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In vitro Regeneration of an endangered medicinal Plant, *Ophiorrhiza mungos* L

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Abstract
Camptothecine (CPT), monoterpene indole alksloid, is a potent inhibitor of eukaryotic DNA topoisomerase-I. CPT and its derivatives are being used in different treatments of several cancers. We have proved that biotechnological potential of *Ophiorrhiza mungos* for mass multiplication from *in vitro* grown plants an efficient protocol for the regeneration of endangered *O. mungos* plants using nodal explants. The nodal explants were inoculated on Murashige and Skoog (MS) medium fortified with different concentrations of plant growth regulator N^6-benzyladenine (BA). The results revealed that the maximum number of shoots (20.06) with 100 % regeneration frequency from nodal explants obtained in MS medium fortified with 2mg/l BA within eight weeks. The present findings indicate that *O. mungos* respond favourably for *in vitro* propagation and these *in vitro* regenerated flowering plants of *O. mungos* can be used for over expression of key genes involved in regulating terpenoid indole alkaloid (CPT) biosynthesis.

Keywords: *Ophiorrhiza mungos*, Camptothecine, Topotecan, *In vitro* propagation, DNA topoisomerase I, Plant growth regulators.

Introduction

Camptothecin, is a modified terpenoidindole alkaloid (TIA), was first isolated from *Camptotheca acuminata* (Wall *et al.,* 1966) [1]. It exhibits excellent anti-tumor activity, due to its ability to inhibits DNA topoisomerase I (topo-I), an enzyme involved in DNA replication and transcription (Ulukan and Swaan 2002) [2]. Later identification of CPT was from the indigenous tree Nothapodytes and certain species of Ophiorrhiza. The major sources of CPTs are *C. acuminata, Nothapodytes foetida, O. mungos and O. pumila* (Lorence and Nessler, 2004) [3]. A number of studies have indicated its therapeutic potential against various cancers, including ovarian and colon cancers (Cragg and Newman 2005) [4]. Two semi-synthetic drugs derived from CPT, namely topotecan and irinotecan, have been approved by the US Food and Drug Administration (FDA) in 1994 and used extensively for the treatment of metastatic colorectal cancer, cervical cancer and small cell lung cancer throughout the world (Venditto and Simanek 2010) [5]. Beside their anti-tumor activity, CPT derivatives have also been found to show good activity against viruses such as the human immunodeficiency virus (HIV) (Priel *et al.*, 1991) [6]. More derivatives of CPT are now in clinical studies, such as 9-nitrocamptothecine, 9-aminocamptothecine and rubitecan (Sankar and Lieberei 2011) [7] which will potentially result in growing demand for these drugs in future. The combined sales of irinotecan and topotecan (CPT analogs) in only 2008 had reached 2.2 billion US dollars and is expected to increase further (Lorence and Nessler, 2004; Lu and *et al*., 2009) [3, 8]. The annual production of CPT throughout the world is only 600kg, while approximately 3000kg of CPT is required in the international markets (Kai *et al*., 2014) [9]. However, all the CPT derivatives which are consumed are synthesized from natural CPT, which is mainly obtained by extraction from the stem bark and fruits of *C. acuminata* (from China) (Wall *et al*., 1966) [1] or from the stem bark of *N. nimmoniana* (from India) (Umashaanker *et al*., 2008). The spatial configuration essential for the pharmacological action of CPTs restricts their easy synthesis. So drug manufacturers have to depend on natural sources for the production of CPT-based drugs. However, all the CPT derivatives which are consumed are synthesized from natural CPT, which is mainly obtained by extraction from the stem bark and fruits of *C. acuminata* (from China) (Wall *et al*., 1966) [1] or from the stem bark of *N. nimmoniana* (from India)

(Umashaanker *et al*., 2008). This alkaloid have also been producing in some other plant species, such as *Ervatamia heyneana* (Apocynaceae) (Gunasekera *et al*., 1979), *Merrilliodendron megacarpum* (Icacinaceae) (Arisava *et al.*, 1981). Since the limited supply of CPT is from the above woody plants with slow growth rates it is an important and urgent task to develop sustainable and alternative production source of CPT in order to resolve the worldwide scarcity of natural sources of CPT.

The genus Ophiorrhiza belongs to the family Rubiaceae, which comprises 150 species. In India it is represented by 49 species, have been used in traditional medicines against snake bite, stomatitis, ulcers and wound healing (Kirthikar and Basu 1975) [13]. *O. mungos* is commonly called mongoose plant, distributed throughout Western Ghats of India. The roots of *O. mungos* have been reported as sources of CPT and 10-methoxycamptothecin (Tafur *et al*., 1976) [14]. Some Ophiorrhiza species have been used in traditional and folk medicine as antitussive, analgesic and for the treatment of ulcers, gastropathy, leprosy and amenorrhea. Ophiorrhiza species are also used against snake bite. Now there is an increasing demand for alternative sources and profitable methods to produce CPT. Due to several advantages such as rapid growth rate, unlimited branching, genetic stability, but lower yields of CPT from intact plant and lack of viable method of production has encouraged us to explore the biotechnological potential of *O. mungos* for establishment of mass multiplication through *in vitro* propagation. The present findings indicate that *O. mungos* respond favourably for *in vitro* propagation and these *in vitro* regenerated flowering plants of *O. mungos* will be used for over expression of key genes involved in regulating terpenoid indole alkaloid (CPT) biosynthesis.

Materials and Methods

Seeds of *O. mungos,* were collected from green house plants, School of Ecology and Conservation, Department of Crop Physiology, University of Agricultural Science, GKVK, Bengaluru. The experiment was conducted with germination of *O. mungos* on Murashige and Skoog's medium (MS) (Murashige & Skoog, 1962) [15]. *O. mungos* seeds were taken and soaked in water for overnight, then treated with 2% Bavistin allowed for 3 minutes followed by rinsing 2-3 times in tap water and treated with surfactant Tween80 with one or two drops followed by rinsing with distill water for 5 times each 3 minutes and then seeds were treated with 4% Sodium hypochlorite allowed for 5 minutes followed by rinsing in sterile distill water for 5 times each of 4 minutes. Seeds were allowed to blotting on tissue paper for 10 minutes. Seeds were inoculated into Murushige and Skoog (MS) basal medium containing 3% sucrose solidified with 0.7% agar was used. The pH of the medium was adjusted to 5.8 prior to the addition of agar. The seeds were germinated 21 days after inoculation. Nodal parts were used as explants and inoculated into culture bottles containing 50ml MS basal medium supplemented with different concentrations of BA (0.5, 1.0, 2.0, 3.0, 4.0 mg/l) alone for multiple shoots induction and experiment was conducted in 5 replicates. Culture bottles were incubated at 25±2 °C under 16 hours photoperiod maintained by cool white fluorescent tubes. Data on shoot multiplication was recorded after 8 weeks of culture. All the cultures were subcultured onto the fresh medium after every four weeks. The frequency with which explants produced shoots and number of shoots per explant were recorded after eight weeks of culture.

Statistical analysis

The data was analyzed by analysis of variance (ANOVA) using SPSS version 19.0 to analyze the influence of the basal media and the concentrations of plant growth regulators on *O. mungos* mass multiplication. Significant difference between means were assessed by Duncan's Multiple Range Test (DMRT) ($P = 0.05$).

Results and Discussion

High quality propagation materials of *O. mungos* having camptothecin, could be produced by asexual methods and therefore *in vitro* mass propagation is considered to be best method for the production of true-to-type plantlets. Aseptic seedlings of *O. mungos* was initially obtained from *in vitro* germinated seeds (Plate 1), which were main source for nodal explants. The full strength MS medium fortified with different concentrations of BA favored shoot bud initiation in nodal explants within one week of inoculation. To induce multiple shoots from nodal explants, explants were cultured on MS media containing different concentrations of PGRs like BA (0.5-4 mg/l) for multiple shoot induction (Plate 2). Effect of different concentration of growth regulators on multiple shoot production from nodal explants of *0. mungos* has shown in Table 1 & Figure 1. An effective multiple shoot production by nodal explants was observed on the MS medium fortified with different concentrations BA alone. An effective shoot initiation was observed on the MS medium fortified with very low concentrations of the growth regulators, BA 2mg/l. The highest number of multiple shoots were produced in MS medium fortified with BA (20.06 shoots/node) at 2mg/l with 100% regeneration frequency. The results showed that very low concentrations of growth regulators in the basal medium were adequate for effective shoot initiation. Further, it has been observed that high concentrations of these growth regulators decreased the culture response considerably. It may be explained that this species has adequate endogenous hormones and does not require high amount of exogenous growth regulators for regeneration. There is a linear correlation between the increments of shoot number and BA concentration to the optimal level. Geetha *et al*. (1998) [16] and Anjusha and Gangaprasad (2016) [17] have already reported the suitability of BA over other cytokinins in producing multiple shoots. Camptotheca acuminata, another CPT-producing plant was also reported to produce multiple shoots in the presence of low BA concentration (Liu and Li, 2001) [18].

The nodal explants were superior for obtaining maximum multiple shoots induction and maximum number of shoots per explant when cultured on MS medium with 2.0 mg/l BA alone. These results are in conformity with the results of a study of *O. alata* Ya-ut *et al*. (2011) [19]. The results suggest that the cytokinin, BA played an important role in multiple shoot production. It is well known that cytokinin stimulate plant cell division and participate in the release of lateral bud dormancy, induction of adventitious bud formation, growth of lateral buds and in cell cycle control. The number of shoots formed per explant or the production efficiency of multiple shoots varied with the concentration of cytokinin alone. Despite the fact that MS media with BA promoted multiple shoot formation, the regenerated shootlets failed to elongate on the same media. This may be due to formation of shoot cultures in clumps. The highest number of multiple shoots was observed in MS medium fortified with BA (20.06 shoots/node) at 2mg/l.

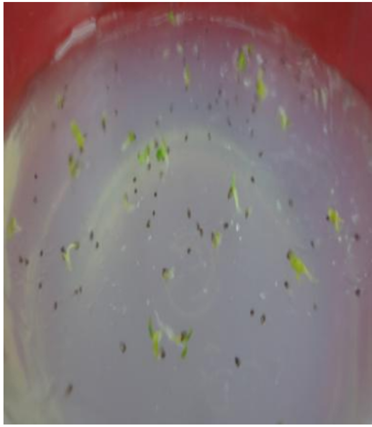

Plate 1: Seed germination

Plate 1: Shoot multiplication

Table 1: Effect of different concentrations of growth regulators on shoot multiplication and shoot Number after 8 weeks of inoculation of nodal explants *Ophiorrhiza mungos*.

BAP(mg/l)	No of shoots/explants(node) (Mean ± Sd)	Regeneration frequency (%)
Control	1.62 ± 0.74[a]	100
0.5	8.46± 1.66[b]	100
1.0	13.26 ± 1.27[c]	100
2.0	20.06 ± 1.27[b]	100
3.0	7.0 ± 1.06[e]	100
4.0	5.13± 0.63[e]	100

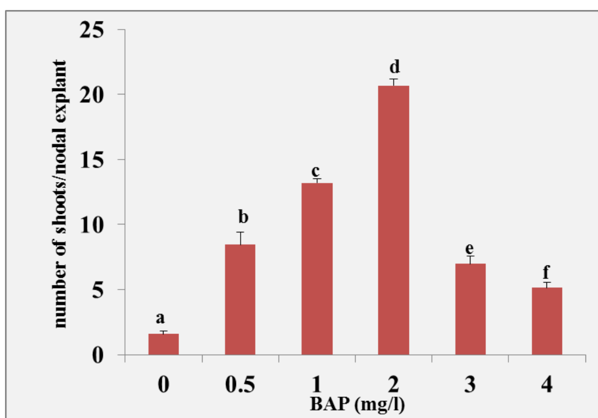

Fig 1: Effect of different concentrations of growth regulators on shoot multiplication and shoot number after 8 weeks of inoculation of nodal explants *Ophiorrhiza mungos*.

Conclusion

Efficient protocol for micropropagation of *O. mungos* from nodal explants has been developed. The results suggested that PGRs at concentration 2.0 mg/l BA was important for inducing shoot proliferation. This finding sets up an important resource base for using multiple shoots for a variety of experiments. Hence, the protocol has been developed in the present study may be useful for the production of any number of plants from nodal explants in short time. It will also provide an efficient method for conserving this valuable medicinal resource in the Western Ghats.

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