International Journal of Agriculture Sciences
ISSN: 0975-3710 & E-ISSN: 0975-9107, Volume 10, Issue 18, 2018, pp.-7259-7262.
Available online at https://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000217

Research Article

STANDARDIZATION OF PROTOCOL FOR THE *IN VITRO* MICROPROPAGATION AND MASS PRODUCTION OF AN ENDANGERED MEDICINAL PLANT, *Ophiorrhiza mungos* L.

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Received: September 20, 2018; Revised: September 25, 2018; Accepted: September 26, 2018; Published: September 30, 2018

Abstract: Camptothecin (CPT), an indole alkaloid compound. Topotecan and irinotecan, are CPT derivatives approved by Food and Drug Administration (FDA) were effectively used for treating various cancer. We have proved that biotechnological potential of *O. mungos* for mass multiplication from in vitro grown plants an efficient protocol for the regeneration of endangered *O. mungos* plants using nodal explants were developed. Initially explants obtained from seed germination of *O. mungos*. Sterilized seeds of *O. mungos* were transferred aseptically into MS medium and seed were germinated after 21 days. The nodal explants were inoculated on Murashige and Skoog (MS) medium fortified with different concentrations of plant growth regulator N^6-benzyladenine (BA) and Thidiazuron (TDZ). The results revealed that the maximum number of shoots (23.06) from nodal explants obtained in MS medium fortified with 2mg/l BA within eight weeks. The maximum shoot elongation (3.76cm) obtained in MS medium fortified with 1.0mg/l Gibberellic acid (GA_3) within four weeks. The regenerated shoots rooted well in the MS medium contain 1mg/l Indole-3-butyric acid (IBA). The rooted plantlets were successfully acclimatized with 100% survival rate in a growth chamber at 25°C, 60 % relative humidity, with 16-hrs photoperiod. 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.

Keywords: *Ophiorrhiza mungos*, Camptothecin, Topotecan, *In vitro* propagation, DNA topoisomerase I

Citation: Nagesha B.V., *et al*., (2018) Standardization of Protocol for the *in vitro* Micropropagation and Mass Production of an Endangered Medicinal Plant, *Ophiorrhiza mungos* L. International Journal of Agriculture Sciences, ISSN: 0975-3710 & E-ISSN: 0975-9107, Volume 10, Issue 18, pp.- 7259-7262.

Introduction

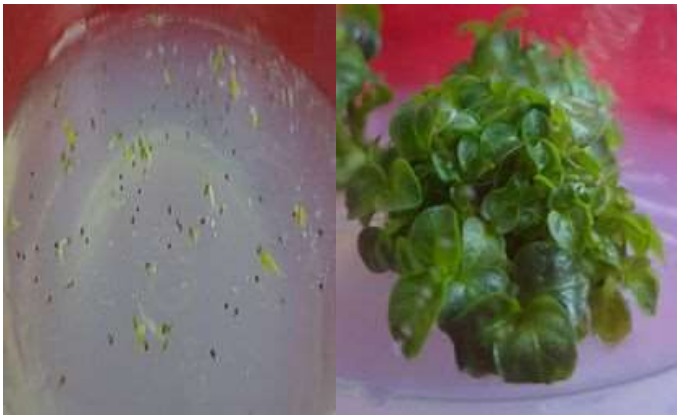
Camptothecin (CPT) is an essential precursor of semisynthetic chemotherapeutic agents (irinotecan, topotecan, etc.) for various forms of cancers throughout the world. Camptothecine, is a modified terpenoidindole alkaloid (TIA), was first isolated from *Camptotheca acuminata* [1]. It exhibits excellent anti-tumor activity, due to its ability to inhibits DNA topoisomerase I (topo-I), an enzyme involved in DNA replication and transcription. 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* [2]. A number of studies have indicated its therapeutic potential against various cancers, including ovarian and colon cancers [3]. 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 [4]. Beside their anti-tumor activity, CPT derivatives have also been found to show good activity against viruses such as the human immunodeficiency virus (HIV) [5]. More derivatives of CPT are now in clinical studies, such as 9-nitrocamptothecine, 9-aminocamptothecine and rubitecan [6] 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 [6, 7]. The annual production of CPT throughout the world is only 600kg, while approximately 3000kg of CPT is required in the international markets. 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) or from the stem bark of *N. nimmoniana* (India) [8]. 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) or from the stem bark of *N. nimmoniana* (from India) [8]. This alkaloid has also been producing in some other plant species, such as *Ervatamia heyneana* (Apocynaceae) [9], *Merrilliodendron megacarpum* (Icacinaceae) [10]. 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* comes under family Rubiaceae, consists 150 species. In India it is represented by 49 species, have been used in traditional medicines against snake bite, stomatitis, ulcers and wound healing [11]. *Ophiorrhiza mungos* L. 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 [12]. 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 the naturally grown plant and lack of new method of production has made us to findout 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 *Ophiorrhiza mungos*, were collected from green house plants, School of Ecology and Conservation, Department of Crop Physiology, University of Agricultural Science, GKVK, Bengaluru. The experiments were conducted with germination of *O. mungos* on Murashige and Skoog's medium (MS) [13]. *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 distil 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 distil 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 seeds were germinated 21 days after inoculation. Nodal parts were used as explants and transferred to culture bottles containing 50 ml MS basal medium supplemented with different concentrations of BA and TDZ (0.5, 1.0, 2.0, 3.0, 4.0 mg/l) separately for multiple shoots induction and experiments were conducted in 5 replicates. Cultures were incubated at 25±2°C under 16 hours photoperiod from cool white fluorescent tubes. Data on shoot multiplication were recorded after 8 weeks of culture. Multiple shoots obtained from these were transferred to MS medium containing different concentrations GA_3 (0.5, 1.0, 2.0, 3.0 mg/l) for elongation of shoots and incubated at 25±2°C under 16 hours photoperiod from cool white fluorescent tubes for shoot elongation. Elongated shoots were transferred to MS medium fortified with various concentrations IBA (1.0, 2.0, 3.0 mg/l) for root formation. For hardening, the rooted plantlets were transferred to thermocol cups containing autoclaved soil rite and grown under fluorescent light for 10 days and kept alternate days under sunlight and fluorescent light and later transferred to bigger pots containing red soil and grown in green house. The survivability rates were determined after 40 days of hardening. All the cultures were sub cultured 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.

Results and Discussion

High quality propagation materials of *O. mungos* L., 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* were initially obtained from in vitro germinated seeds (Fig A) were main source for nodal explants. To induce multiple shoots from nodal explants, explants were cultured on MS media containing different concentrations of PGRs like BA and TDZ separately (0.5 - 4 mg/l) for multiple shoot induction [Table-1] & Fig-B]. The effect of different concentrations of growth regulators on multiple shoot production from nodal explants of *O. mungos* is shown in [Table-1]. An effective multiple shoot production by nodal explants was observed on the MS medium fortified with different concentrations BA and TDZ. An effective shoot initiation was observed on the MS medium fortified with very low concentrations of the growth regulators, BA and TDZ separately at 2mg/l and1mg/l respectively. 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. [14] reported the multiple shoot formation in an endangered medicinal plant, Citrullus colocynthis when cultured in the basal medium fortified with low levels of the growth regulators BA, 2 4-D and TDZ. The results suggest that the cytokinin, BA played an important role in multiple shoot production followed by TDZ. 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. The highest number of multiple shoots was produced in MS medium fortified with BA (23.06 shoots/node) and TDZ (18.13 shoots/node) at 2mg/l and 1mg/l respectively. The shoots kept for elongation in different concentrations of GA_3 [Table-2] & Fig C). The shoot length was observed higher (3.76 cm) at 1mg/l of GA_3. In vitro grown shootlets were separated and transferred to rooting media. Rooting of elongated shoots was achieved on MS medium supplemented with different concentrations of the auxin, IBA [Table-3] & Fig D). All the treatments were induced roots, where maximum number of roots produced by individual shoot was observed in the MS medium containing auxin, IBA at 1.0mg/l [Table-3]. The results indicate that different concentrations of auxin, IBA alone is ideal for rooting of in vitro derived shoots of *O. mungos*. [15] also reported that the two auxins (NAA and IBA) were the most effective growth regulators for the induction of roots from shoots of the medicinal plant, *Cestrum diurnum*. The micropropogated plantlets with well-developed roots were successfully acclimatized to ex-vitro conditions; approximately 100% of plantlets survived the transition from tissue culture to the experimental plot. The hardening experiment revealed that the survivability [Table-4] & Fig E) rate of plantlets was higher (100%) in the hardening medium that contained red soil.



A. Seed germination B. Shoot multiplication

C. Shoot elongation D. Root induction

E. Hardening of in vitro plants

Table-1 *Effect of different concentrations of growth regulators on shoot Multiplication and shoot number after 8 weeks of inoculation of nodal explants O. mungos.*

BA(mg/l)	TDZ(mg/l)	No of shoots/explants (node) (Mean ± Sd)	Regeneration frequency (%)
Control	-	2.62 ± 0.74	100
0.5	-	9.46± 1.66	100
1.0	-	14.26 ± 1.27	100
2.0	-	23.06 ± 1.27	100
3.0	-	11.0 ± 1.06	100
4.0	-	6.13± 0.63	100
-	0.5	11.26 ± 1.27	100
-	1.0	18.13 ± 1.30	100
-	2.0	8.0 ± 1.36	100
-	3.0	5.86 ± 0.83	100
-	4.0	4.73 ± 0.70	100

Table-2 Effect of different concentrations of growth regulator (GA_3) on Shoot elongation after four weeks of *O. mungos* shoots.

Treatment-GA_3(mg/l)	Shoot length(cm) (Mean ± Sd)
Control	1.61 ± 0.19
0.5	2.78 ± 0.20
1.0	3.76 ± 0.26
2.0	2.41 ± 0.20
3.0	1.91 ± 0.21

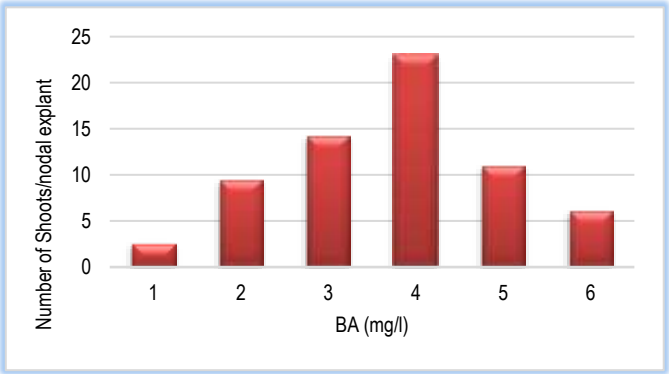
Table-3 Effect of different concentrations of growth regulators on root induction After four weeks of *O. mungos.*

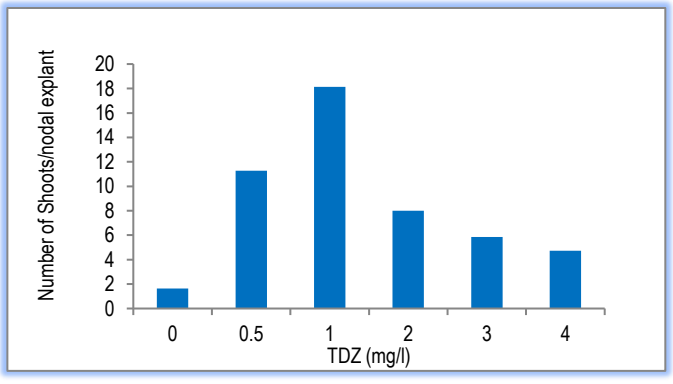
IBA (mg/l)	Response
1	+++++
2	+++
3	++

+++++ = Good +++ = Medium ++ = Poor

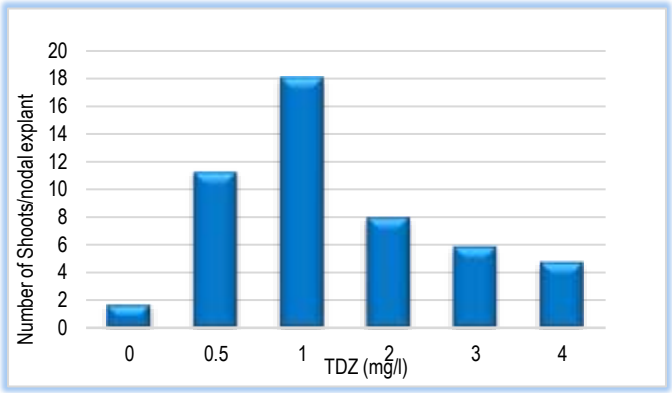
Table-4 Effect of different composition of hardening medium on survivability of Plantlets of *O. mungos.*

Hardening medium composition	No of plantlets under hardening	No of plantlets survived	Percentage of survivability (%)
Red soil	25	25	100





Graph-1 Effect of different concentrations of growth regulators (BA and TDZ) on shoot multiplication and shoot number after 8 weeks of inoculation of nodal explants *O. mungos.*



Graph-2 Effect of different concentrations of growth regulator (GA_3) on Shoot elongation after four weeks of *O. mungos* shoots.

Conclusion

Efficient protocol for micro propagation of *O. mungos* from nodal explants has been developed. The results suggested that PGRs at a combination of 2.0 mg/l BA and TDZ 1mg/l was important for inducing shoot proliferation. This finding sets up an important resource base for using multiple shoots for a variety of experiments. The in vitro plants after they were acclimatized to ex vitro conditions, the micropropagated plants eventually displayed similar leaf and flower morphology. Hence, the protocol 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.

Application of research: This research have made an attempt to develop regeneration protocol and conservation of endangered medicinal plant

Research Category: Medicinal Plant

Abbreviations: N^6-benzyladenine (BA) and Thidiazuron (TDZ), Gibberellic acid (GA_3, Indole-3-butyric acid (IBA)

Acknowledgement / Funding: Author thankful to University of Agricultural Sciences, GKVK, Bengaluru, 560 065, India.

***Research Guide or Chairperson of research**: one name
University: University of Agricultural Sciences, GKVK, Bengaluru, 560 065, India
Research project name or number: PhD Thesis

Author Contributions: All author are equally contributed
Author statement: All authors read, reviewed, agree and approved the final manuscript

Conflict of Interest: The authors declare that there is no conflict of interest regarding the publication of this research paper.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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