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In Vitro Micropropagation of *Anisochilus carnosus* (L) Wall

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ABSTRACT

The present study describes a protocol for the *in vitro* mass propagation of *Anisochilus carnosus* (L) Wall – an anticancer ethnomedicinal herb. Young and healthy leaf segments were inoculated on to the solid Murashige and Skoog (MS) medium supplemented with different auxins and cytokinins individually and in combination. Maximum callusing percentage (97%) was achieved in MS medium fortified with 2,4-Dichlorophenoxyacetic acid (2,4-D) at 5 mg/l. Multiple shoots were achieved in benzylaminopurine (BAP) and Indole-3 acetic acid (IAA) combination with maximum of 14±0.23 shoots per culture at 7 mg/l BAP + 3.5 mg/l IAA concentration. For *in vitro* rhizogenesis, elongated micro shoots were aseptically excised and transferred to the half strength MS liquid medium enriched with different auxins. Out of the different auxins tested, indole butyric acid (IBA) was found to be the best for rhizogenesis at 2 mg/l with maximum of 11±0.45 roots per shoot. The well established rooted plantlets were hardened under laboratory conditions for two weeks by transferring to the poly cups containing vermicompost and sterile soil in the ratio of 1:1. The hardened plantlets were transferred to the green house for two weeks and finally to the filed with 90 % survivability.

INTRODUCTION

India is a treasure house of 47,000 species of Angiosperms, out of which a total of 560 plant species have been recognised as threatened species by IUCN (Bapat *et al.*, 2008). Health consciousness and harsh side effects of non-natural products have made the natural plant products a common man's preference. Different traditional health care systems exist among different tribes and geographical regions. Among the continents, Asia is a treasure of knowledge with regard to the use of plants in treatment of various ailments. In India, among the major systems of indigenous medicines, i.e. Ayurveda, Siddha, Unani and Folk (tribal), Ayurveda is developed most and widely accepted and practiced (Kunwar and Rainer, 2008). In recent years, traditional medicine has become a topic of global importance as the modern man prefers herbal medicine over others. The surge of public interest in herbal medicine tied with rapid expansion of pharmaceutical industries and more importantly the unrestricted collection of medicinal plants from wild in destructive and unsustainable manner has put the wild medicinal plant population in dwindling mode. The wild population of medicinal plants has drastically come down which is the grave issue to be addressed (Thomas, 1997). It is being held that number of multipurpose ethnomedicinal plants have become either endangered or are under extinction threat in tropical ecosystems of developing countries.

Owing to the high demand of medicinal plants and a subsequent threat, a lot of attention is being paid to the plant tissue technique to arrest this threat. The over exploitation of medicinal plants stipulates the establishment of *in vitro* tissue culture conditions for the rapid and mass propagation of medicinal plants. Plant tissue culture is the most convenient and effective strategic way for germplasm conservation and rapid multiplication of medicinally important plant species to cater the present pharmaceutical demands and ensures no damage is done to the natural plant population.

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A. carnosus (L) Wall.is an annual herb, 30-60 cm tall, robust and branched with fleshy leaves. It grows in the Asia continent of Hong Kong, Macao, Shangai, Tianjin, India, Myanmar and Sri Lanka. It is an multipurpose ethnomedicinal plant used for the treatment of different ailments like gastrointestinal disorders, respiratory disorders, cough, cold and fever (Kamble *et al.,* 2008). Its formulation with *Ocimum basilicum*, *Mentha piperita* and *Alpinia galanga* has been reported to treat skin inflammation and influenza (Subramanian and Nair, 1972). Essential oils have been extracted by hydrodistillation from the leaves and have been reported to be antimicrobial in nature (Senatore *et al.,* 2003). The plant has been reported to have anti-inflammatory activity (Grover *et al.,* 2001), antiulcer activity (Mohammed *et al.,* 2008), antifungal property (Kulandhaivel *et al.,* 2011) and anticancer property (Muthuraman *et al.,* 2012). Due to multipurpose medicinal nature of *A. carnosus* and the stress from pharmaceutical industries, the present study was aimed to standardize a protocol for its *in vitro* mass propagation.

MATERIALS AND METHODS

Anisochilus carnosus plants were collected from Western Ghats, Tamil Nadu, India and are being maintained in the medicinal plant garden of the Department of Studies in Botany, University of Mysore, Mysuru. Young and healthy leaf explants were collected from the garden and were washed under running tap water for ten minutes to remove the soil and dust exudates. The explants were then treated with `fungicide bavistin (5% w/v) for 5 minutes followed by dipping in 70% alcohol for 30-40 seconds and were then washed with sterile double distilled water. Inside the laminar air flow explants were treated with 0.01% $HgCl_2$ for 5 minutes followed by washing thrice with sterile double distilled water. The surface sterilised leaf explants were cut into small pieces (1-2 sq.cm) and inoculated on to the solid MS medium containing 3% sucrose, gelled with 0.08 % agar and fortified with different combinations and concentrations of growth regulators. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121^0C for 15 minutes. The cultures were incubated at 22 ± 2^0C with 16 hour photoperiod under light florescent tubes with light intensity of 25µmol/s^2/m^2 for 4 weeks. *In vitro* regenerated shoots after attaining a height of 3-5 cm were aseptically transferred to half strength MS liquid medium supplemented with different auxins. Root length and number of roots per shoot data was collected after 4 weeks. After 4 weeks well rooted micro shoots were hardened under laboratory conditions for 2 weeks by transferring to the polycups containing vermicompost and sterile soil in the ratio of 1:1. The hardened plantlets then were transferred to the green house for two weeks and then to the garden with 90% survivability.

RESULTS

Callus induction

After two weeks of inoculation, callus initiation was reported along the cut edges of leaf explants. When leaf explants were placed onto the MS medium either on its adaxial or abaxial surface, callus was first reported from the abaxial surface. Explants showed curling and along the cut edges turned into creamy colour and the callus was induced. Explants were tested on MS medium supplemented with different concentrations of auxins (0.5-5 mg/l) for callus induction**(Fig. 1a)**.

Almost all the auxin concentrations tested produced callus on MS medium. Best callusing response was reported in MS medium supplemented with 2, 4-D at 5 mg/l concentration. On combination of BAP with different auxins, callusing was achieved in all combinations of BAP with 2,4-D, IAA, NAA (naphthalene acetic acid) and IBA. Effect of different auxins alone and in combination with BAP callus induction of leaf explants of *A. carnosus* is given in **Table 1.**

Induction of multiple shoots

Leaf explants callused moderately and then produced shoot buds which later proliferated into multiple shoots with maximum number of 14±0.76 shoots per culture (**Fig.1b; 1c**). The best combination for multiple shoot induction was found to be BAP in combination with IAA and NAA. The effect of BAP in combination with auxins on multiple shoot induction is presented in **Table 2**.

Table 1: Effect of growth regulators on callus induction of leaf explants in *A. carnosus*.

Conc. mg/l	2,4-D	IAA	NAA	IBA	BAP	Conc. mg/l	BAP+2,4-D	BAP+IAA	BAP+NAA	BAP+IBA
	Percentage Callusing						Percentage Callusing			
0.5	38	--	--	--	--	1+0.5	40	20	15	10
1	46	--	--	--	--	2+1	66	22	10	18
1.5	55	--	--	--	15	3+1.5	80	30	23	20
2	68	--	--	21	18	4+2	85	46	27	32
2.5	82	50	36	32	35	5+2.5	88	53	35	35
3	87	62	41	32	48					
3.5	93	70	50	45	55					
4	90	70	54	52	60					
4.5	92	72	59	55	68					
5	97	80	64	61	78					

*All the growth regulator treatments to leaf explant were in 6 replicates and were repeated thrice; callusing percentage = (Number of explants callused/ Total number of explants inoculated) x 100

Table 2: Effect of growth regulators on multiple shoot induction in *A. carnosus* leaf explants.

Growth Regulator	Concentration (mg/l)	Mean shoot number	Mean Shoot length
BAP	1	--	--
	3	--	--
	5	--	--
	7	--	--
BAP+2,4-D	1+0.5	--	--
	3+1.5	--	--
	5+2.5	--	--
	7+3.5	--	--
BAP+IAA	1+0.5	--	--
	3+1.5	6±0.56	4.25±0.58
	5+2.5	14±0.76	4.88±0.42
	7+3.5	7±0.43	4.45±0.65
BAP+NAA	1+0.5	--	--
	3+1.5	5±0.52	3.24±0.54
	5+2.5	5±0.41	4.41±0.38
	7+3.5	4±0.51	4.67±0.21
BAP+IBA	1+0.5	--	--
	3+1.5	--	--
	5+2.5	--	--
	7+3.5	--	--

*All the growth regulator treatments to leaf explant were in 6 replicates and were repeated thrice. Each value represents Mean ± S.D. Statistical analysis using DMRT ($P \leq 0.5$).

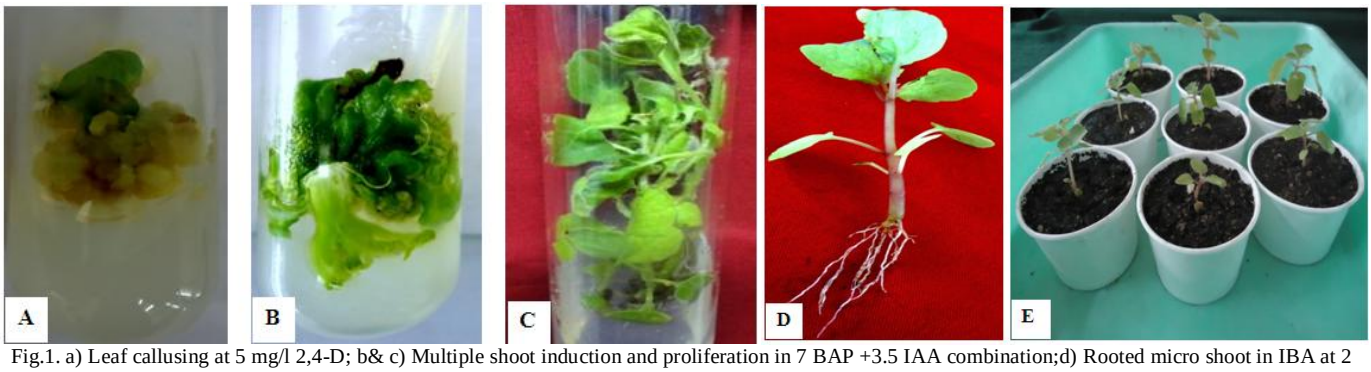

Fig.1. a) Leaf callusing at 5 mg/l 2,4-D; b& c) Multiple shoot induction and proliferation in 7 BAP +3.5 IAA combination;d) Rooted micro shoot in IBA at 2 mg/l; e) Acclimatised plantlets.

Table 3: Effect of auxins on *in vitro* rhizogenesis of regenerated shoots from *A. carnosus.*

Growth regulator	Concentration mg/l	Mean no. of roots per shoot	Mean root length	Percent shoots forming roots
IAA	0.5	--	--	--
	1	--	--	--
	1.5	--	--	--
	2	--	--	--
	2.5	--	--	--
	3	--	--	--
IBA	0.5	--	--	--
	1	--	--	--
	1.5	3.6±0.56	2.42±0.63	82
	2	11.21±0.69	3.67±0.41	95
	2.5	4.5±0.34	2.02±0.48	70
	3	--	--	--
NAA	0.5	--	--	--
	1	--	--	--
	1.5	--	--	--
	2	--	--	--
	2.5	--	--	--
	3	--	--	--

*All the growth regulator treatments to micro shoots were in 6 replicates and were repeated thrice. Each value represents Mean ± S.D. Statistical analysis using DMRT ($P \leq 0.5$).

Rhizogenesis

Well elongated *in vitro* regenerated micro shoots were aseptically removed from the culture flasks and transferred to the MS half strength liquid medium supplemented with different auxins. Micro shoots developed *in vitro* roots in MS medium fortified with IBA at 2 mg/l, inducing roots in 3 weeks with maximum of 11±0.21 roots per shoot (Fig.1d; Table 3). At higher concentrations of IBA (above 2mg/l), basal callusing was reported with stunted shoot growth.

Hardening of regenerated plantlet

Well developed *in vitro* rooted plantlets were taken out carefully from the culture tubes and were transferred to the poly cups containing vermicompost and sterile soil in the ration of 1:1 and were hardened under laboratory conditions for two weeks **(Fig. 1e)**. After hardening, plantlets were transferred to the green house for two weeks. Fully acclimatised and grown plantlets were finally transferred to the bigger pots and maintained under natural conditions in the garden where 90% of the plantlets survived.

DISCUSSION

The aim of the tissue culture studies is to standardise the *in vitro* conditions for the rapid and mass propagation of the medicinally important plants. In our study, leaf explants responded to callusing differently on different growth regulator concentrations and combinations. Best callus induction was achieved from leaf explants on MS medium containing either auxins alone or in combination with BAP. Out of the auxins used, 2,4-D was found to be the best for callus induction. Similar response of higher callus induction from leaf explants in 2,4-D supplemented MS medium has been reported earlier in *Cichorium intybus* (Nandagopal and Ranjithakumari, 2006) and *Clematis gouriana* (Raja and Krishna, 2007). 2,4-D has been reported to be the determining factor in active cell division and is the most effective auxin in callus induction as reported earlier by Sudarshana and Shanthamma, (1991), Pretto and Santarem, (2000) and Vengadesan *et al*., (2000). MS medium supplemented with BAP in combination with different auxins produced callusing, however BAP + 2,4-D proved to be the best callus inducing combination. Similar results have been reported earlier in *Beloperone plumbaginifolia* (Shameer *et al.,* 2009), *Momordica dioica* (Davendra *et al* 2009), *Arnica montana* (Petrova *et al*., 2011) and C*occinia abyssinica* (Tola *et al.,* 2015); however our studies contradict with the Chaitali *et al*., (2014) who reported BAP in combination with IBA as the best combination for callus induction in *Spilanthes acmella.*

Multiple shoot induction was achieved in MS medium supplemented with BAP in combination IAA and NAA. Higher concentration of cytokinins (BAP) and lower concentrations of auxins promote organogenesis as described earlier by Kelkar *et al.*, (1996) in *Piper colubrinum*, Kavitha *et al.*, (2001) in *Pearl millet* and Matheew *et al.* (2015) in *Vanilla tahitensis*. Primarily, cytokininins have major role on plant development, such as regulation of shoot formation, multiplication and the promotion of cell division and expansion (Mok and Mok, 2001).Our results concur with the studies carried out earlier in *Zingiber officinale* (Kirmal *et al*., 1992), *Curcuma longa* (Neeta *et al*., 2000) and *Gynandropsis pentaphylla* (Nagarathnamma *et al*., 2013). In the present study our results revealed that the effect of BAP and auxin combination on multiple shoot induction is more compared to BAP or auxins when used separately. This is in confirmation with the earlier studies in *Bambusa glaucescens* (Shirin and Rana, 2007) and *Lagenaria siceraria* (Saha *et al*., 2007). For *in vitro* rhizogenesis, IBA at 2 mg/l concentration induced maximum number of roots while simultaneously improving the growth of micro shoots. Our results are in accordance with the studies carried out on *Thapsia garganica* (Mukunga *et al.,* 2006), *Pseudarthria viscid* (Vinothkumar *et al.,* 2010), *Cassia angustifolia* (Parveen and Shahzad, 2011) and *Solanum nigrum* (Sridhar and Naidu, 2011). The superiority of IBA over other auxins for root induction has been earlier reported in *Cajanus cajan* (Dayal *et al.,* 2003), *Murraya koenigii* (Rout, 2005) and *Quill resiliencies* (Fleck *et al.,* 2009).

CONCLUSION

The present study describes the standardized protocol for the callus induction and *in vitro* propagation of *A. carnosus* using leaf explants. The study revealed that leaf is the useful explant for the mass propagation of this plant. The standardized protocol in the present study could be employed for the mass propagation of such highly valuable medicinal plant and could negate the threat to the wild existence while simultaneously will meet the pharmaceutical demands.

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