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HORTICULTURE

Adventitious Shoot Regeneration from *in vitro* Leaf Explants of *Ophiorrhiza mungos* L. var. *angustifolia* (Thw.) Hook. F.

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ABSTRACT

Ophiorrhiza mungos var. *angustifolia* is a herbaceous medicinal plant belonging to the family Rubiaceae. It is a rich source of potent anticancer compound camptothecin (CPT). Massive uprooting of the species from its natural habitat for the extraction of CPT along with poor seed viability has led to the destruction of its population. At this context, the present study was proposed to develop *in vitro* protocol from leaf explant for the propagation and production of CPT from the species. *In vitro* multiple shoot cultures maintained in half strength Murashige and Skoog medium with 3% sucrose and 0.8% agar supplemented with 8.88 μM Benzyl Amino purine (BA) were served as the explant source. Multiple shoots were induced on leaf explant cultures on half strength MS solid medium fortified with different concentrations of (4.44-22.19 μM BA, 4.67- 23.23 μM Kinetin, 0.49- 2.45 μM 2 isopentanyladinine, 4.57- 22.85 μM Zeatin) either individually or in a combination. Medium supplemented with 13.31 μM BA showed the best result with 52.33±3.06 shoots/explant. The synergic effect of BA with other cytokinins also favoured shoot induction in a higher rate but they were very minute in nature. The shoot buds initiated were repeatedly sub cultured and multiple shoot cultures were developed. The CPT content of thus produced multiple shoots were checked (0.13 ± 0.1 mg/g DW) and found comparable with that of the field grown plants (0.14 ± 0.1 mg/g DW). The authenticity of CPT was checked with HPLC analysis. Protocol developed in the present study can be used for the regeneration of adventitious shoots from leaf explant of *O. mungos* var. *angustifolia.*

Highlights

- *In vitro* regeneration protocol has been developed for the adventitious shoot regeneration from leaf explants of *O. mungos* var. *angustifolia*
- Developed protocol can also be used for the *in vitro* production of CPT from multiple shoot cultures raised from leaf explants

Keywords: *Ophiorrhiza,* shoot regeneration, Murashige and Skoog, camptothecin, cytokinins

Ophiorrhiza mungos var. *angustifolia* is a herbaceous medicinal plant belonging to the family Rubiaceae. The natural population of the genus is well demanding since they contain high value secondary metabolite, camptothecin (CPT) – a potent anticancer agent. CPT was isolated by Wall and Wani in 1966. Later on CPT was isolated from other genus also, which include *Nothapodytes foetida* (Govindachari and Viswanathan 1972), *Chonemorpha grandiflor*a (Kulkarni *et al.* 2010) and the genus *Ophiorrhiza* (Tafur *et al.* 1976). The species *munogs* var. *angustifolia* commonly called Indian snake root or mongoose plant is an annual herb distributed widely in southern Western Ghats region of India with an average height of 30 cm with small white flowers in corymbose cyme and elliptic-lanceolate leaves.

Massive uprooting of the species has led to the destruction of natural population of the species as

well as other species of the genus. At this context, many workers have developed different in vitro culture systems for the propagation and production of CPT from the genus. (Jose and Satheeshkumar 2004; Roja 2008; Ya-ut *et al.* 2011).

The present investigation was aimed to develop an efficient in vitro regeneration system for mass propagation from *in vitro* leaf explants of *O. mungos* var. *angustifolia* and estimation of camptothecin.

MATERIALS AND METHODS

Explant source

Plant was collected from southern Western Ghats region of India during June 2016 and authentically identified using available literature. Plant was maintained in the green house, department of Botany, University of Kerala, Thiruvananthapuram, Kerala and a specimen was deposited in the department herbarium (KUBH 6164). The nodal explants (1 cm) from the mother plant was surface sterilised and cultured on half strength Murashige and Skoog medium (1962) fortified with 8.88 µM BA and 3% sucrose for raising multiple shoot cultures. This is the standard medium for maximum multiple shoots that was developed in our laboratory (unpublished data). The cultures were maintained by sub culturing onto fresh medium with the same composition in every 3 weeks and was served as the explant source for *in vitro* leaves in further experiment.

Culture initiation

Adventitious shoots were induced on the *in vitro* leaf explants of *O. mungos* var. *angustifolia.* Leaves from 60 days old multiple shoot cultures were dissected and cut into suitable size of 1cm^2, they were inoculated onto half strength MS solid medium with 3% sucrose and 0.8 % agar and fortified with different concentrations of cytokinins (4.44- 22.19 µM Benzyl Aminopurine, 4.67- 23.23 µM Kinetin, 0.49- 2.45 µM 2 isopentanyladinine, 4.57- 22.85 µM Zeatin) either individually or in a combination. Explant cultured on media without plant growth regulators (PGRs) served as the control. pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 18 minutes under 1.5 kg cm^2 pressures. All the cultures were kept under 16 hrs. photoperiod (35-50 µEm^2/s) provided with white fluorescent tubes (Philips Ltd., Mumbai) at 25±2°C. Results were taken after 4 weeks of inoculation. Each treatment was repeated three times with 15 explants per treatment.

Shoot multiplication

After 4 weeks of shoot bud initiation, the cultures were transferred onto half strength MS medium fortified with 8.88 µM BA for further shoot multiplication. Cultures were repeatedly sub cultured 3 times onto the fresh medium with the same composition in an interval of 4 weeks.

Quantification of Camptothecin

Camptothecin content of the *in vitro* shoots regenerated from *in vitro* leaf explant in cultures were quantified and compared with the field grown plants. Micro shoots were harvested from *in vitro* cultures and shoots were collected from field grown plants, washed in tap water and rinsed with distilled water and dried at 60 °C for 16 hrs. in a hot air oven. Two grams of dried samples were powdered and extracted with 40 ml methyl alcohol in Soxhlet apparatus as described by Renjith *et al.* (2013). The extracts were concentrated under vacuum (Rotavap PBU-7) and the concentrate was dissolved in methyl alcohol, filtered using a 0.22 µm filter (Millipore) and used for HPLC analysis as described by (Fulzele *et al.* 2001). Isocratic analytical HPLC was performed on a Shimadzu HPLC system using a Luna 5u C18: 250x4.6mm (Phenomenex) column. The column was maintained at room temperature. The mobile phase was acetonitrile: water (45:55 v/v) at a flow rate of 1 ml/ min. CPT was identified at 254 nm in a detector (Shimadzu UV/Vis Photodiode array detector: SPD-M10 Avp) attached to the HPLC system based on a co-chromatography with authentic CPT (Sigma Aldrich, USA). The CPT quantification was done by comparison with the standard curve of authentic CPT in methanol (HPLC grade, Merck). Five different concentrations (2.5, 5, 10, 20 and 40 µg/ml) of authentic CPT sample were used for plotting and calibrating the standard HPLC chromatogram of CPT to which the unknown samples were compared and quantified.

Statistical Analysis

All the experiments were replicated 3 times with 15 cultures per treatment. One way ANOVA

(IBM SPSS- 20) was performed to determine the significance of treatment and also the interaction between factors. The mean separation was done according to Duncan's Multiple Range Test ($p<0.05$)

RESULTS AND DISCUSSION

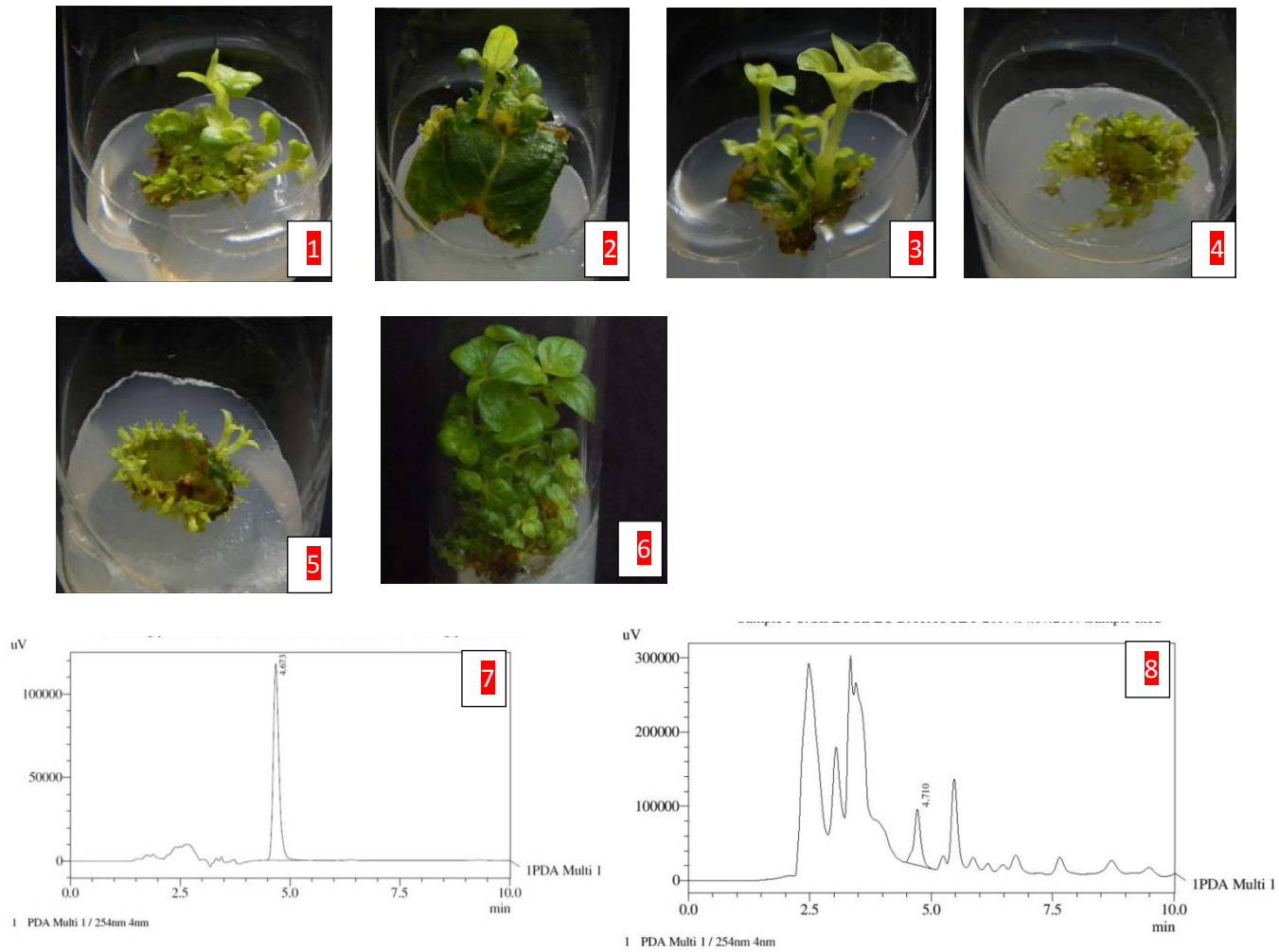
Shoot buds were initiated on *in vitro* leaf explants cultured on half strength MS medium fortified with different concentrations of cytokinins within 2 weeks of inoculation (Table 1). Leaves inoculated on half strength MS basal medium did not induce shoot buds. Of the cytokinins tested in the present study, 13.31 µM BA was found to be the best PGR concentration favouring shoot induction. Beegum *et al.* (2007) has already reported BA as the most effective cytokinin for the induction of shoots from leaf explant. Averages of 52.33±3.06 shoot buds were induced on a single leaf explant in the presence of 13.31µM BA (Fig. 1). But the shoot length was very less compared with that of other cytokinins produced shoots (0.1±0 cm). At the same time shoots with an average length of 0.4±0.0 cm was produced in the presence of 22.19 µM kinetin (Fig. 2), but only a lower number of shoots could be proliferated from a single explant (15.67±0.58). The highest number of shoots produced in the presence of zeatin was 18.67±1.53 (Fig. 3) and that of 2ip (Fig. 4) was 22±1.73 in a concentration of 18.28 µM and 1.47 µM respectively.

Since, 13.31 µM BA was found to be the optimum hormonal concentration for maximum number of multiple shoot production, different concentrations of other cytokinins were tried in a combination with the optimum BA concentration in the half strength MS medium and were found very effective. The synergic effect of BA with other cytokinins tested

Table 1: Effect of different cytokinins in shoot induction from *in vitro* leaf explants in half strength MS medium with 3% sucrose and 0.8% agar after 4 weeks of inoculation

Medium	BAP µM	KIN µM	Zeatin µM	2ip µM	Mean number of shoots/ explant ± SD	Mean shoot length± SD(cm)
Half Strength MS	—	—	—	—	0.00	0.00
	4.44	—	—	—	19 ± 3.61^{efgh}	0.1 ± 0^{f}
	8.88	—	—	—	34.33 ± 1.15^{c}	0.1 ± 0^{f}
	13.31	—	—	—	52.33 ± 3.06^{a}	0.1 ± 0^{f}
	17.16	—	—	—	50.33 ± 5.51^{a}	0.1 ± 0^{f}
	22.19	—	—	—	38.33 ± 1.53^{b}	0.1 ± 0^{f}
	—	4.65	—	—	20 ± 1^{ef}	0.3 ± 0.1^{cde}
	—	9.29	—	—	30 ± 2.65^{d}	0.1 ± 0^{f}
	—	13.94	—	—	20.67 ± 1.15^{ef}	0.37 ± 0.06^{ab}
	—	18.58	—	—	20 ± 1^{ef}	0.33 ± 0.15^{bc}
	—	23.23	—	—	15.67 ± 0.58^{hij}	0.4 ± 0.0^{a}
	—	—	4.57	—	12.33 ± 0.58^{j}	0.13 ± 0.06^{f}
	—	—	9.14	—	16 ± 1^{ghi}	0.17 ± 0.06^{f}
	—	—	13.71	—	18.67 ± 1.53^{efgh}	0.2 ± 0.1^{ef}
	—	—	18.28	—	21 ± 1^{ef}	0.17 ± 0.06^{f}
	—	—	22.85	—	19.67 ± 1.53^{efg}	0.13 ± 0.06^{f}
	—	—	—	0.49	13.67 ± 1.53^{ij}	0.37 ± 0.03^{ab}
	—	—	—	0.98	17.67 ± 0.58^{fgh}	0.23 ± 0.04^{def}
	—	—	—	1.47	22 ± 1.73^{e}	0.33 ± 0.01^{bc}
	—	—	—	1.96	21.33 ± 0.58^{ef}	0.34 ± 0.06^{bc}
	—	—	—	2.45	20 ± 1^{ef}	0.23 ± 0.06^{def}
Treatment Df (n-1)					94.031***	10.570***

*Means within a column followed by same letters are not significantly ($p<0.05$) different as determined by Duncan's multiple range test. NS-Non-Significant, ***F value is highly significant at $p<0.001$, **Significant at $p<0.01$ level, * Significant at $p<0.05$ level*



Multiple shoots induced on *in vitro* leaf explant in half strength MS medium fortified with; 1. 13.31 µM BA **2.** 22.19 µM kinetin 3**.** 18.28 µM zeatin **4.** 1.47 µM 2ip **5.** 9.29 µM kinetin + 13.31 µM BA **6.** Multiple shoots after 3 subcultures. **7.** HPLC chromatogram of standard CPT **8.** HPLC chromatogram of *in vitro* cultures of multiple shoots.

gave a positive result. The highest number of shoot buds induced were in a combination of 9.29 µM kinetin with 13.31 µM BA and an average of 73±4.36 shoot buds/ leaf was produced (Fig. 5). A maximum of 44±2 shoots were produced in the presence of 18.28 µM Zeatin with 13.31 µM BA. The highest number of shoot produced in the combination of 2ip and BA was 31±1 (0.98 µM 2ip + 13.31 µM BA). All these results are merely higher than the results obtained when leaf explants were cultured in individual concentrations of cytokinins. But the shoot buds were very small and were having a mean shoot length of 0.1 cm in all the cases. Thus combination of cytokinins were not preferred for shoot induction from the leaf explant.

Direct organogenesis have been considered as one of the most reliable and applicable method for true-to type high rate clonal multiplication of *O. mungos* var. *angustifolia.* Direct organogenesis from leaf explants were reported in many other species also. Shoot initiation on the leaf disc of strawberry was reported by Nehra *et al.* (1989). Somatic embryogenesis was induced on *in vitro* leaves of *Phaleanopsis amblis* (Chen and Chang 2006). In all the cases, BA was found to be the appropriated PGR for direct regeneration or the synergic effect of BA along with plant hormones gave best results. Other cytokinins produced more number of shoots which were minute or stunted in nature.

The shoot buds after 4 weeks were sub cultured onto fresh MS solid medium fortified with 13.31 µM BA and were found effective with shoot multiplication

Table 2: Effect of different cytokinins in shoot induction from *in vitro* leaf explants in half strength MS medium with 3% sucrose and 0.8% agar after 4 weeks of inoculation

	KIN µM	ZEATIN µM	2ip µM	Mean number of shoots/ explant ± SD	Mean shoot length ± SD (cm)
	4.65	—	—	48.33±7.64^{c}	0.1
	9.29	—	—	73±4.36^{a}	0.1
	13.94	—	—	56.33±7.77^{b}	0.1
	18.58	—	—	37±2.65^{de}	0.1
	23.23	—	—	24.67±4.51^{fg}	0.1
BA 13.31	—	4.57	—	23±2.65^{g}	0.1
µM	—	9.14	—	35.33±4.73^{e}	0.1
	—	13.71	—	35±5^{e}	0.1
	—	18.28	—	44±2^{cd}	0.1
	—	22.85	—	37.33±2.52^{de}	0.1
	—	—	0.49	23.33±1.53^{fg}	0.1
	—	—	0.98	31±1^{ef}	0.1
	—	—	1.47	27.67±1.53^{fg}	0.1
	—	—	1.96	24.67±1.53^{fg}	0.1
	—	—	2.45	23.33±4.51^{fg}	0.1
Treatment Df(n-1)	—	—	—	35.419***	

*Means within a column followed by same letters are not significantly ($p<0.05$) different as determined by Duncan's multiple range test. NS-Non-Significant, ***F value is highly significant at $p<0.001$, **Significant at $p<0.01$ level, * Significant at $p<0.05$ level*

rate. The shoot multiplication was continued until the medium got fully exhausted. Multiple shoot cultures were established by repeatedly sub culturing the shoot clumps onto fresh medium with same combination in an interval of 4 weeks.

Estimated CPT content of multiple shoots after 3 subcultures (4 months old) was 0.13 ± 0.1 mg/g DW similar to that of field grown plant (4 months old), which is estimated as 0.14 ± 0.1 mg/g DW. This was validated by the report of Renjith *et al.* (2013), about the screening of CPT content of *Ophiorrhiza* species in the southern Western Ghats. The HPLC analysis was done to authenticate the presence of CPT in the *in vitro* cultures (Fig. 7 & 8).

CONCLUSION

It can be concluded that *in vitro* leaves can be used as explants for raising multiple shoot cultures of *Ophiorrhiza mungos* var. a*ngustifolia* for the production of camptothecin in half strength MS solid medium fortified with 13.31 µM BA. The protocol that the present study has come up with is the first time report of the use of leaves as explants for the propagation of this species.

REFERENCES

Chen, J. and Chang, W.C., 2006. Direct somatic embryogenesis and plant regeneration from leaf explants of Phalaenopsis amabilis. *Biologia Plantarum,* **50**: 169-173.

Govindachari, T. and Viswanathan, N. 1972. Alkaloids of Mappia foetida. *Phytochemistry,* **11**: 3529-3531.

Jose, B. and Satheeshkumar, K. 2004. *In vitro* mass multiplication of Ophiorrhiza mungo Linn.

Kulkarni, A., Patwardhan, A., Lele, U. and Malpathak, N. 2010. Production of camptothecin in cultures of Chonemorpha grandiflora. *Pharmacognosy research,* **2**: 296.

Nehra, N.S., Stushnoff, C. and Kartha, K. 1989. Direct shoot regeneration from strawberry leaf disks. *Journal of the American Society for Horticultural Science* (USA).

Renjith, R., Varghese, S.C., Kurup, R., Gopalakrishnan, R., Venkataraman, R., Satheeshkumar, K. and Baby, S. 2016. HPTLC-based quantification of camptothecin in Ophiorrhiza species of the southern Western Ghats in India. *Cogent Chemistry,* **2**(1): 1275408.

Roja, G. 2008. Micropropagation and production of camptothecin from in vitro plants of *Ophiorrhiza rugosa* var. decumbens. *Natural product research,* **22**: 1017-1023.

Shahanaz Beegum, A., Poulose Martin, K., Zhang, C.L., Nishitha, I., Slater, A. and Madhusoodanan, P. 2007. Organogenesis from leaf and internode explants of *Ophiorrhiza prostrata*, an anticancer drug (camptothecin)

producing plant. *Electronic journal of Biotechnology,* **10**: 114-123.

Tafur, S., Nelson, J., DeLong, D. and Svoboda, G. 1976. Antiviral components of *Ophiorrhiza mungos*. Isolation of camptothecin and 10-methoxycamptothecin. *Lloydia,* **39**: 261.

Wall, M.E., Wani, M., Cook, C., Palmer, K.H., McPhail, A.A. and Sim, G. 1966. Plant antitumor agents. I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from camptotheca acuminata1, 2. *Journal of the American Chemical Society,* **88**: 3888-3890.

Ya-ut, P., Chareonsap, P. and Sukrong, S. 2011. Micropropagation and hairy root culture of *Ophiorrhiza alata* Craib for camptothecin production. *Biotechnology letters,* **33**: 2519-2526.