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Rapid Shoot Regeneration from Thin Cell Layer Explants of an Endangered Medicinal Asclepiad *Ceropegia spiralis* L.

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Abstract

The thin cell layers of nodes and internodes of *Ceropegia spiralis* L. were cultured on MS supplemented with BAP 13.32 μM/l + NAA 0.537 μM/l induced 17.34 ± 0.55 shoots showing extensive growth. Later on the organogenesis was also induced on MS containing BAP 13.32 μM/l + 2, 4-D 1.130 μM/l, whereas the medium with BAP 13.32 μM/l + 2, 4-D 4.52 μM/l has the highest callus producing ability in recalcitrants as well as in normal explants. Shoots developed were rooted best on 0.5 MS with NAA 10.74 μM/l. Optimum shoot and root multiplication was obtained within eight weeks. *In vitro* plantlets were successfully weaned and transferred to soil with about 90 per cent survival rate. So far, more than 650 weanlings have been produced successfully and reintroduced into nature for their recovery.

Introduction

The genus *Ceropegia* (Asclepiadaceae) was reported by 200 species distributed in the tropical and subtropical Asia, Africa, Australia, Malaysia and in the Canary and Pacific islands (Bruyns 2003). In India 48 spp. were found of which 28 are endemic to the Peninsular India (Ansari 1984, Ahmedullah and Nayar 1986). *Ceropegia spiralis* L. an annual herb grown wildly in south India is an endangered species (Nayar and Sastry 1987, Madhav Gadgil 2004) popularly known to villagers, herbalists, as "Nimmati gadda". A slender erect or slightly twining herb up to 50 cm long, with depressed tubers. Flowering and fruiting are in between May and October months.

Tuberous roots of many *Ceropegia* species are edible. The root tubers are the officinal parts contain an alkaloid called "Ceropegin" (Nadkarni 1976) bitterness of the tubers was eliminated by boiling and then consumed (Mabberley 1987).

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The *Ceropegia spiralis* root tubers are also known to contain starch, sugars, gum, albuminoids, fats, crude fiber and valuable constituents which are used in many traditional Indian Ayurvedic drug preparations active against many diseases especially diarrhoea, dysentery. The starchy tubers are useful as a nutritive tonic (Kirtikar and Basu 1935, Reddy et al. 2006, Chopra et al. 1956). In this genus *C. bulbosa* and *C. candelabrum* also have medicinal properties (Jain and Defillips 1991). Several reports were published on the *in vitro* studies of *Ceropegia* species i.e., *C. candelabrum* (Beena and Martin 2003, Beena et al. 2003), *C. bulbosa* var. *bulbosa* (Britto et al. 2003). *C. bulbosa* (Goyal and Bhadauria 2006) *C. jainii*, *C. bulbosa*. *C. bulbosa* var. *lushii* (Patil 1998), *C. sahyadrica* (Nikam and Savant 2007). However, to date, there are no reports on the micropropagation of *C. spiralis* though it is an important edible tuberous asclepiad in the Southern Peninsular India. The aim of the present investigation was to develop systems for *in vitro* propagation to conserve and domesticate the wild endemic taxa *C. spiralis*.

Present investigation describes a shoot regeneration system using transversal thin cell layer (tTCLs) isolated from the *C. spiralis*. The TCL technology originated long ago. Since then, TCLs have been successfully used in the micropropagation of many plants including some recalcitrants such as *Lupinus* species (Mulin and Bellio-Spataru 2000) and *Spinacea oleracia* (Leguillon et al. 2003).

Cell divisions is a critical activity during the growth and development of a plant providing the building blocks for the differentiation of *in vitro* thin cell layers. Moreover, TCL technology is a solution to many of the issues currently hindering the efficient progress of medicinal, ornamental and floricultural crop improvement, since it addresses the issue of plant breeding at the first stage of the problem. Since the regeneration of specific organs may be effectively manipulated with TCLs, in conjunction with specific controlled *in vitro* conditions and exogenously applied plant growth regulators

Materials and Methods

An *in vivo* growing *Ceropegia spiralis* L. (Nimmati gadda) was collected from Akashaganga of Tirumala hills, Eastern Ghats, India. The voucher specimen KSM 14878 was deposited in the department of biotechnology herbarium, Montessori Mahila Kalasala, Vijayawada, Andhra Pradesh, India. The nodes containing axillary buds were washed in the running tap water followed by a fungicide and bactericide each 0.3% for 10 min and with tween 20 (5% v/v for 4 min). Then with surface disinfectant $HgCl_2$ (0.1% w/v for 2 min) after repeated washes in double distilled water, the sterilized segments were then washed thoroughly with sterilized distilled water, cut into appropriate sizes, and cultured on MS solidified with agar 0.9% (w/v) HiMedia Laboratories Pvt. Ltd.

Mumbai and different growth regulators (BAP, NAA, IAA, IBA) at different concentrations either alone or in combinations were added to the medium. In the present investigation all the media were autoclaved at 121ºC and 15 lbs pressure for 20 min after adjustment of the pH to 5.7 ± 1. However, in present study, TCL explants were excised transversely from *in vitro* grown six months old plants.

All the cultures were maintained at 24 ± 2ºC under 16 hrs photoperiod with 3000 lux light intensity using fluorescent lights (Philips India Ltd.) and 90 - 95% relative humidity within 250 ml Bottles and 25 × 150 mm culture tubes and the test tubes were covered with the aluminum foil. When the hormones failed to induce a specific response (callus, adventitious shoots/roots) at the end of the first cycle, it was not considered as suitable combination. Twenty cultures were raised for each treatment and all experiments were repeated thrice.

Explants (tTCLs of 1-2 mm thickness) were excised from the nodal and internodal regions of *in vitro* grown plantlets were placed in contact with the medium. The explant showed an excellent response within a week. Both transverse TCLs and longitudinal TCLs were employed in the present investigation, the explants were excised from the nodal and internodal meristematic regions, four TCLs (two from each side) were taken from either sides of the node. Only tTCLs response is considerable. The sections of the explant were placed on the MS supplemented BAP in combination with different auxins to induce the callus, organogenesis and somatic embryos. The synthetic auxins IBA and NAA are quite active in induction of rooting. They were tested individually and in combination. IBA and NAA were induced and elongated the roots, respectively when they used in combination.

Microshoots with well-developed root system were transferred directly to small pots containing sterile vermiculite and coco peat in (1 : 1) ratio rejuvenated growth within 20 days. Survival rate of the plantlets is 90 per cent and plantlets successfully established in the field exhibited morphology similar to that of mother plants.

Results and Discussion

The TCL system is quite suitable for the regeneration of plantlets from the explants. With the help of this method, the problem created by the latex can be avoided in normal cultures. The TCLs contain less amount of latex in them, which facilitate the correct contact with the medium. In the current investigation, the shoot regeneration was noticed using tTCLs isolated from the nodal explants, the tTCLs explants were excised from *in vitro* grown plants swelled after four/five days of culture, due to small amount of light green callus proliferation on the sub epidermal area. Shoot regeneration occurred from tTCL explants that appeared green and formed a peripheral crown of buds which elongated rapidly

within a week. The optimum number of shoots (17.34 ± 0.55) (Fig. 1B) were observed in the medium containing BAP 13.32 μM/l + NAA 0.537 μM/l and showed maximum regeneration capacity by producing shoots within a week (Table 1). The TCLs showed remarkable response by producing light green callus

Table 1. Effect of BAP and auxins on hoot regeneration from *Ceropegia spiralis* thin cell layer.

BAP μM/l	Auxins in μM/l				No. of shoots (Mean ± SD)	Length of shoots (Mean ± SD)	Basal callusing
	2,4-D	IAA	IBA	NAA			
13.32	0.452				6.79 ± 1.52 ^{c}	4.50 ± 0.18 ^{d}	++++
13.32	1.130				11.35 ± 2.56 ^{b}	4.25 ± 0.36 ^{d}	++++
13.32	2.26				6.42 ± 0.57 ^{c}	1.10 ± 0.33 ^{d}	++++
13.32	4.52				3.58 ± 0.23 ^{d}	1.28 ± 0.33 ^{d}	++++
13.32		0.577			5.17 ± 0.32 ^{d}	11.90 ± 0.84 ^{b}	+++
13.32		1.44			3.59 ± 0.35 ^{d}	8.20 ± 0.24 ^{c}	+++
13.32		2.88			5.89 ± 0.35 $^{c\,d}$	5.05 ± 0.14 ^{c}	++
13.32		5.77			7.01 ± 0.53 ^{c}	8.56 ± 0.19 ^{b}	+++
13.32			0.49		2.04 ± 0.3 ^{d}	0.59 ± 0.18 ^{d}	+
13.32			1.23		5.56 ± 0.58 ^{c}	5.43 ± 0.52 ^{c}	+++
13.32			2.46		6.05 ± 0.57 ^{c}	15.09 ± 0.66 ^{a}	++
13.32			4.92		10.50 ± 0.62 ^{b}	8.29 ± 0.12 ^{bc}	+
13.32				0.537	17.34 ± 0.55 ^{a}	10.32 ± 0.25 ^{b}	++++
13.32				1.342	6.00 ± 0.48 ^{c}	9.87 ± 0.50 ^{b}	+++
13.32				2.685	7.34 ± 0.36 ^{c}	6.53 ± 0.14 ^{c}	+++
13.32				5.37	5.44 ± 0.42 ^{d}	7.04 ± 0.33 ^{c}	++++

Mean following by the same letter was not significantly different by Tukey test at 0.05% probability. Degree of callusing: + = Very scanty; ++ = Scanty; +++ = Medium; ++++ = Profuse.

which in turn gave a crown of shoots at its periphery. These results are comparable with studies on *Ceropegia bulbosa* (Raghuramulu and Pullaiah 1999), *Cryptolepis buchanani* (Venkateswara et al. 1987) of traditional explants (nodes, internodes, leaves) and from TCLs, in *Brassica napus* (Klimaszewska and Keller 1995); in *Nicotiana tabaccum* (Nhut et al. 2003a, Heylen and Vendrg 1991) based on these last two references using TCL explants provides a method for efficient bud regeneration. On the other hand, the medium containing BAP 13.32 μM/l + IBA 0.49 μM/l exhibited very low number of shoots (2) but they show an excellent rate of growth. The results stated that the BAP along with the NAA had a very good ability in induction of the shoots, all other auxins are inferior to the NAA in the vegetative bud differentiation. Well-developed shoots (2 - 3 cm)

were inoculated on the different media containing auxins alone. Half strength of MS with 3% sucrose supplemented with NAA 10.74 µM/l and 8.44 ± 0.07 (Fig. 1C) the maximum root initiation (85%) was observed. *In vitro* rooted plants were successfully weaned and transferred to mist chamber, after the acclimatization, the weanlings were transferred to soil with about 90 per cent survival rate (Fig. 1E).

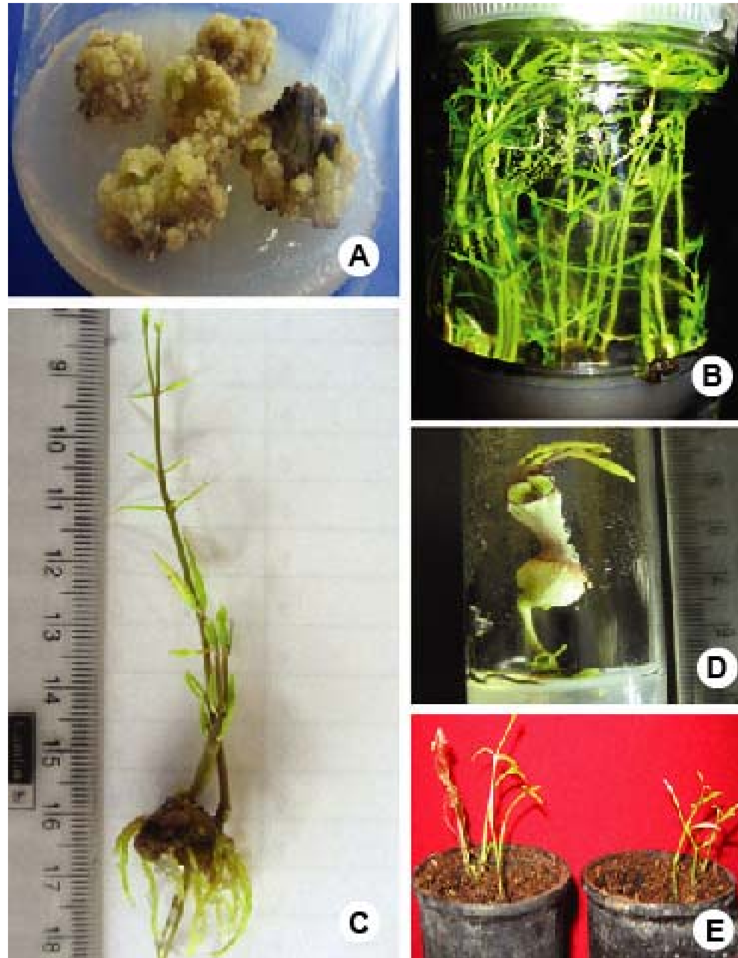

Fig. 1. Regeneration from thin cell layer explants of an endangered medicinal Asclepiad *Ceropegia spiralis*. A. Embryogenic callus development from the tTCLs cultured on MS fortified with BAP 13.32 µM/l +2,4-D 1.103 µM/l after 30 days. B. Initiation of multiple shoots from the tTCLs on the medium containing BAP13.32 µM/l + NAA 0.537 µM/l after 15 days. C. Rooting from *in vitro* raised shoots cultured on half strength of MS with 3% sucrose supplemented with NAA 10.74 µM/l after 16 days. D. *In vitro* flowering from the tTCLs cultured on the medium supplemented with BAP 13.32 µM +IAA 5.77 µM/l showed enlarged flower after 7 days. E. Acclimatized plants after ten days.

In the present investigation, tTCLs are quite active in callogenesis. Some of the explants showed extensive callusing, which iwas friable and cream to light green. 2,4-D at (0.452, 1.130, 2.26, 4.52 μM/l) in combination with BAP produced noticeable amounts of callus. The combination (BAP 13.32 μM/l + 2, 4-D 0.452 μM/l or 1.130 μM/l) produced friable, extensive callus of light green colour and granular in its nature. Different stages of somatic embryos were noticed in these combinations. Similar types of observations were observed in TCL explants of *Pelargonium* (Gill et al. 1992), *Heliconia psittacorum* (Goh et al. 1995), *Musa* spp. (Nhut et al. 2003a), *Manihot esculenta* (Nhut et al. 2003b,c). In the present investigation similar observations were noticed in the nodal explants of *C. spiralis* (data not shown) and some other species of this family, such as *Asclepias curassavica,* (De Bagga et al. 1986; Pramanik et al. 1986) *Calotropis gigantea* (Roy and De 1990), *Tylophora indica*, (Sharma and Chandel 1992) *Holostemma ada-kodien* (Martin 2002, 2003).

The pre-existing hormone in the explant plays a crucial role in the stimulation of the shoot initials. We have noticed a problem of bleached shoots just because of the fast growth of the shoots. Moreover, some of the shoots necrosed and turned pale due to the presence of ethylene in the bottles/test tubes as in case of *C. candelabrum* (Beena et al. 2003). Chlorophyll formation rate is a bit slow, when compared to that of the formation of shoots, due to which the plants turned pale in their color. All the plants were pretreated in the medium containing auxins at various levels so that they all became a bit bulged in their girth by accumulating starch.

Some of the plants became non-responsive for a long time (Recalcitrants) as a result they died or did not grow for long time. These plants were used as explants for the tTCL culture. Recalcitrancy might be due to the production of free radicals, lipid peroxidases, and toxic, aldehydic lipid peroxidation products (Benson 2000) levels of these compounds vary in response to different tissue culture manipulations. Kaai et al. (2008) found a close relation between the recalcitrancy and the abscisic acid. However, recalcitrancy was found when plants were subcultured *in vitro*. It was defined as the inability of plant tissue culture to respond to *in vitro* manipulations. We have excised the explants from the either sides of the nodes, aiming at getting the meristematic tissue in sections where the metabolically and physiologically active tissues were expected. These tissues were stimulated when they were cultured on the medium suitable for the proliferation of callus and organogenesis finally becomes normal plant.

When TCL explants cultured on medium it may choose to show the multiple morphogenetic programmes like callusing, shoot regeneration, rooting, flowering, somatic embryogenesis etc., or individual morphogenetic programmes organogenesis as determined by the shoot apical meristem and as

per the signal provided from the external plant growth regulators. Same results were observed in *Saintpaulia ionantha,* calluses, roots, somatic embryos, were achieved (Ohki 1994). Shoots and embryo like structures obtained from tTCLs of *Amaranthus edulis* when epidermis was cultured on the MS with a cytokinin (Bui et al. 1998). It is quite effective to use thin cell layers rather than using a complete node. TCLs have been successfully used in the micropropagation of *Lilium longiflorum* (Bui et al. 1999) and *Oryza sativa* (Nhut et al. 2001), including some recalcitrant ones, such as *Lupinus* spp. (Mulin and Bellio-Spataru 2000) and *Spinacea oleracea* (Leguillon et al. 2003).

The response of TCL explants excised from the nodes and inter nodes of recalcitrant plants was noted. The medium containing the low levels of 2,4-D had the less callusing ability, as the concentration increases, the callusing degree also increases up to 4.52 μM/l. Whereas, IAA and IBA had no significant effect on callusing of the recalcitrants. Slight to high levels of callusing was noticed in the medium containing high levels of NAA. For further improvements, other factors were taken into account, such as hormonal and light pretreatments just like (Julliard et al. 1992, Nhut et al. 2000) the addition of $AgNO_3$ (Aksaka-Kennedy et al. 2005) or of various sugars, but also more specific factors such as tTCLs explants thickness or position along the organ (Nhut et al. 2001).

The role of internal and external hormones on the organogenesis had notable effect in this particular investigation. As the concentration of the auxin (2,4-D) increases, the organogenic efficiency also increases, (Table 2). 2,4-D at 1.130 μM/l along with the BAP had optimum embryogenic callusing affinity wherein somatic embryos were observed (Fig. 1A). Homologous explants display discrepant differentiation in medium with deferent exogenous phytohormones. Exogenous hormones act as triggers in regulating explant differentiation, which in turn results in the production of extensive and friable callus. It was observed that the endogenous and exogenous hormonal supplies stimulated the bud initials to differentiate into either vegetative or floral buds.

Floral bud differentiation was initiated from explants of nodes in pre-flowering status when exogenous growth regulators IAA, NAA and IBA along with BAP were supplied in the medium. The TCL explants growing in the medium augmented with only auxins like NAA and IBA did not show any flowering, whereas the explants cultured on the medium supplemented with BAP along with IAA had a tremendous ability to induce and enlarge the flowers. The explants placed on the medium containing BAP 13.32 μM/l + IAA 5.77 μM/l showed enlarged flowers (Fig. 1D), whereas, IBA and NAA in combination with BAP had the ability to induce flower buds, but they did not mature properly and remained as such. In contrast, the explants on the medium with NAA+BA differentiated into floral buds, whereas IAA + BA were quite active in the

vegetative bud formation. (Li Ying-Zhan and Han Bi-Wen 1995). In contrast with the findings by Van der Krieken et al. (1990) and Heylen (1991), endogenous contents in TCLs had significant role during floral bud differentiation and were significantly related to floral bud differentiation along with the external PGRs.

Table 2. Effect of BAP and different auxins concentrations on callusing of the recalcitrant thin cell layer of *Ceropegia spiralis*.

BAP μM/l	Auxins in μM/l				Degree of callusing (Mean ± SD)
	2,4-D	IAA	IBA	NAA	
13.32	0.452				1.67 ± 0.29 [cd]
13.32	1.130				3.76 ± 0.36 [a]
13.32	2.26				3.70 ± 0.44 [a]
13.32	4.52				3.88 ± 0.29 [a]
13.32		0.577			0.53 ± 0.08 [d]
13.32		1.44			2.00 ± 0.54 [c]
13.32		2.88			2.06 ± 0.53 [c]
13.32		5.77			1.67 ± 0.49 [cd]
13.32			0.49		1.50 ± 0.50 [cd]
13.32			1.23		1.92 ± 0.21 [c]
13.32			2.46		1.95 ± 0.42 [c]
13.32			4.92		1.72 ± 0.40 [cd]
13.32				0.537	1.60 ± 0.27 [cd]
13.32				1.342	2.14 ± 0.28 [c]
13.32				2.685	2.12 ± 0.15 [c]
13.32				5.37	2.84 ± 0.64 [b]

Mean following by the same letter was not significantly different by Tukey test at 0.05% probability.

The source of the tTCLs was also a critical factor in our experiment. The tTCLs isolated from the nodal meristems responded well compared to any other explants, exhibited the highest shoot regeneration frequency. In our investigation, factors evaluated which had the influential role in morphogenesis.

This TCL technique could be used in the fundamental regeneration studies and for crop improvement through mutagenesis or transgenics in addition to being an efficient regeneration process (Teixeira da Silva and Fucai 2003) provided evidences of the capacity to efficiently produce non-chimeric transgenic plants using similar methods. Thus, tTCLs should provide good system for the study of fundamental and applied aspects of regeneration and transformation of this valuable, endangered, medicinal plant.

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