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ORIGINAL ARTICLE

Tissue culture studies and estimation of camptothecin from *Ophiorrhiza prostrata* D. Don

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Abstract Camptothecin is an important molecule in the synthesis of some of major anticancer agents such as irinotecan and topotecan. Traditional sources of camptothecin are mainly woody plants, some of which are being overexploited to a level that threatens the existence of the respective species. Alternate sources are being identified. In the current study, a tissue culture protocol was developed for the mass multiplication of *Ophiorrhiza prostrata*, an herbaceous camptothecin-containing plant, using seed explants, in order to evaluate its potential as an alternate source of commercial camptothecin. The germination of seeds was standardized on half-strength solid basal MS medium (94.8 ± 2.65% success rate). Seedlings subcultured on half-strength MS agar medium supplemented with 1.5 mg l^{-1} benzylaminopurine (BAP) produced the most shoots (28.0 ± 2.58) from the nodal region of a single seedling. Mass multiplication of in vitro shoots was achieved by subculturing a single shoot as well as shoot clumps. Subculturing shoot clumps containing a minimum of 4–6 shoots yielded fewer shoots per node (32.2 ± 2.03) than when single shoots were subcultured (25.0 ± 2.0). Shoot elongation was achieved on half-strength MS basal medium in a period of 2–3 weeks. Various concentrations of auxins were tested for rooting, and 1 mg l^{-1} NAA gave the highest number of roots (44.0 ± 4.13). Rooted shoots were successfully established under field conditions. High-performance thin-layer chromatography (HPTLC) was employed to measure the camptothecin levels in wild-grown plants (1.46 ± 0.02 µg g^{-1} dry wt), in-vitro-regenerated plants (1.10 ± 0.02 µg g^{-1} dry wt), and in-vitro-derived hardened plants (1.83 ± 0.02 µg g^{-1} dry wt). The results of this study indicate that in vitro methods could be a potential path to the rapid expansion of plant biomass, which could serve as a potential source of commercial camptothecin.

Keywords *Ophiorrhiza prostrata* D. Don · Camptothecin · Tissue culture

Introduction

Camptothecin is an important molecule in the commercial synthesis of some major anticancer agents such as topotecan and irinotecan (Pommier et al. 1995). The traditional sources of camptothecin are typically woody plants such as *Camptotheca accumata* (Wall et al. 1966) and *Nothapodytes foetida* (Arisawa et al. 1981), but these are slow-growing tree species. Due to the increasing demand for camptothecin, these plants are being overexploited, threatening their survival. While alternate sources of camptothecin exist, some of which are herbaceous plants with rapid growth characteristics, their yield per kilogram of body mass is much lower than for traditional sources, hindering their commercial viability. However, biotechnological methods, including in vitro mass multiplication, potentially offer the means to rapidly generate the alternate source plants, implying that commercial-level cultivation could be attempted in order to produce more feedstock, which could compensate for the lower yield per kilogram of plant mass. The high demand for camptothecin and other

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active pharmaceutical ingredients, coupled with the over-exploitation of source plants, has led to the development of various in vitro culture protocols and the production of camptothecin using *Ophiorrhiza* species (Van-Hengel et al. 1994; Wiedenfeld et al. 1997; Lorence and Nessler 2004). *Ophiorrhiza* is an Indo-Malaysian genus that comprises about 150 species distributed across tropical and subtropical Asia, Australia, New Guinea, and the Pacific Islands and belongs to the dicotyledonous family Rubiaceae (Mabberly 2008). *Ophiorrhiza* species are either herbaceous or understorey shrubs that are readily amenable to in vitro tissue culture. *Ophiorrhiza prostrata* D. Don produces CPT mainly in its roots and shoots, but has been largely unexploited for the bioproduction of camptothecin. In vitro clonal propagation of *Ophiorrhiza pumila* (Aimi et al. 1989), *O. mungos* (Tafur et al. 1976; Roja 2006), *O. alata* (Pornwilai et al. 2011), *O. liukiuensis*, *O kuroiiwai* (Kitajima et al. 2005), *O. rugosa* var. *decumbens* (Vineesh et al. 2007; Roja 2008), *O. rugosa* var. *prostrata* (Gharpure et al. 2010), *O. japonica* (Kai et al. 2008), and *O. filistipula* (Arbain et al. 1993) has been reported previously. A few attempts have been made to produce CPT via *O. prostrata*, but its low yield limits this approach (Beegum et al. 2007; Martin et al. 2008). The present work represents the first report of the in vitro germination of seeds in order to establish a tissue culture protocol for the mass multiplication of *O. prostrata*, together with the quantification of the camptothecin produced by this plant.

Materials and methods

Plant materials

Flowering plants of *O. prostrata* (Rubiaceae) were collected during August–November from the Agastyamala Hills of Western Ghats, Thiruvananthapuram, Kerala, India, and a voucher specimen was deposited in the institute herbarium under the number TBGT 69942.

Culture initiation

Mature fruits (capsules) 2–2.25 × 4–4.5 mm in size were collected from the plants (Fig. 1A) and maintained in the shade in a net house. The shade-hardened fruits were then washed for 10–15 min with 0.5% (v/v) commercial detergent (Teepol), followed by thorough washing under running tap water. Surface sterilization of the fruits was carried out in a laminar airflow hood. In order to standardize the surface sterilization process, the capsules were initially treated with different concentrations of sodium hypochlorite solution (0.5–1% v/v) for 7–10 min and mercuric chloride ($HgCl_2$) (0.05–0.1% w/v) for 3–7 min individually. The capsules were then washed with sterile distilled water 3–4 times. The fruits were pressed with sterile forceps to release the minute seeds [0.3 × (0.3μ–0.35 mm)]. Thereafter, the seeds were again washed in sterile distilled water 2–3 times before being transferred to various growth media, including solid and liquid media with full-strength, half-strength, and quarter-strength MS salts to optimize seed germination. In each case, 3% sucrose (w/v) was added to the medium. The seeds inoculated on liquid medium were kept on a Gyrotory shaker (New Brunswick, USA) at 60 rpm and data were recorded at different times over the course of 3–5 weeks. The pH of the medium was adjusted to 5.8 before agar was added. The mixture was then autoclaved at 121 °C and a pressure of 1.5 kg cm^{-2} for 18 min. All cultures were incubated at 26 ± 2 °C under a 16/8 h day/night photoperiod imposed using white fluorescent tube lights.

Shoot multiplication

After 2 weeks of seed germination, seedlings of size 2.0–2.5 cm were carefully removed from solid and liquid media and placed in sterile petri dishes. Their roots were removed. Shoots with a single node and a shoot apex of length 1.0–1.5 cm were separated from the seedlings and inoculated onto half-strength solid MS medium containing 3% sucrose and supplemented with various concentrations and combinations of 6-benzylaminopurine (BAP) (0.25–2.5 mg l^{-1}), kinetin (Kn) (0.5–2.5 mg l^{-1}), indole-3-acetic acid (IAA) (0.1–1.5 mg l^{-1}), and naphthaleneacetic acid (NAA) (0.5–2.5 mg l^{-1}). The number of shoots produced per shoot and their corresponding lengths were recorded periodically.

After 5 weeks, multiple shoots formed on the shoot segment on medium supplemented with 1.5 mg l^{-1} BAP. Shoots 1–2 cm long were separated and transferred individually for further multiplication. Multiplication of shoots was achieved through repeated subculturing of shoots 2.0–2.5 cm long on medium of the same composition. Each subculture lasted for 3 weeks. Three subcultures were performed in total, and the influence of repeated subculturing on shoot multiplication was analyzed.

Shoot elongation and rooting

For shoot elongation, shoots (1.5–2.5 cm long) obtained from seedlings were dissected out, either individually or in clusters, and inoculated onto half-strength solid MS medium devoid of hormones.

For root induction, elongated shoots (3.0–5.0 cm) obtained after 3 weeks were dissected out, either individually or in clusters, and transferred onto half-strength solid MS medium containing different concentrations of IAA (0.5–2.0 mg l^{-1}), indole 3-butyric acid (IBA) (0.5–2.0 mg l^{-1}), and naphthaleneacetic acid (0.5–2.0 mg l^{-1}).

Fig. 1 In vitro seed germination and mass multiplication of *O prostrata* D. Don: **A** seeds of *O. prostrata,* **B** formation of root, **C** formation of root and shoot, **D** and **E** complete seedlings, **F** germination of seeds in solid media, **G** germination of seeds in liquid media, **H** multiple shoot in solid media, **I** multiple shoot in liquid media, **J** elongation of shoot, **K** rooting of shoot, **L** rooted shoot ready for acclimatisation, **M** in vitro plants in green house and **N** acclimatised plants in field

Deflasking and hardening

After 3 weeks of shoot rooting, the plantlets were carefully removed from the culture vessel and washed repeatedly under running tap water to remove the remnants of the medium. The thoroughly washed plantlets were then planted in poly bags (6 × 4 cm) filled with a mixture of sand and soil (1:1) and kept in a shade net house (50% shade), where they were watered once a day.

Estimation of camptothecin

Whole-plant material of *O. prostrata* was harvested from the shade net house and washed under running water to remove soil particles and then dried in complete shade. Shoots derived from in vitro cultures were weaned away from the culture vessels and washed thoroughly in distilled water before drying for camptothecin analysis. Similarly, in-vitro-derived plantlets established in polythene bags were uprooted after 1 month and dried in the shade for

camptothecin analysis. Shoots and 1-month-old in-vitro-derived plantlets were harvested and dried in the shade. All dried samples were powdered separately. Ten grams of dried powder were extracted with methanol, and camptothecin was estimated using HPTLC.

A stock solution of camptothecin (100 μg ml^{-1}) was prepared by transferring 10 mg standard camptothecin to a 100-ml standard volumetric flask, and this was further diluted to a volume of 100 ml with methanol. Thereafter, 0.01 ml of this stock solution were transferred to a 10-ml standard volumetric flask, and the volume was made up with methanol to furnish a working standard solution of 0.01 μg ml^{-1} camptothecin. Different volumes of the stock solution (2, 3, 4, 5, 6, 7, 8, 9, and 10 μl) were spotted onto the TLC plate to obtain concentrations of 0.0002, 0.0003, 0.0004, 0.0005, 0.0006, 0.0007, 0.0008, 0.0009, and 0.0010 μg $spot^{-1}$ of camptothecin, respectively. A calibration graph was obtained by plotting peak areas against corresponding concentrations. The resulting calculated correlation coefficient (r) was 0.9981.

Estimation of camptothecin

Twenty microliters of sample solution (1 mg ml^{-1} methanol extract) were applied in triplicate to a pre-coated silica gel 60 F_{254} HPTLC plate 0.2 mm in thickness (E. Merck, Mumbai, India) using a Camag Linomat IV automatic sample applicator. The samples were applied as 7-mm bands in duplicate, 7 mm apart and 10 mm from the bottom edge of the plate. The plate was developed to a distance of 80 mm with toluene:acetonitrile:glacial acetic acid (65:35:1 v/v/v) used as the mobile phase in a Camag glass twin-trough chamber at room temperature (25 ± 2 °C). After development, the plate was dried for 10 min and then scanned in fluorescence mode at λ = 366 nm (Dighe et al. 2007) using a Camag TLC Scanner III with a mercury lamp and the CATS 3 software package. To check the identity of the camptothecin band, the UV absorption spectrum of the sample was overlaid with that of the standard. To verify the purity of the band from the sample extract, the absorption spectrum was overlaid at the start, middle, and end positions of the band. Peak areas were recorded for each camptothecin concentration. The data-pairing technique was used to determine whether there was any significant difference in peak area between the sample and the standard solution of camptothecin, and the same concentration was recorded in duplicate. The amount of camptothecin in each sample was calculated using the linear regression equation derived from the calibration curve.

Results and discussion

Culture initiation

The genus *Ophiorrhiza* is of immense economic importance due to the presence of camptothecin in many of the species in this genus (Renjith et al. 2013), so the main objective of the present investigation was to establish an efficient and reproducible tissue culture system for *O. prostrata*, permitting the mass production of plantlets and the estimation of camptothecin. Mature fruits treated with 1% (v/v) sodium hypochlorite (NaClO) for 9 min and with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 4 min gave the greatest response. However, the response of the fruits treated with NaClO (45.4 ± 1.12%) was much lower than the response of the fruits sterilized with $HgCl_2$ (87.8 ± 1.193%). The capsule surfaces sterilized with high sterilant concentrations were completely free from contamination during the initial period of 10–15 days; the seeds subsequently became necrotic and died without showing any sign of germination. Optimizing the surface sterilization was an important aspect of the investigation, as this optimization ensures that as many clean explants as possible survived sterilization. Using $HgCl_2$ (0.1%), the highest percentage of contamination-free explants was obtained along with the optimal rate of response, which was comparable to the results published by Yao and Krikorian (1981) and George and Sherrington (1984). Similar results were also observed in the case of *O. rugosa* var *decumbens*, in which 0.1% mercuric chloride helped to reduce the rate of contamination of leaf, stem, tuberous root, and petiole explants (Vineesh et al. 2007).

Various responses in terms of *O. prostrata* seed germination were observed with different medium formulations. Seed germination involves a series of events that commence with the uptake of water by the seed and terminate with the elongation of the shoot (Bewley and Black 1994). Species-specific media for seed germination have been reported for many plants (Arditti and Ernst 1984; Kauth et al. 2008). Although more than 50 medium formulations have been used for the in vitro culture of tissues of various plant species (Gamborg et al. 1976; Huang and Murashige 1977), the formulation described by Murashige and Skoog (1962) (MS medium) is most commonly used, often with relatively minor changes (Uduebo 1971; Wakhlu et al. 1990; Zhou et al. 1994; Saxena et al. 1998). In the present study, the responses of seeds cultured on media containing different salt concentrations varied; the highest rate of germination (84.8 ± 2.65%) was obtained on half-strength MS basal nutrient medium, followed by quarter-strength (74.33 ± 0.29%) and full-strength (57.6 ± 0.20%) media. Seeds also germinated and grew on the solid as well as the

liquid media within a period of 3–5 weeks (Fig. 1A–E). Germination on the solid media took 3–4 weeks (germination percentage: 94.8 ± 2.65), while in the liquid media it took 4–5 weeks (germination percentage: 91.67 ± 3.23) (Fig. 1F, G). The seedlings fully grown from seeds in the solid or liquid media attained a length of 2–2.5 cm with two leaves and 2–3 roots within 2 weeks after germination. Seed germination of *O. prostrata* was more pronounced with half-strength MS salts than with the full- and quarter-strength salt concentrations (Fig. 2A, B). It is evident from the results that the salt strength of the MS medium had a strong influence on the seed germination. However, it is interesting that both the full-strength and the quarter-strength media gave suboptimal results, with the half-strength medium yielding the best results. Similar seed germination behavior with respect to medium strength has also been noted for other species, such as *O. liukiuensis* (Asano et al. 2009) and *Swertia chirata* (Chaudhuri et al. 2007). On the other hand, the adequacy of a relatively weak nutrient composition (as in the half-strength medium) for germination and initial growth also hints that *O. prostata* may be utilizing the nutrients stored in its seeds for up to several days after germination, as has been noted elsewhere in other species (Elena et al. 2011). Likewise, the ability to germinate on both liquid and solid media may represent an adaptation to the seed dispersal mechanism (dispersal mainly occurs via rainwater flow in the hilly and marshy terrains where the plant is naturally found), as has been reported in other species (Arditti and Ernst 1984; Kauth et al. 2008).

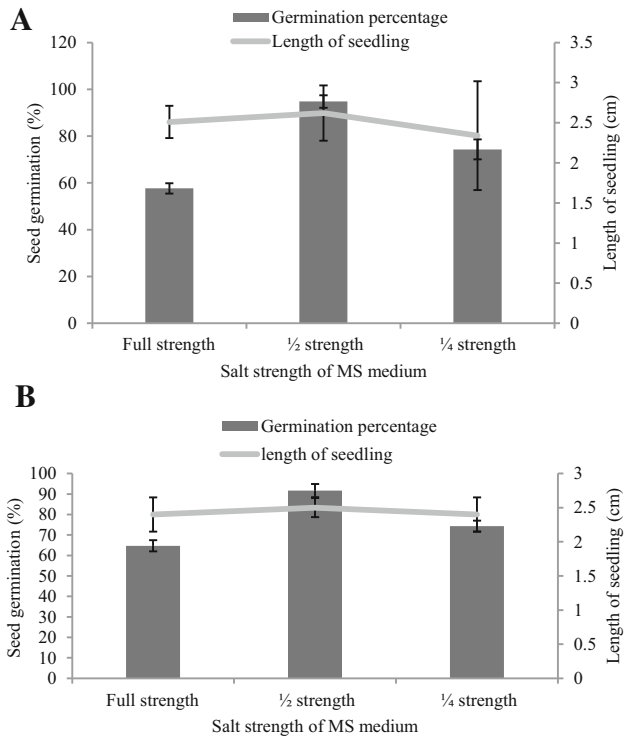

Fig. 2 **A** Germination of mature seeds in solid MS media with different salt strengths. **B** Germination of seeds in liquid MS media with different salt strengths. Observations were performed after 5 weeks, and the *error bars* correspond to ± 1SD

Multiplication of shoots

Multiple shoots obtained without a callus phase on MS medium supplemented with BAP alone is not uncommon, as demonstrated by the presence of many similar reports for medicinal plants in the literature. The effects of auxins and cytokinins on shoot multiplication in various medicinal plants have also been reported (Vincent et al. 1992; Sharma and Singh 1997). Cytokinin levels were shown to be the most important influence on multiplication in many medicinal plants (Roja et al. 1987; Krishnan and Seeni 1994).

Upon subculturing, 80% of the initiated shoots/shoot buds grew to an average length of 2.48 ± 0.466 cm, with no sign of additional shoot bud formation in half-strength solid MS medium devoid of hormones after 3 weeks. Shoots subcultured in media containing different concentrations and combinations of kinetin (Kn), benzylaminopurine (BAP), indole-3-acetic acid (IAA), and naphthaleneacetic acid (NAA) resulted in the proliferation of multiple shoots at varying rates. Shoots were found to proliferate from the nodal region of the subcultured shoot. Comparatively high multiplication rates were observed with all treatments, but not in the control. The best results were recorded on medium containing 0.5–2.5 mg l^{-1} BAP. On the medium containing 1.5 mg l^{-1} BAP alone, more than 90% of the shoot buds proliferated, with an average of 28.0 ± 2.58 shoot buds and an average length 1.81 ± 0.102 cm obtained within 3 weeks of culture (Table 1). The concentrations and combinations of hormones tested in the media yielded between 10.1 ± 1.79 and 28.0 ± 2.58 shoot buds per original bud. On the contrary, there was little difference in the shoot lengths obtained with different hormone concentrations and combinations. A linear increase in the number of shoots induced with increasing BAP concentration was noticed up to 1.5 mg l^{-1} BAP; above this concentration, shoot proliferation decreased as the concentration of hormone increased. Upon increasing the culture duration (for example up to 8 weeks), the proliferation of shoot buds in the various media continued, reaching a rate as high as 36.0 ± 4.2 shoots per explant (data not presented). In these cases, only 60% of the shoots reached a harvestable length, and the growth of other shoots remained suppressed. The efficacy of 6-benzylaminopurine (BAP) at inducing multiple shoots has been demonstrated by many researchers. The potency of BAP at inducing shoot buds in the present

Table 1 Effects of different concentrations of plant growth regulators in half-strength MS medium on shoot multiplication from seedling-derived explants of *O. prostrata*

Plant growth regulators (mg l^{-1})				Average number of shoot buds	Average length of shoot bud (cm)
Kn	BAP	IAA	NAA		
0.0	0	0	0	1.8 ± 1.13^{k}	2.29 ± 0.119^{a}
0.5	0	0	0	$12.7 \pm 1.70_{hij}$	1.70 ± 0.25820^{d}
1.0	0	0	0	10.1 ± 1.79^{j}	1.46 ± 0.217^{fg}
1.5	0	0	0	17.5 ± 2.17^{g}	1.55 ± 0.302^{ef}
2.0	0	0	0	14.0 ± 1.76^{h}	1.31 ± 0.066^{hi}
2.5	0	0	0	11.8 ± 2.04^{hij}	1.37 ± 0.067^{gh}
0	0.25	0	0	18.1 ± 4.38^{fg}	1.92 ± 0.058^{bc}
0	0.50	0	0	25.0 ± 3.46^{bc}	1.81 ± 0.062^{bc}
0	1.00	0	0	27.0 ± 3.05^{ab}	1.36 ± 0.066^{ghi}
0	1.50	0	0	28.0 ± 2.58^{a}	1.81 ± 0.102^{bc}
0	2.00	0	0	23.0 ± 1.82^{de}	1.26 ± 0.093^{i}
0	2.50	0	0	22.0 ± 1.15^{de}	1.13 ± 0.066^{j}
0.25	0	0.1	0	14.0 ± 2.16^{h}	1.64 ± 0.089^{de}
1.50	0	0.5	0	12.0 ± 2.58^{hij}	1.42 ± 0.125^{gh}
2.50	0	1.5	0	13.0 ± 3.46^{hi}	1.93 ± 0.027^{b}
0.25	0	0	0.5	20.0 ± 2.58^{ef}	1.55 ± 0.089^{ef}
1.50	0	0	1.5	17.0 ± 1.76^{g}	1.63 ± 0.071^{de}
2.50	0	0	2.0	10.9 ± 2.80^{ij}	1.63 ± 0.071^{de}
0	0.25	0	0	11.0 ± 2.16^{ij}	1.93 ± 0.071^{b}
0	1.50	0	0	10.6 ± 2.22^{ij}	1.89 ± 0.068^{bc}
0	2.50	0	0	12.0 ± 1.82^{hij}	1.85 ± 0.059^{bc}
0	0.25	0	0	25.0 ± 3.33^{bc}	1.90 ± 0.058^{bc}
0	1.50	0	0	20.5 ± 4.27^{ef}	1.47 ± 0.067^{fg}
0	2.50	0	0	22.0 ± 2.90^{de}	1.80 ± 0.092^{c}

Observations were made after 4 weeks of culture

Kn kinetin, *BAP* benzylaminopurine, *IAA* indole-3-acetic acid, *NAA* naphthaleneacetic acid

Mean values with the same superscripts within columns are not significantly different ($p \leq 0.05$) according to the LSD test

study compared to other cytokinins (KN, IAA) is in agreement with the findings of other researchers (Cheong and Pooler 2003; Jose and Satheeshkumar 2004). Shoot induction and regeneration has been reported in many species of *Ophiorrhiza*, including *O. mungos* (Namedo et al. 2012), *O. decumbens* (Roja 2008), *O. pumila* (Saito et al. 2001), *O. alata* (Pornwilai et al. 2011), and *O. prostrata* (Beegum et al. 2007; Martin et al. 2008). Explants from nodes, leaves, auxenic seedlings, and roots from the same genotype of *Ophiorrhiza* are known to exhibit diverse regeneration responses depending on the medium and PGR supplementation applied (Beegum et al. 2007). An absence of a synergistic influence of cytokinin and auxin combinations has also been reported for some other species, indicating that the influence of BAP on shoot bud induction depends on the plant considered (Benjamin et al. 1987).

The production of shoots by subculturing individual shoots as well as shoots in clumps showed that the degree of proliferation was dependent on the inoculum size. Subcultured shoot clumps containing a minimum of 4–6 shoots yielded a maximum of 32.20 ± 2.03 shoots, whereas subcultured single shoots yielded fewer (25.01 ± 2.0) shoots (Fig. 3). It is interesting to note that shoot cultures that were maintained on the medium during multiplication for more than 5 weeks exhibited profuse rooting on shoots, mainly from the node and intermodal regions, which made isolating individual shoots difficult. Therefore, separate rooting was required for individual shoots. The microshoots were subcultured on the same medium for further multiplication, and healthy shoots were carefully selected for rooting.

Shoot elongation and rooting

In *O. prostrata*, comparatively high shoot elongation was noted in MS medium without hormones. Half-strength MS medium devoid of hormones was sufficient for shoot

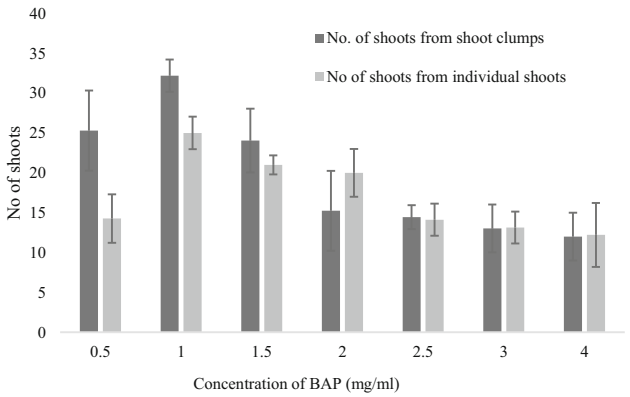

Fig. 3 Shoot multiplication of shoot clumps and individual shoots subcultured on half-strength solid MS medium supplemented with various concentrations of BA. Observations were performed after 4 weeks

elongation of 5–6 cm within a period of 2–3 weeks (Fig. 1J). During this period, no root formation occurred up to 4 weeks, but prolonged incubation for 5–6 weeks resulted in root formation. In our study, not much effort was needed to induce shoot elongation in *O. prostrata*; half-strength basal MS medium was effective for this purpose. Cuenca et al. (1999) reported that increasing the concentration of cytokinins inhibits shoot elongation. Generally, elongation has been observed to occur in *Ophiorrhiza* shoot cultures in hormone-free basal medium or in medium containing low concentrations of auxins (Jose and Satheeshkumar 2004).

Shoot rooting occurred at different rates in half-strength solid MS media with different concentrations of IAA, NAA, and IBA. Generally, 5–12 roots emerged from the base of an individual shoot. Among the various hormones tested, NAA was shown to be the strongest positive influence on root formation on shoots (Fig. 1K). The number of roots produced varied significantly with the concentration of NAA used (Table 2). Rooting is the most important step during the production of complete plantlets and the greatest influence on their survival. The growth regulators in and the nutrient content of the medium play a vital role in the rooting process (Reddy et al. 2001; Thomas and Sankar 2009). Alagumanian et al. (2004), Giridhar et al. (2004), and Baksha et al. (2005) suggest that half-strength MS medium gives the best results in terms of root formation. In the present study, half-strength medium with the plant growth regulator NAA at 1 mg l^{-1} was found to be more effective at encouraging root formation (44.0 ± 4.13 roots were obtained in this medium) than IAA and IBA. This result is in agreement with the findings of Agrawal and Sardar (2007) for *Cassia angustifolia*, and those of Sharma (2004) and George et al. (2004) for *Centella asiatica*.

Deflasking and hardening

The transition period during the hardening process after transfer from the in vitro to the ex vitro environment is considered to be the most important step in tissue culture (Faisal et al. 2007). Rooted shoots (Fig. 1L) of length 3.5–5.0 cm with 3–4 pairs of leaves were planted in poly bags (8 × 4 inches) filled with a sand and soil (1:1) mixture and kept in a shade net house, where they were periodically watered and exhibited a survival rate of >90%. After 3 weeks, new leaves emerged in almost 90% of the plantlets (Fig. 1M). The plants that were established in the poly bags were transplanted to the field. The established plants exhibited a uniform morphology (Fig. 1N). Afolayan and Adebola (2004) pointed out that micropropagated plants can be used to supplement the natural stocks of plants in wild populations as well as to provide a ready supply to the herbal medicinal trade. An efficient in vitro protocol would be able to produce a large number of plantlets with proper rooting in a short period of time along with acclimatization in the field. The results of the present study certainly facilitate the propagation, conservation, and sustainable utilization of this species.

A schematic of the tissue culture protocol for the mass multiplication of *O. prostrata* is given Fig. 4.

Estimation of camptothecin

Camptothecin contents were estimated using HPTLC, and the results revealed significant levels of camptothecin in the in-vitro-derived plants (Figs. 5, 6). The level of camptothecin was found to differ significantly between the in-vitro-derived multiple shoots and the in-vitro-derived hardened plants. The camptothecin content was observed to be highest in the in-vitro-derived hardened plants after 4 weeks (1.83 ± 0.02 μg g^{-1} dry wt), followed by the field-grown plants (1.46 ± 0.02 μg g^{-1} dry wt), whereas the lowest content was observed in the in-vitro-grown multiple shoots (1.10 ± 0.02 μg g^{-1} dry wt). Previous studies performed in our own laboratory (Krishnakumar et al. 2012) identified relatively low levels of camptothecin (1.47 μg g^{-1} dry wt) in *O. prostrata* compared to those in traditional sources. The plants grown using the tissue culture protocols in our study had 1.10 ± 0.02 μg g^{-1} DW (in-vitro-grown multiple shoots) and 1.83 ± 0.02 μg g^{-1} DW (in vitro grown and established in the field), though the content varied depending on the method used to grow the plants. Such variations in the secondary metabolites in plants raised using different in vitro methods have also been noted previously, as in the case of *Catharanthus roseus* (Constable et al. 1981), *Cephaelis ipecacuanha* (Teshima et al. 1988), *Azadirachta indica* (Srividya et al. 1998), and *Rauvolfia serpentina* (Ihsan et al. 2007).

Table 2 Rooting responses of shoots of *O. prostrata* in half-strength MS medium supplemented with different concentrations and types of auxins

Plant growth regulators (mg ml^{-1})			Rhizogenic response	
IAA	IBA	NAA	Average no. of roots	Average length of root (cm)
0.0	0	0	20.0 ± 2.58^{f}	5.00 ± 1.763^{abc}
0.5	0	0	25.1 ± 4.74^{e}	4.00 ± 1.054^{def}
1.0	0	0	25.0 ± 3.46^{e}	3.60 ± 0.843^{ef}
1.5	0	0	36.0 ± 3.71^{bcd}	3.35 ± 0.709^{f}
2.0	0	0	28.0 ± 1.15^{e}	4.05 ± 0.643^{def}
0	0.5	0	26.0 ± 2.58^{e}	4.98 ± 0.762^{abc}
0	1.0	0	33.1 ± 3.38^{d}	4.50 ± 0.707^{cd}
0	1.5	0	38.0 ± 3.59^{b}	4.65 ± 0.474^{bcd}
0	2	0	37.0 ± 4.16^{bc}	5.50 ± 0.333^{a}
0	0	0.5	34.0 ± 1.76^{cd}	3.35 ± 0.411^{f}
0	0	1.0	44.0 ± 4.13^{a}	3.40 ± 0.516^{f}
0	0	1.5	39.0 ± 3.43^{b}	5.40 ± 0.459^{ab}
0	0	2	34.0 ± 1.56^{cd}	4.35 ± 0.474^{cde}

Observations were performed after 3 weeks of culture

Mean values with the same superscripts within columns are not significantly different ($p \leq 0.05$) according to the LSD test

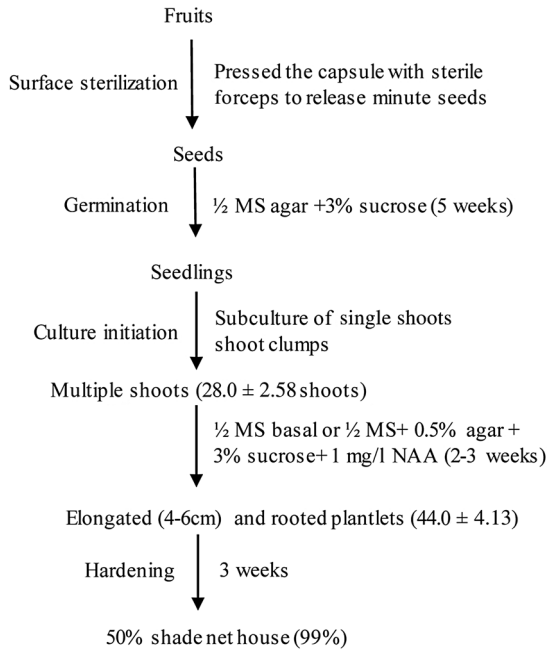

Fig. 4 A schematic of the tissue culture protocol for the mass multiplication of plants through seedlings of *O. prostrata*

High yields of camptothecin (0.3 mg g^{-1} DW) have reportedly been obtained from *Nothopodites foetida*, while yields from *Camptotheca accuminata* range from 0.2 to 5.0 mg g^{-1} DW depending on the tissue analysed. However, it should be noted that both of these are slow-growing tree species (Ramesha et al. 2008; Yan et al. 2003), so they accumulate biomass over a period of years, unlike rapid biomass-generating methods that could be employed to compensate for the relatively low levels (per unit dry mass) of camptothecin content. It might also be possible that increased production of camptothecin is obtained by optimizing other conditions, akin to the enhanced production of secondary metabolites in other plant species (Bhojwani and Razdan 1983). *O. prostata*, like other species of the genus, is easy to cultivate and harvest under in vitro and in vivo conditions (Renjith et al. 2013). The present study highlights the methods used for the large-scale production of the source plants by tissue culture. Another aspect to be noted is that *O. prostata* has remarkable antioxidant and anticancer activities, as has been noted in previous in vitro pharmacological evaluations (Krishnakumar et al. 2012). While some of its pharmacological action may be due to its camptothecin content, the relatively low (per unit dry mass) level of camptothecin present in *O. prostata* may not explain its full spectrum of biological activities. It should be noted that this species is mainly used as an anti-inflammatory (and as an anti-snake venom decoction) (Krishnan et al. 2014), which may not be linked to the camptothecin content. Thus, the species is an interesting target for exploring further biological activities of interest. Our protocol for the rapid generation of *O. prostata* by tissue culture, which makes it very amenable for further studies in this regard.

Conclusions

The genus *Ophiorrhiza* has the potential to be a viable alternate source of camptothecin, but detailed pharmacological investigations of individual species are only just

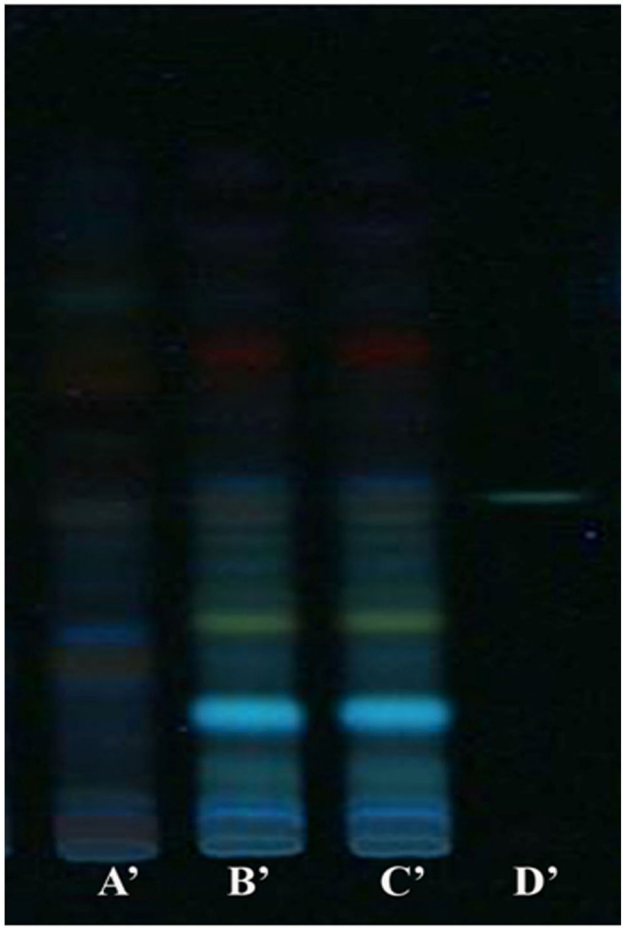

Fig. 5 HPTLC chromatograms of methanol extracts of *O. prostrata*. **A'** In-vitro-derived 1-month-old hardened plants; **B'** field-grown plants; **C'** in-vitro-grown multiple shoot; **D'** standard camptothecin

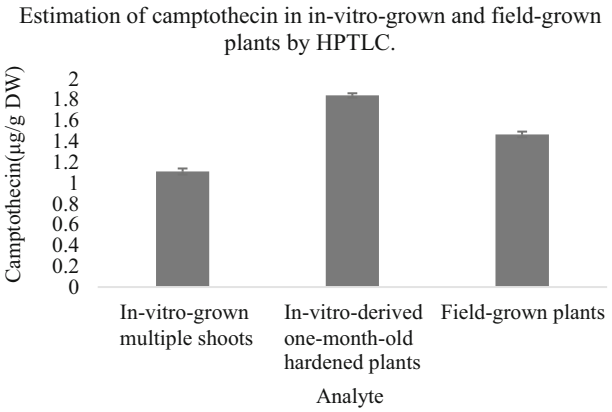

Fig. 6 Estimation of camptothecin in in-vitro-grown and field-grown plants using the HPTLC method

emerging. On the other hand, camptothecin and its derivatives have been investigated in detail, and increasing numbers of novel anticancer derivatives are being identified, which threatens the existence of current sources of camptothecin (mainly woody plants). Our work generated an in vitro tissue culture system that could be utilized to scale up the biomass in a species which harbors camptothecin, though this approach is not commercially viable at present. We succeeded in generating a tissue culture protocol for the rapid multiplication of the targeted species, *O. prostata.* However, the content of camptothecin is still not sufficient to rely on the said plants as a commercially viable alternative for producing camptothecin. Further optimization studies to enhance the content of camptothecin and the development of cell-line/callus models which can be used to rapidly expand the biomass to compensate for relatively low levels of camptothecin are required in *O. prostata* in order to project the species as an alternate source of camptothecin. Efforts in this direction are currently underway in our laboratory.

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