increase in shoot number (in-folds) and number of elongated shoots produced after 21 d of inoculation. For each experiment a minimum of 5 replicates were taken and repeated thrice. Shoots could be easily rooted and plantlets were successfully hardened using procedures described by Bilochi (2001).

RESULTS

In vitro adventitious shoot buds, without an intervening callus phase, could be induced directly from the leaf (both PL and DL) in *C. paniculatus* on a variety of treatments (Fig. 1). PL explants were more responsive in adventitious shoot bud differentiation. Adventitious shoot buds mostly differentiated from the petioles while occasional shoot buds also developed from leaf apex and margins of the leaf lamina. In case of DL explants, buds were induced from leaf tips and margins of leaf lamina, though most regeneration events occurred from midrib and its petiolar base. Differentiation of shoot buds could be first observed within 3 weeks of culture in PL while after 6 weeks in DL explants.

The induction of adventitious shoot buds from leaf explants was dependent on the addition of cytokinin to the medium. Kinetin could induce adventitious shoot buds from both the explants. However, this response improved when Kn was replaced with BAP in the medium. The number of shoot buds regenerated per explants increased with increasing concentration of BAP, reaching maximum at 2.0 mgl^{-1} and thereafter, decreased with further increase in BAP concentration. At 2.0 mgl^{-1} concentration of BAP, PL and DL explants produced *ca.* 11.0 and 6.0 shoot buds per explants, respectively. Here, all the PL explants produced shoot buds while DL explants showed *ca.* 66% response (Table 1).

The combination of both BAP and Kn was more effective in induction of more number of adventitious shoot buds in PL explants as compared to BAP and Kn used alone. However, maximum number (*ca.* 13.0) of shoot buds were produced when the medium was supplied with 0.5 mgl^{-1} BAP and Kn each. Although shoot buds were differentiated in DL explants at all the combinations of BAP and Kn tested, it could not supercede the response that was obtained at 2.0 mgl^{-1} BAP used alone (Table 1). The addition of auxins (NAA, 2, 4-D and IAA) individually in the medium either caused callusing or induced rooting alone with callus in some cases. The incorporation of different concentrations (0.1 - 1.0 mgl^{-1}) of auxins either with BAP (2.0 mgl^{-1} each for DL explants) or a combination of BAP and Kn (0.5 mgl^{-1} each for PL explants) could not improve regeneration response.

Table 1 Effect of cytokinins (BAP and Kn) on in vitro shoot bud differentiation from in vitro derived leaf explants in *Celastrus paniculatus* (Observations were recorded after 63 days)

Cytokinin	Concentration [mgl^{-1}]	Average number of shoot buds [$explant^{-1}$]**		Explant response [in per cent]	
		PL explants	DL explants	PL explants	DL explants
BAP	0.0	0.0^{g}*	0.0^{e}*	NR	*NR*
	0.5	4.0^{f}	2.25^{cd}	50	*66.67*
	1.0	7.83^{c}	1.33^{d}	100	*50*
	2.0	11.0^{b}	6.0^{a}	100	*66.67*
	5.0	7.4^{ce}	4.8^{ab}	83.33	*83.33*
Kn	0.5	0.0^{g}	0.0^{e}	NR	*NR*
	1.0	5.75^{def}	0.0^{e}	66.67	*NR*
	2.0	7.5^{cd}	1.0^{d}	66.67	*33.33*
	5.0	5.50^{df}	1.75^{d}	66.67	*66.67*
BAP + Kn	0.5 + 0.5	13.17^{a}	3.5^{bcd}	100	*66.67*
	1.0 + 1.0	8.0^{c}	4.6^{ab}	100	*83.33*
	2.0 + 2.0	8.83^{c}	4.0^{ac}	100	*66.67*
	5.0 +5.0	6.0^{def}	3.0^{bcd}	83.33	33.33

** Only responsive explants were considered to calculate average number of shoot buds per explant*

Means followed by different letters differ significantly at $p \leq 0.05$.

NR- No response.

Higher concentration ((0.5 - 1.0 mgl^{-1}) of auxins in combination with cytokinin adversely affected regeneration response in both PL and DL explants.

Table 2 Effect of BAP on *in vitro* growth and multiplication of shoots of *C. paniculatus* cultured on MS medium supplemented with 0.8% agar and 3.0 % sucrose

BAP (mgl^{-1})	Rate of shoot multiplication (in folds)	Number of elongated shoots produced per cluster
0.0	1.40	5.00
0.5	3.00	7.16
1.0	3.45	4.83
2.0	3.66	4.50

Differentiated shoot buds along with leaf explants, when cultured on fresh medium supplemented with different concentrations of BAP (0.0 - 2.0 mgl^{-1}) for further shoot multiplication and elongation, evoked responses at 2.0 mgl^{-1} of BAP. This concentration produced maximum number of shoots (*ca.* 12.0) and the shoots multiplied at a rate of *ca.* 3.0-fold. Least number of elongated shoots was produced at this concentration (2.0 mgl^{-1}) of BAP. MS medium devoid of BAP favoured only shoot elongation and did not induce new shoots. However, 0.5 mgl^{-1} of BAP promoted both shoot multiplication and elongation. At this concentration *ca.* 2.0-fold rate of shoot multiplication was observed and produced maximum number (5.0) of elongated shoots per explants. Therefore, all the cultures were subsequently transferred to this medium for further shoot growth and elongation (Table 2).

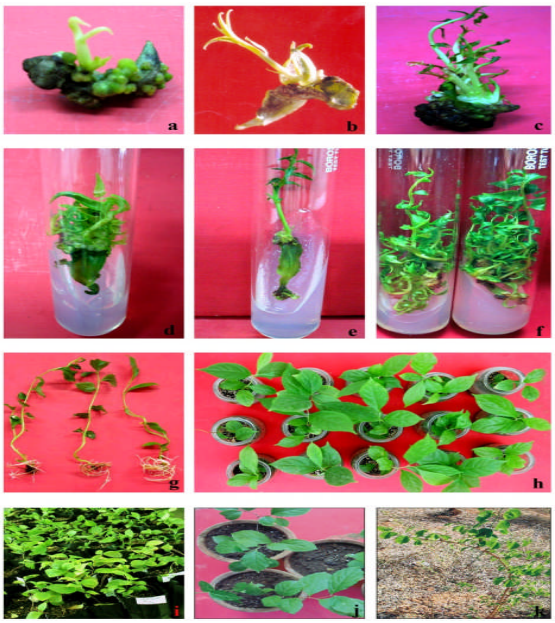

Figure legends

Figure 1 Different stages of *in vitro* adventitious shoot bud differentiation and plantlet regeneration from culture derived leaf explants in *C. paniculatus*. Early stages of multiple shoot bud induction from leaf explants (**a and b**). Multiple shoot regeneration from midrib (**c**), petiolar leaf base (**d**) and leaf tip (**e**). Shoot elongation and multiplication on MS medium containing 0.5 mgl^{-1} BAP (**f**). Induction of roots in pulse treated (500 mgl^{-1} IBA for 10 min) shoots after 21d (**g**).
Hardened plants of *C. paniculatus* grown on Soilrite™ moistened with 1/4th MS salt solution (**h**).
Tissue culture raised plants growing in polybags (**i**).and earthen pots (**j**) nursery
Field grown tissue culture raised plant (k)

In vitro regenerated shoots measuring an average length of *ca.* 3.0 cm were rooted successfully on 1/4th MS medium containing 1.0% sucrose and 0.6% agar after pulse treatment of IBA (500 mgl^{-1}) for 10 min. This treatment could induce callus-free rooting in *ca.* 80% shoots in same manner as do shoots derived from axillary buds. All rooted shoots were successfully hardened under greenhouse conditions. During *in vitro* hardening, shoots became elongated, leaves turned greener and expanded. Consequently, the plants appeared much healthier after *in vitro* hardening. Of 200 plants transferred in polybags containing sand and farmyard manure (1:1), 160 plants (*ca.* 80%) survived under nursery shed conditions.

DISSCUSSION

Direct regeneration from leaf as another alternative step for clonal propagation and germplasm conservation is well established phenomenon. The present study demonstrated potential of shoot bud regeneration from culture derived leaf explants in *C. paniculatus*. Although occasionally, in some explants, shoot buds differentiated directly from leaf apices, midribs and margins of leaf lamina, but most buds developed on petioles or petiolar leaf bases. Similar results have been observed in the leaves of *Solanum nigrum* (Sridhar and Naidu, 2011) and *Tylophora indica* (Verma *et al.,* 2010). The presence of BAP in culture medium was found to be the key factor governing the *in vitro* response. Various workers have reported the role of cytokinins in shoot bud formation (Sivanesan and Jeong, 2007; Sadaheeshna *et al.,* 2009; Sadaheeshna *et al.,* 2010). In present studies BAP appeared to be more effective and Kn for inducing adventitious shoot buds from leaf explants in *C. paniculatus.* Among the different levels of BAP tested, 2.0 mgl^{-1} BAP produced maximum number of shoot buds from DL explants. The positive effect of combined cytokinins (BAP and Kn) on adventitious shoot bud differentiation has also been reported in several other plants (Dayal *et al.*, 2003; Bhaskaran and Jayabalan, 2005; Sreedhar *et al.*, 2008; Jain *et al.*, 2011). In the present case, combination of BAP and Kn at 0.5 mgl^{-1} each, proved to be best in induction of shoot buds from PL explants. Exogenous supply of auxins alone caused callus formation in cultured leaf explants while their combination with cytokinins suppressed shoot bud differentiation. Similar antagonistic auxin-cytokinin effect on adventitious shoot bud differentiation has been reported in a number of cases (Tanimoto and Harada, 1982; Purohit *et al.*, 2004). *In vitro* regeneration of adventitious shoots is a preferred system for genetic transformation and therefore the protocol described here will be useful in this respect. System could also be used for conservation of this important threatened taxon.

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