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Original article

Elicitors act as a signal transducer in the enhancement of camptothecine production from *in vitro* cultures of *Ophiorrhiza mungos* L.

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Abstract

Ophiorrhiza mungos L. is a member of Rubiaceae family, can be a gold standard for the *in vitro* production of high-value anticancer compound, camptothecine (CPT) to meet the increasing market demand for medicine. The axillary bud and leaf explants were inoculated on Murashige and Skoog (MS) medium, fortified with different concentrations and combinations of plant growth regulators (PGRs) like N^6-benzyladenine (BA), linetin (Kn) and indole-3 acetic acid (IAA). The best morphogenic response was observed on MS medium fortified with 8.87 µM l^{-1}BA, 4.65 µM l^{-1}Kn and 2.69 µM l^{-1}IAA, which exhibited the hundred percent regeneration frequency and maximum number of shoots from nodal and leaf explants (63.56 ± 0.28 and 45.03 ± 0.38, respectively) within four weeks. Fortification of 0.87 µM l^{-1} of gibberellic acid (GA_3) along with 4.44 µM l^{-1} BA enhanced shoots elongation by 7.06 ± 0.19 in 100% of shoot cluster cultures within four weeks. A high percent frequency of rooting (75%) was achieved within 15 days of shoot implantation on half strength MS medium fortified with 4.9 µM l^{-1}, 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 h photoperiod. The content of CPT (0.23% w/w) was significantly increased in regenerated plants treated with elicitors compare to the naturally grown plants. The present results evidently showed comparable chemical profile, suggesting that *in vitro* regenerated plants could be an alternative approach to obtain CPT. These *in vitro* regenerated flowering plants of *O. mungos* will be used by floral dip method for over expression of key genes involved in regulating terpenoidindole alkaloid (CPT) biosynthesis.

Key words: *Ophiorrhiza mungos* L., camptothecine, HPLC, *in vitro* propagation, *ex vitro* flowering

1. Introduction

Camptothecine (CPT) is one of the most propitious anticancer compounds of the 21st century; widely used against ovarian, pancreatic and colorectal cancer (Vlad *et al.*, 2000; Polu *et al.*, 2015; Das *et al.*, 2015; Raisuddin *et al.*, 2018). CPT specifically inactivates topoisomerase-I leading to cell death, thus acting as antineoplastic agent (Wright *et al.*, 2015). The compound was initially identified and isolated from the Chinese tree, *Camptotheca acuminata*. Later in 1962, the structure of the compound was elucidated (Wall *et al.*, 1966). The worldwide medical demand of topotecan and irinotecan (derivatives of CPT) is 3 billion US dollars per year, making CPT the third most promising alkaloid of the twenty-first century. A number of studies have indicated its therapeutic potential against various cancers, including ovarian and colon cancers (Cragg and Newman, 2005). 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). Beside their antitumor activity, CPT derivatives have also been found to show good activity against viruses such as the human immunodeficiency virus (HIV) (Priel *et al.*, 1991). More derivatives of CPT are now in clinical studies, such as 9-nitro camptothecine, 9-amino camptothecine and rubitecan (Sankar and Lieberei, 2011) 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 *et al.*, 2009). The annual production of CPT throughout the world is only 600 kg, while approximately 3000 kg of CPT is required in the international markets (Kai *et al.*, 2014). 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) or from the stem bark of *N. nimmoniana* (from India) (Uma *et al.*, 2008). Apart from *C. acuminata*, many other plant species are also reported to contain CPT. *Nothapodytes foetida* (Roja and Heble, 1994), *Merrilliodendron megacarpum* (Arisawa *et al.*, 1981), *Pyrenacantha klaineana* (Zhou *et al.*, 2000) and many species of

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Ophiorrhiza (Shahanaz Begum *et al*., 2007) are other major sources of CPT. Patwardhan (2006) has reported that the concentration of CPT in tree species increases with increase in the age of tree until a point and later, it remains proportionate to the thickness of bark. The whole plant of camptotheca contains CPT ranges from 0.2 to almost 5.0 mg/g (dw), depending on the tissue analysed (López-Meyer *et al*., 1994), while that of Nothapodytes is reported to be 0.3 mg/g (dw) (Ramesha *et al*., 2008). The tree needs 8-10 years to reach the mature stage from seedling and start flowering. It needs 1000-1500 tons of wood chip every year to meet the market demand of CPT (Watase *et al*., 2004). To meet the market demand of CPT due to the increase in cancer cases around the world, and to conserve the populations of these plants, an economical and alternative sustainable system is necessary. Plant cell tissue and organ culture have been used as an efficient method for commercial production of high-value plant secondary metabolites when natural resources are limited (Vanisree *et al*., 2004). 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). *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 (Watase *et al.*, 2004). Luteolin-7-O-Glucoside (LUT7G), isolated from leaves of *O. mungos*, can be a potent anticancer drug for colon carcinogenesis (Arul *et al.*,2011). At this scenario, the present study aims enhanced production of CPT from the multiple shoot cultures of *O. mungos*. Multiple shoots culture of the species is successfully established using mature leaf and nodal explants from the *in vitro* grown plants and CPT accumulation was studied. To enhance the CPT production, cultures were treated with different concentrations of various elicitors, namely; methyl jasmonate and salicylic acid. These *in vitro* regenerated flowering plants of *O. mungos* will be used by floral dip method for over expression of key genes involved in regulating terpenoidindole alkaloid (CPT) biosynthesis.

2. Materials and Methods

2.1 Plant material and mass multiplication

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. Seeds were taken into autoclaved eppendorf tube (2 ml) treated with 2 $mgl^{-1}GA_3$ to overcome the dormancy and then washed 2-3 times with water, followed by Tween 80 (1%, v/v) for 5 min and surface-sterilized with 4% sodium hypochlorite solution. The seeds were washed thoroughly to remove every trace of the sterilant, with sterile distilled water at least 4 times (5 min each) and then inoculated onto MS (Murashige and Skoog, 1962) medium for *in vitro* germination. Leaf and nodal explants from 28-day-old *in vitro* seedlings were cultured on MS medium containing different concentrations and combinations of plant growth regulators (PGRs) for multiple shoot proliferation. The precise concentrations and combinations are outlined in the Table 1. In all these experiments, the chemicals and PGRs used were of analytical grade (Himedia, India). All media were adjusted to pH 5.8 with 0.1 N NaOH and/or 0.1 N HCl before gelling with 8 $g\ l^{-1}$ agar and autoclaved at 121°C, 15 lbs pressure for 15 min. All the cultures were incubated under light intensity of 75-100 $\mu mol\ m^{-2}\ s^{-1}$ provided by cool white fluorescent lamp for a period of 16 h at 25 ± 2 ^{0}C and 70-80 % relative humidity. *In vitro* regenerated plantlets with 2-3 nodes each were excised and transferred to half of different concentration/combination (S) was used as a secondary medium for elongation of the shoots. 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. The percentage of regeneration frequency was calculated as the total number of initial explants, which gave response to hormonal treatment per total number of explants multiplied by 100. Half of the cultured shoots were multiplied and maintained for further elicitation experiment and phytochemical analysis. The other half of shoots rooted and transferred to *ex vitro* conditions.

2.2 Effects of elicitors on CPT accumulation

2.2.1 Effects of methyl jasmonate (MeJA) and salicylic acid (SA)

Four month old *in vitro* grown *O. mungos* plants were elicited with methyl jasmonate (MeJA) and salicylic acid (SA) for CPT accumulation. Filter-sterilized stock single shoots were inoculated into MS medium supplemented with 2 mgl^{-1} BA and different concentrations of MeJA (50 µM, 100 µM and 150 µM) and SA (50 µM, 100 µM and 150 µM) and allowed to grow in elicitor media for four weeks. After four weeks, the plants were harvested and CPT accumulation was estimated.

2.2.2 *In vitro* rooting and acclimatization

In vitro regenerated shootlets (longer than 3.0 cm having 4-5 leaves) were cultured in half strength MS medium containing various auxins like NAA, IAA and IBA for root induction as revealed in the Table 3. Media devoid of growth regulators were used as control. All the rooted shoots were separated after 6 weeks and transferred to plastic cups containing sterile soilrite and covered with transparent polyethylene bags to maintain humidity. After 25 days, the hardened plants were transplanted to earthen pots filled with potting mixture 2:1:1 (garden soil, sand and farmyard manure), allowed to grow in greenhouse and percentage of survival was recorded after three to four week's of transplantation. These plants were irrigated with tap water every second day. These plantlets were maintained in the greenhouse (25 ± 2°C under natural photoperiod conditions) for acclimatization *ex vitro*.

2.3 Extraction of camptothecine

Fresh samples of *in vitro* regenerated and natural plants were dried to constant moisture content and ground to a fine powder using mortar and pestle. Each of the sample (approximately 100 mg of powder) was extracted in 61 % ethanol (3 ml) at 45°C for 3 h in a shaking water bath. The extract, after cooling to room temperature was centrifuged at 10,000 rpm for 10 min at 10°C. The supernatant was passed through a 0.2 µm filters (Tarson, India) and subjected to HPLC analysis.

Table 1: Effects of various concentrations of BA, kn individually and combination with IAA on shoot proliferation in mature leaf and nodal explants of *O. mungos* cultured on MS medium with 3% sucrose

Plant growth regulators at different concentrations (mg l^{-1})			Regeneration frequency (%)		Mean shoot number per explants ± SE	
BA	**Kn**	**IAA**	**Node**	**Leaf**	**Node**	**Leaf**
0.5	--	--	100	75	22.46 ± 0.79^{m}	12.10 ± 0.55^{m}
1.0	--	--	100	78	31.56 ± 1.06^{gh}	22.50 ± 0.78^{hi}
2.0	--	--	100	90	45.86 ± 1.51^{d}	39.33 ± 1.20^{d}
3.0	--	--	100	85	39.80 ± 1.28^{e}	30.60 ± 0.96^{f}
5.0			100	80	31.10 ± 0.92^{hi}	22.9 ± 0.66^{h}
--	0.5	--	95	70	24.50 ± 0.87^{kl}	22.46 ± 0.78^{ij}
--	1.0	--	100	80	35.16 ± 1.18^{f}	32.20 ± 1.07^{e}
--	2.0	--	95	72	31.76 ± 0.97^{g}	26.00 ± 0.86^{g}
--	3.0	--	90	70	25.63 ± 0.80^{j}	22.16 ± 0.69^{ijk}
--	5.0	--	90	70	24.90 ± 0.74^{k}	17.43 ± 0.53^{l}
2.0	1.0	0.1	100	90	52.73 ± 0.33^{c}	40.93 ± 0.36^{bc}
2.0	1.0	0.5	100	90	63.56 ± 0.28^{a}	45.03 ± 0.38^{a}
2.0	1.0	1.0	100	90	59.90 ± 0.42^{b}	41.13 ± 0.27^{b}

Mean ± SE, n = 30. Means followed by the same letter are not significantly different by the DMR test at 0.05% probability level. Regeneration frequency was calculated by (number of explants sprouted / total number of explants inoculated) × 100

Table 2: Effect of various combination of benzyl aminopurine (BA) and gibberellic acid (GA_3) on length of shoots of *O. mungos*. Data were collected after three months of culture in secondary medium

Plant growth regulators (mg l^{-1})		Average length of shoots (cm)
GA_3	BA	
0.1	-	2.86 ± 0.19^{f}
0.2	-	3.30 ± 0.18^{ef}
0.3	-	5.23 ± 0.19^{c}
0.4	-	4.53 ± 0.20^{de}
0.5	-	4.23 ± 0.17^{ef}
0.3	0.5	6.26 ± 0.17^{b}
0.3	1.0	8.05 ± 0.19^{a}
0.3	2.0	5.83 ± 0.21^{b}
0.3	3.0	4.90 ± 0.16^{cd}
0.3	5.0	4.66 ± 0.18^{de}

Means ± SE followed by the same letter are not significantly different at *p=0.05* according to the Duncan's Multiple Range Test (DMRT)

Table 3: Rooting response *in vitro* regenerated shoots of *O. mungos* on half strength MS medium containing IBA, NAA and IAA in various concentrations with 1% sucrose after 6 weeks of culture

Plant growth regulators (mg l^{-1})			Percentage of response	Mean number of roots per explants ± SE
IBA	NAA	IAA		
0.5	-	-	62	22.56 ± 0.81^{e}
1.0	-	-	75	52.20 ± 1.74^{a}
2.0	-	-	60	15.10 ± 0.52^{j}
3.0	-	-	66	13.73 ± 0.46^{kl}
-	0.5	-	45	17.93 ± 0.64^{h}
-	1.0	-	65	32.43 ± 1.08^{b}
-	2.0	-	58	32.50 ± 1.08^{bc}
-	3.0	-	55	18.86 ± 0.81^{g}
-	-	0.5	2.85	13.86 ± 0.51^{k}
-	-	1.0	5.71	23.60 ± 0.78^{d}
-	-	2.0	11.4	20.50 ± 0.73^{f}
-	-	3.0	17.11	17.76 ± 0.58^{hi}

Mean ± SE, n=30. Means followed by the same letter are not significantly different by the DMR test at 0.05% probability level.

2.4 HPLC-DAD analysis

The extracted samples (20 µl) was analyzed by reverse phase HPLC (Shimadzu, Japan) using RPC-C18 column (250 mm × 4.6 mm, 5 µm size: Phenomenex) with UV absorbance at 254 nm. The separation was performed at a flow rate of 1.5 ml/min with mobile

phase-pump A: 25% acetonitrile and pump B: 75% water + 0.1% trifluro-acetic acid (TFA) in an isocratic mode. The CPT standard was prepared using DMSO and methanol in 1:3 (v/v) ratio, respectively at a concentration of 1.0 mg/ml. The working solution was prepared in the concentration range of 0.1-0.5 mg/ml from stock solution and 20 µl of the solution was injected into the HPLC system for preparation of standard curve.

2.5 Statistical analysis

The data were analyzed by analysis of variance (ANOVA) using SPSS version 19.0 Windows (Chicago, USA) 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) ($p = 0.05$) (Gomez and Gomez, 1976).

3. Result and Discussion

3.1 Establishment of multiple shoots by PGRs

High quality propagation materials of *O. mungos*, having camptothecine, 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. Species belong to the genus *Ophiorrhiza* display endogenous, morphological seed dormancy; the seeds become dormant soon after harvest. In this study, 100% of seed germination of *O. mungos* was achieved when seeds were treated with gibberellic acid (2 mgl^{-1} GA_3) for 48 h (Figure 1A). Aseptic seedlings of *O. mungos* were initially obtained from plants germinated from sterilized seeds, were main source for explants like leaf and nodes. To induce multiple shoots from nodal and leaf explants, explants were cultured on MS media containing different concentrations and combinations of PGRs like BA (0.5 - 4 mg l^{-1}); Kn (0.5 - 4 mg l^{-1}) and IAA (0.1; 0.2 and 0.3 mg l^{-1}) for multiple shoot induction (Table 1 and Figure 1). The highest regeneration frequency of nodal explants that formed shoots was 100% on the MS medium supplemented with a BA alone, followed by 95% on the medium containing Kn alone, while proximal leaf explants showed 90% regeneration frequency when inoculated on the same medium (Table 1).

The concentration of cytokinins influenced the number of shoots that were formed. In the present study, using BA alone, the number of shoots per explant increased with increase of BA concentration upto 2.0 mg l^{-1}. Further increase in BA concentration, reduce the number of shoots per explant. Aziemah *et al*. (2016) obtained similar results in *Oscimum basilicum* from shoot tip explants. Reduction of shoot number might be caused by the toxicity of higher concentrations of BA. The effect of BA on multiple shoot bud differentiation has been demonstrated in CPT producing plants (Ugraiah *et al*., 2016; Pradeep *et al*., 2015; Begum *et al*., 2007). The number of days taken to initiate shoots and number of shoots developed were recorded at regular intervals until 8 weeks. Multiple shoots initiated within a week of inoculation from the nodal ends followed by leaf explants. The maximum number of shoots was 63.56 per nodal explant, obtained on the medium fortified with 2.0 mg l^{-1} BA, 1.0 mg l^{-1}kn and 0.5 mg l^{-1} IAA (Figure 1B), followed by 45.86 shoots per explant, in the medium consisting of 2.0 mg l^{-1} BA alone. For leaf explant, the highest number of shoots per leaf explant was 45.03, obtained using same combination of medium used for nodal explants (Figure 1C) and, followed by 39.33 in the medium containing 2.0 mg l^{-1}. By contrast, the least shoots (12.10 per leaf explant) were obtained with the medium supplemented with 0.5 mg l^{-1} BA. These results recommended that culturing nodal explants on MS medium containing 2.0 mg l^{-1} BA, 1.0 mg l^{-1}Kn and 0.5 mg l^{-1} IAA was the best combination for obtaining maximum multiple shoot induction and the greater number of shoots per explant. Similar results have also been reported for *Ophiorrhiza alata* (Ya-ut *et al*., 2011); *O. prostrata* (Begum *et al*., 2007); *Leptadenia reticulata* (Sudipta *et al*., 2011); *Pogostemon cablin* (Swamy *et al*., 2014) and in *Ophiorrhiza mungos* (Pradeep *et al*., 2015). According to Roja (2008), the medium containing 4 mg l^{-1} BA and 0.5 mg l^{-1} NAA was effective in regeneration of shoot cultures in *O. rugosa* var. *decumbens*. This is conflicting to our findings that increased concentrations of cytokinins exhibited a decreased number of shoot buds coupled with callus proliferation. This may be due to faster cell division leading to profuse callus proliferation and resulting in the encumbrance of morphogenesis.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^{-1} BA alone. These results are in agreement with the results of a study of *O. alata* (Ya-*ut et al*., 2011) and *Boucerosiatruncato coronata* (Ugraiah *et al*., 2015). Mhaveer *et al*. (2014) reported that the MS basal medium supplemented with 6-BA (4.0 ppm) + IAA (0.5 ppm) was found to be best for the development of shoot in *Acorus calamus* Linn. Namdeo *et al*. (2012) consummate a maximum mean of 25 shoots per explants of *O. mungos* grown on MS medium fortified with Picloram + Thidiazoran + Gibberilicacid (1:2:1) after 60 days of incubation.

3.2 Shoot elongation

Despite the fact that MS media with BA, Kn and IAA in combination 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. Purkayastha *et al*. (2008) reported that use of BA stimulates shoot proliferation while inhibiting shoot elongation. Transferring culture to media without PGRs also did not support the shoot elongation. So, developing a suitable media can promotes both proliferation and elongation of shoot clumps. To achieve shoot elongation, individual proliferated shoots were separated from shoot clumps and transferred to shoot elongation medium containing different concentrations and combinations of PGRs (Table 2). A significant difference in elongation was observed at different concentrations and combinations of PGRs wherein a combination of BA and GA_3 was found to be the best. Highest elongation (8.05 cm) was observed on the medium containing 0.3 mg $l^{-1}GA_3$ and 1.0 mg l^{-1}BA (Figure 1D). In the absence of GA_3, shoot growth was reduced, as GA_3 has vital role in shoot elongation have been reported in several plants species *Nothapodytesnim moniana* (Ugraiah *et al*., 2016; Pradeep *et al*., 2015; Paul *et al*., 2010; Deepak *et al*., 2015). The explants continuously produced shoots during successive subcultures on MS medium containing 2.0 mg l^{-1} BA, 1.0 mg l^{-1}Kn and 0.5 mg l^{-1} IAA.

3.3 *In vitro* rooting, acclimatization and *ex vitro* flowering

In vitro grown shootlets were separated and transferred to rooting media (Table 3). All the treatments induced rooting, where root induction rate varied significantly between the treatments after 6 weeks of culture (Table 3). The IBA (1.0 mg l^{-1}) was most effective treatment and produced significantly more roots per shootlet (52.20

± 1.74) with 75 percentage of response after 6 weeks of culture (Figure 1E). The results are in agreement with the findings in *Ophiorrhiza japonica* where IBA was found to be better than NAA to induce the formation of maximum number of roots (Kai *et al*., 2008) and in *Alternanthera sessilis* (Singh *et al*., 2009). Similar results were shown in *Cosciniumfene stratum* (Gaertn.) Colebr in inducing rooting in callus (Kuntal Das *et al*., 2018). The effectiveness of IBA in inducing rooting has also been reported in several other medicinal plants (Purohit and Dava, 1996; Namdeo *et al*., 2012; Sayeed *et al*., 2015).

The crucial step of micropropagation is acclimatization of *in vitro* obtained plantlets. *In vitro* regenerated plants of *O. mungos* with no morphological abnormalities were transplanted into pots with survival rate of 100% (Figure 1F). The micropropogated plantlets with well-developed roots were successfully acclimatized to *ex-vitro* conditions; approximately 90% of plantlets survived the transition from tissue culture to the experimental plot. The micropropagated plants were morphologically uniform and grew well in the greenhouse (Figure 1G). In comparison, the survival rate of *O. mungos* plantlets was 82 % (Senthilkumar *et al*., 2009). After about 12 weeks of acclimatization, normal flowers developed from the *ex vitro* plants (Figure 1G). These flowers were similar in morphology to the *in vivo* flowers in *O. mungos*. The *ex vitro* flowers exhibited complete floral structures with shapes, sizes and colours similar to the *in vivo* flowers.

3.4 Seed germination test

The seeds obtained from *ex vitro* and *in vivo* plants. Each *ex vitro* and *in vivo* flower produce plenty (∞) per spike and were visually similar. The seeds formed *ex vitro* and *in vivo* showed equivalent (100%) rates of germination (Figure 1H).

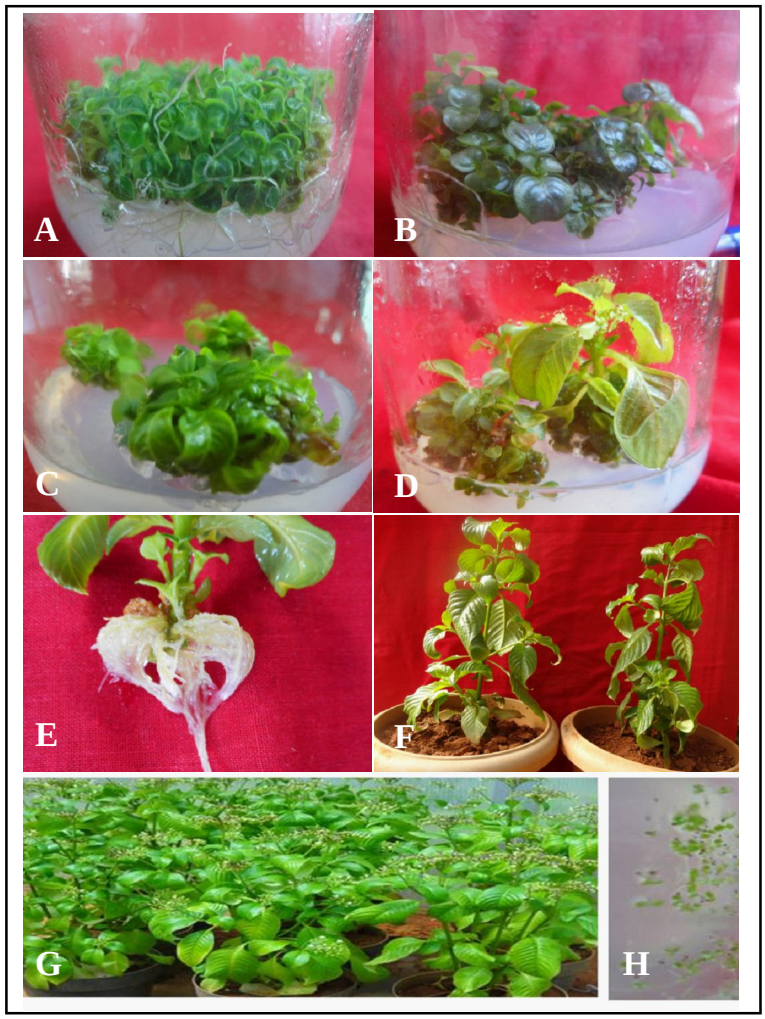

Figure 1: Mass multiplication and *ex vitro* flowering of *O. mungos*
A. Seed germination after 3 weeks on media
B. Emerging of multiple shoots from single node after 6 weeks of inoculation
C. and D emerging of multiple shoots from leaf after 8 weeks of inoculation
D. *In vitro* rooting of plantlets after 6 weeks of inoculation on MS medium with 1 mg/l IBA
E. Acclimatized plants established 6 weeks after transfer to soil in the field
F. *In vitro* established plants with flowers in full bloom developed after 8 week of acclimatization in the field soil
G. Germinated seeds from an *ex vitro* plants (micropropagated)

3.5 Effects of elicitors on CPT production

Elicitation is an effective strategy to enhance the secondary metabolite production in low yielding plants. Elicitors are used to modify cellular metabolism in order to enhance the secondary metabolite production. Treatment with biotic or abiotic elicitors has been useful strategy to enhance secondary metabolite production in plant tissue and cell cultures. The induction mechanism of elicitors is generally regarded as inducing the expression of defence-related genes and activating defense-related secondary metabolite pathways (Qian *et al*., 2006). One of the abiding rationale of the choice of these elicitors has been their ability to transduce sub-cellular signals, often mimicking that set off by various stressors, both abiotic and biotic (Zhang *et al.*, 2000). The response of a particular elicitor may vary from plant-to-plant and between different cell lines; therefore, it becomes crucial to determine suitable concentrations of elicitors for product optimization (Namedo, 2007). In this context, we studied the effort of MeJA and SA elicitors on camptothecine accumulation in *O. mungos* shoot cultures. However, there are no studies on the investigation of effects of these elicitors on camptothecine production in *in vitro* culture of *O. mungos*.

Jasmonic acid and methyl jasmonate are produced in plants in response to many biotic and abiotic stresses. These signal the stress to plants and elicit secondary responses in plants. Thus, MeJA has been used to elicit the production of secondary metabolites in plants. In fact, it has been realized that MeJA treatment to plant cell cultures is an effective strategy to improve the production of secondary metabolites in *in vitro* culture conditions (Vasconsuelo and Boland, 2007). Earlier reports have shown noradrenalin content increased by 8 folds in *Portulaca oleracea* MeJA treated hairy roots (Pirian and Piri, 2012). Similarly, recent studies shown that MeJA treated hairy root cultures has increased rhinacanthin content by 1.7 folds (Meena *et al.*,2014); increased saponin content by 3.3 folds in *Calendula officinalis* (Ghanati and Bakhtiarian, 2014). *In vitro* grown plants of *O. mungos* accumulated significantly higher level of CPT when treated with MeJA compared to untreated plants (Figures 4 and 5 B). *In vitro* plants treated with 150 µM MeJA produced highest CPT (0.23 %) compared to untreated plants (0.11 %), followed by 100 µM MeJA (0.21 %) and 50 µM MeJA (0.18 %). Exogenous application of SA has been shown to elicit a variety of plant responses mimicking those due to disease and pest infestation and also due to abiotic stressors. To investigate the effect of SA on the production of camptothecine, we employed the same method used for MeJA. In this context, several reports have shown the elicitation of a number of plant secondary metabolites subsequent to exogenous application of SA even without the imposition of stress. Meena *et al*. (2014) showed that rhinacanthin content increased by 3 folds in SA treated hairy root cultures of *Rhinacanthus nasutus*. Recently, Bhuvaneswari *et al*. (2015) reported a 4.9-fold increase in gymnemic acid in *Gymnema sylvestre* suspension cultures treated with SA. However, contrary to these studies, in the present study, SA treated plants failed to show significant increase in CPT content compared to untreated plants (Figures 4 and 5 A).

3.6 Quantification of camptothecine in *in vitro* and *in vivo* plants

Data on quantitative analysis of camptothecine in *in vitro* and *in vivo* plants are summarized in Figure 2. Variation was observed in the camptothecine content among various plant materials tested. The amount of CPT content in *in vitro* leaves 0.08 % and in stems 0.14 % was more than the *in vivo* grown leaves 0.04 % and stems 0.06 % (Figure 2). These results support the recent findings of Namdeo *et al*. (2012) who showed a higher accumulation of CPT in *in vitro* plants of *O. mungos* compared to that of naturally grown plants. Lijie *et al*. (2015) reported on enhancement of CPT production in *O. pumila* employing a metabolic engineering strategy. Similarly, higher production of CPT and 9-MCPT has been reported in *in vitro* plants of *N. nimmoniana* (Karwasara and Dixit, 2013; Devanand *et al.*,2015) and *O. rugosa* var. *decumbent* (Roja and Bhavani, 2014). It is likely that the added growth regulators in the *in vitro* grown plants might have a role in inducing the CPT production in the *in vitro* regenerated plants.

The HPLC profile of camptothecine in different parts of *in vitro* regenerated and wild plants are shown in Figure 3. Traditionally, camptothecine has been extracted from stem, roots, stem/root bark and fruits and content of the compound in the natural plants may vary depending on the population, growth conditions, place and time of collection (Padmanabha *et al*., 2006). Meager amount of camptothecine accumulation was reported in callus, somatic embryos and seedling explants of *Nothapodytesnim moniana* (Roja and Heble, 1994; Dandin and Murthy, 2012). Significantly, accumulation of camptothecine was observed in this study when using PGRs and elicitors combination. A combination of 2.0 mg l^{-1} BA, 1.0 mg l^{-1}Kn, 0.5 mg l^{-1} IAA and 150 µM MeJA yielded the highest percentage (0.23 %) (Figure 4 B) in tissue culture of *O. mungos*. Similar results were reported that elicitation of YE and $AgNO_3$ in cell suspension cultures of *O. mungos* (Deepthi and Sasheeshkumar, 2016). However, shoots obtained from cultures in the present study showed significantly higher CPT than *in vivo* plants. These results suggest that the shoot biomass produced through *in vitro* culture could have implications for a sustainable production of CPT, without dependence on the natural populations of *O.mungos*.

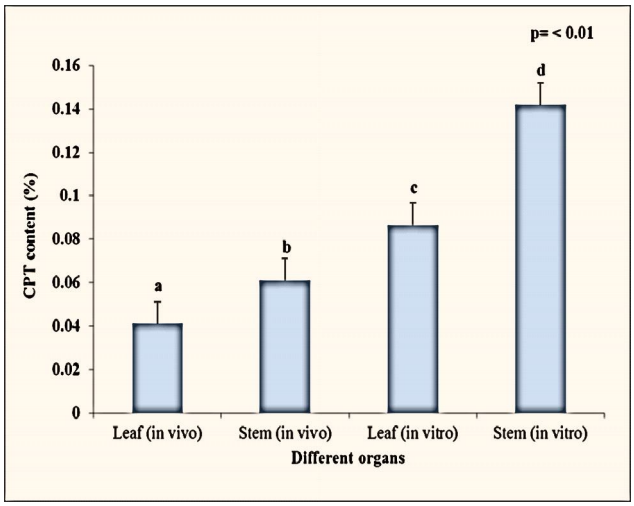

Figure 2: Camptothecine (CPT) content in different parts of *in vitro* regenerated and *in vivo* plants of *O. mungos* (mean of three experiments).

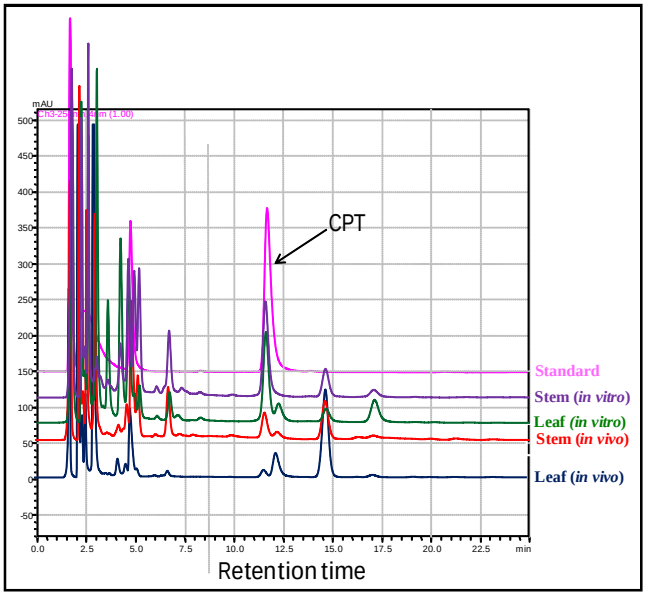

Figure 3: HPLC profile showing CPT in different parts of *in vitro* regenerated and *in vivo* plants of *O. mungos*.

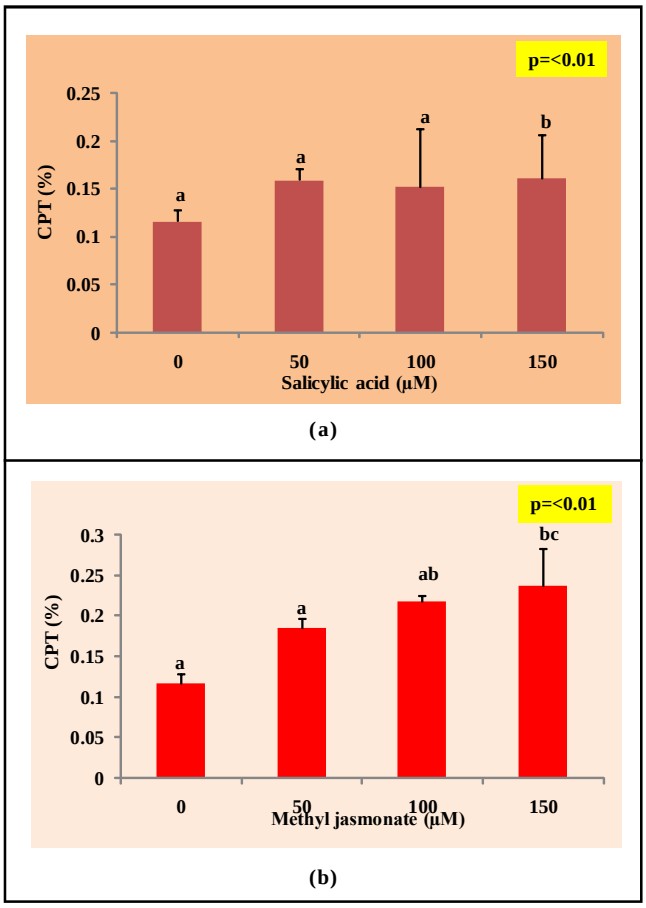

Figure 4: Effect of elicitors on CPT production: (a) Effect of SA at different concentrations (0, 50, 100 and 150 µM) on CPT production, (b) Effect of MeJA at different concentrations (0, 50, 100 and 150 µM) on CPT production.

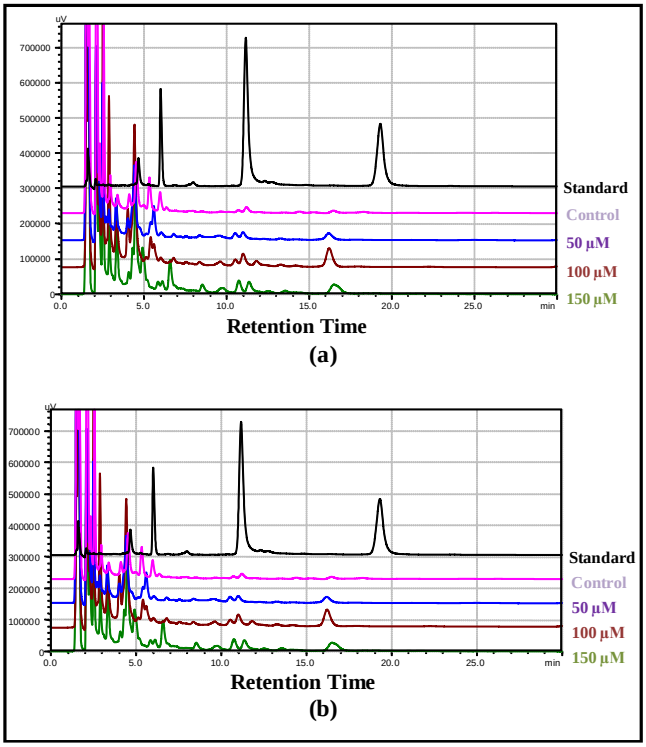

Figure 5: HPLC chromatogram: (a) Effect of SA at different concentrations on CPT production, (b) Effect of MeJA at different concentrations on CPT production.

4. Conclusion

In conclusion, efficient protocol for micropropagation and *ex vitro* flowering of *O. mungos* from nodal and leaf explants were developed. The results suggested that PGRs at a combination of 2.0 mg l^{-1} BA, 1.0 mg l^{-1}Kn and 0.5 mg l^{-1} IAA was important for inducing shoot proliferation. This finding sets up an important resource base for using multiple shoots for a variety of experiments. *In vitro* regenerated plants accumulated significantly higher CPT in their leaves and stem compared to *in vivo* grown plants. Though, the exact reasons are not known. It is likely that the use of growth regulators in the *in vitro* grown plants could have induced the production of CPT. *In vitro* regenerated plants of *O. mungos* were treated with elicitors such as methyl jasmonate and salicylic acid. Among them, significant elicitation was obtained in plants treated with methyl jasmonate. The *in vitro* plants after they were acclimatized to *ex vitro* conditions, the micropropagated plants eventually displayed similar leaf and flower morphology, seed germinability and high camptothecine content as the *in vivo* mother plants.

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Conflict of interest

We declare that we have no conflict of interest.

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