Indian Journal of Biotechnology
Vol 7, April 2008, pp 260-265

Rapid *in vitro* multiplication of *Drosera burmanii* Vahl.: A vulnerable and medicinally important insectivorous plant

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Received 16 October 2006; revised 30 May 2007; accepted 20 August 2007

A protocol has been stardardised for the rapid and large-scale *in vitro* multiplication of the vulnerable medicinal herb, *Drosera burmanii* Vahl. by enhanced axillary bud proliferation from shoot tip explants. In order to standardize *in vitro* multiplication, the effects of different strengths of Murashige and Skoog (MS) medium (1/4, 1/3, 1/2, and full strength), different percentages of sucrose (1%, 2% and 3%), various *p*H (3.7, 4.7, 5.7 and 6.7) and MS basal medium fortified with different concentrations of kinetin (Kn) and 6-benzylaminopurine (BAP) (0.1, 0.5, 1.0 and 2.0 mg L^{-1}) were tried on shoot tip explants. Maximum number of multiple shoots developed on MS medium supplemented with Kn (1.0 and 2.0 mg L^{-1}) and BAP (0.5, 1.0 and 2.0 mg L^{-1}) separately. Direct plantlet regeneration from the leaves and *in vitro* flowering were also observed. Rooting was best achieved on MS basal medium. This protocol could be useful for large-scale production of biomass for quercetin, plumbagin bioprospection and long term *in vitro* conservation.

Keywords: *Drosera burmanii*, *in vitro* multiplication, plumbagin, quercetin, shoot tips

Introduction

Drosera burmanii Vahl. (Family Droseraceae) is an annual insectivorous plant. Owing to its insectivorous nature, it vividly grows in nitrogen deficient, acidic, sandy and swampy soils. It is spread throughout South East Asia, China, Japan and India. The genus *Drosera* is known to contain phytochemicals such as plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone), 7-methyljuglone (5-hydroxy-7-methyl,4-naphthoquinone)[1], quercetin[2] and glucosides of free quinones[3-5]. These phytochemicals act as immunomodulators, anti-spasmodics, anticancer, antimicrobial, antiviral, anti-inflammatory, antioxidants and are used for curing various infectious diseases. They have also been observed to enhance *in vitro* phagocytosis of human granulocytes[6-9].

Plants that are isolated and restricted to specific habitats are highly vulnerable to extinction[10]. Many species of *Drosera* are threatened due to their restricted habitat and indiscriminate usage in herbal industries. In India, though *D. burmanii* has been categorized as vulnerable according to IUCN[11], stringent conservation measures have not yet been established. Infact evidence suggests that all over Asia the export of *D. burmanii* is happening in an unsustainable manner. Also many European countries are importing *D. burmanii* for "Herba Droserae" instead of using native endangered European *Drosera* species[12]. The restricted natural habitat of *D. burmanii* is being affected by invasive species, climatic shifts, urbanization, agricultural pollutants and its population is getting dwindled primarily because of collection from wild for herbal industries apart from the above uses. On the other hand, during heavy rains the minute seeds of *D. burmanii* get carried away from its natural to unsuitable habitats[13]. Also the demand is exceeding the natural availability, its survival is being threatened by dwindling natural habitats. Thus, the conservation and multiplication of *D. burmanii* should be on high priority to prevent its local extinction.

Plant tissue culture is an important technique used in the multiplication and conservation of threatened species. Earlier, several research groups worked on establishing multiplication and conservation strategies in various species of *Drosera* using different parts of plant as explants such as seeds in *D. rotundifolia*[14-20], *D. intermedia*[21], *D. pygmaea*[22], *D. aliciae*[14,23], *D. peltata*[24], *D. anglica*, *D. cuneifolia*[25] and *D. spathulata*[26], leaves in *D. rotundifolia*[27-32], *D. hilaria, D. regia*[33], *D. natalensis*[28,34],

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D. spathulata[35,36], *D. capenesis* and *D. binata*[25,29], shoot tips in *D. rotundifolia*[19,27], *D. natalensis*[34] and *D. binata*[25], axillary shoots and internodes in *D. rotundifolia* and *D. inetrmedia*[27], flower buds and flower stalks in *D. natalensis*[34], rhizomes in *D. binata*[25] and *D. natalensis*[34], for utilization of plant material for bioprospecting and their long term *in vitro* conservation.

The authors have attempted to establish conservation and multiplication strategies for this vulnerable medicinal plant. Initially to establish *in vitro* cultures, attempts were made to germinate seeds in *in vivo* and *in vitro* conditions by various treatments, out of which only few seeds germinated. Even after several repetitions it was difficult to predict the percentage of germination. According to International Carnivorous Plant Society (ICPS) in many of the *Drosera* species, seeds need a cold treatment (4°C) and GA_3 (at various ppm) to break the dormancy but inspite of adopting the above measures satisfactory results were not obtained[13]. Hence, we have attempted to establish reliable direct plantlet regeneration protocol by using shoot tip explants for large-scale production of *D. burmanii* biomass, such that it can be used for isolation of quercetin, plumbagin and other useful secondary metabolites and also for long term *in vitro* germplasm storage.

Materials and Methods

Plant Material and Sterilization

Plants of *Drosera burmanii* Vahl. were collected from the University of Hyderabad Campus, where it grows luxuriantly around marshy lake fringes in a few restricted places. The plants were washed thoroughly for 30 min under running tap water followed by removal of older leaves covered with insect exoskeleton and other debris. Shoot tips of about 1.0 cm excised from the stem were soaked in 1% (w/v) bavistin (BASF India) solution for 30 min with regular shaking at every 5 min. Surface sterilization was carried out by using 35% ethanol and 0.05% of $HgCl_2$ for 30 sec each, followed by rinsing for 3-5 times with sterile double distilled water.

Culture Medium and Conditions

Initially, the shoots were raised on Murashige and Skoog (MS) basal medium[37] containing 3% (w/v) sucrose, 0.3% (w/v) agar (Hi-Media) at *p*H 5.7. The cultures were incubated at 25±2°C under 16/8 h photoperiod with a light intensity of 50 µmol m^{-2} s^{-1}. Once the cultures were established, shoot explants from these cultures were used in a series of experiments by using (i) different strengths of MS medium (1/4, 1/3, 1/2, and full strength), (ii) MS basal medium with different percentage of sucrose (1%, 2% and 3%), (iii) MS basal medium with various *p*H (3.7, 4.7, 5.7 and 6.7) and (iv) MS basal medium fortified with plant growth regulators like kinetin (Kn) and 6-benzyl amino purine (BAP) (0.1, 0.5, 1.0 and 2.0 mg L^{-1}).

The shoots, separated after 8 weeks of subculture, were transferred on MS basal medium for root initiation. All the cultures were incubated at 25±2°C under 16/8 h photoperiod with a light intensity of 50 µmol m^{-2} s^{-1}. Each treatment consisted of 20 replicates and each experiment was repeated thrice.

Acclimatization of Regenerated Plants

Regenerated plants having well developed roots were removed from culture bottles and washed free of agar media and transferred to plastic pots containing sterilized soilrite (Kel Perlite, Vishwasnagar, Karnataka) which were maintained on MS medium. The pots were covered with transparent plastic covers to ensure high humidity. The growth chamber was maintained at 23±2°C with light intensity of 50 µmol$m^{-2}s^{-1}$ on a 16/8 h photoperiod and gradually opened during acclimatization period of 2 weeks. After acclimatization, plantlets were transferred to greenhouse through simulated habitat and the percentage of survival was observed.

Each experiment was repeated a minimum of three times and data were recorded after 8 weeks on the number of shoots per explant and percentage of response from the axil of bract was recorded. The mean number and standard error was analyzed with Sigma Plot Statistical Software Version 6.2.

Results and Discussion

In vitro multiplication of various *Drosera* plants has proved to be difficult due to fungal and bacterial contaminants on the surface of the leaves[28,29]. Even in *D. burmanii*, brief (1 min) sterilization of the shoot explants using 40-70% ethanol and 0.1% $HgCl_2$, deteriorated the tissue. Lower concentrations, viz. 35% ethanol and 0.05% $HgCl_2$ for 30-45 sec to be effective for elimination of contaminants without harming the explants to a significant extent. Leaf explants were also tried initially but due to necrosis of tissue during sterilization proved to be too difficult to continue to use.

Effect of Different Strengths of MS Medium

According to Crouch and van Staden[34] and Perica and Berljak[38] the best medium for *in vitro* multiplication of *Drosera* species is MS medium. Since *Drosera* plants grow normally in nutrient poor habitats, the effects of the strength of MS medium on shoot proliferation and growth were tested. In *D. burmanii*, shoot proliferation was not influenced by any of the tested strengths of the MS medium. The number of shoots were similar in all strengths of MS medium, but number of shoots induced from the axil of bracts was greater when full strength MS medium was used (Table 1, Fig. 1A). Similar results were observed in *D. indica*[39]. However, shoot proliferation was greatly influenced as the number of shoots increased comparatively in *D. rotundifolia*[19,29,31], *D. capensis* and *D. binata*[29] and in *D. peltata*[24] by different strengths of MS medium. In all the strengths of MS medium black, unbranched roots completely covered with root hairs were observed.

Effect of Different Sucrose Concentrations

For *in vitro* cultures the main source of carbohydrate is generally sucrose and, therefore, the effects of sucrose concentration on shoot proliferation of *D. burmanii* were examined. There did not appear to be any effect of different concentrations of sucrose on shoot proliferation. Almost all shoots produced roots but the percentage of response for rooting was better at 3% sucrose (Table 2). Roots were black, unbranched and totally covered with root hairs. Similar results were also observed in *D. indica*[39].

Effect of Various *p*H

All the insectivorous plants grow in acidic soils (*p*H 3 to 5)[40]. Since *p*H affects nutrient uptake and shoot proliferation[41], the effect of *p*H levels on shoot proliferation of *D. burmanii* was examined. The shoot proliferation from the shoot tip explants was not influenced by variation in *p*H but as the *p*H decreased, there was a gradual increase in the number of shoots developed from the axil of bracts though the percentage of response was low (Table 3). Similar results were observed in *D. indica*[39]. Whereas in *D. peltata* and *D. rotundifolia,* the effect of different *p*H greatly influenced shoot proliferation and was severely inhibited in higher acidic media[7,24].

Effect of Cytokinins

Cytokinins generally inhibit root development and promote shoot growth[42]. In *D. burmanii*, multiple shoots were developed within 50-60 d upon 1.0 and 2.0 mg L^{-1} Kn and 0.5, 1.0 and 2.0 mg L^{-1} BAP, respectively (Table 4; Fig. 1B). High concentration (>0.5 mg L^{-1}) of Kn and BAP retarded growth of the developed shoot buds. Their stunted nature made it difficult to distinguish separate individual shoots. The suppression increased with increasing concentration of BAP or Kn, along with enhanced red pigmentation

Fig. 1—*In vitro* multiplication of *D. burmanii* (A) regeneration of plantlet from axil of bract (B) multiple shoots production on MS + 2.0 mg L^{-1} Kn after 8 weeks (C) *in vitro* flowering and rooting on MS basal medium (D) rapidly multiplied shoots in magenta boxes on different concentrations of Kn and BAP.

Table 1—Effect of different strengths of MS medium on shoot proliferation from shoot tips and bracts of *D. burmanii* after 8 weeks culture

MS medium concentration	No. of shoots/explant Mean ± SE	No. of shoots from the axils of bracts Mean ± SE	% of response of shoots induced from axil of bract
¼ MS	1.2 ± 0.5	3.2 ± 1.8	76.0
½ MS	2.2 ± 1.7	5.8 ± 3.0	70.0
⅓ MS	1.3 ± 0.7	5.1 ± 2.8	63.3
MS basal	1.5 ± 1.2	5.1 ± 2.6	53.3

Table 2—Effect of different concentrations of sucrose on shoot proliferation from shoot tips and bracts of *D. burmanii* after 8 weeks cultures on MS medium

Conc of sucrose %	No. of shoots/explant Mean ± SE	No. of shoots from the axils of bracts Mean ± SE	% of response of shoots induced from axil of bract
1	1.2 ± 0.6	4.0 ± 2.2	23.3
2	1.0 ± 0.1	4.0 ± 1.8	76.6
3	1.7 ± 1.3	5.3 ± 2.4	83.3

Table 3—Effect of *p*H on shoot proliferation from shoot tips and bracts of *D. burmanii* after 8 weeks cultures on MS medium

*p*H	No. of shoots/explant Mean ± SE	No. of shoots from the axils of bracts Mean ± SE	% of response of shoots induced from axil of bract
3.7	1.5 ± 1.2	5.2 ± 2.5	63.3
4.7	1.3 ± 0.7	5.0 ± 2.4	80.0
5.7	1.3 ± 0.6	4.4 ± 2.6	70.0
6.7	1.3 ± 0.7	3.3 ± 1.5	70.0

Table 4—Effect of cytokinins on shoot proliferation from shoot tips and bracts of *D. burmanii* after 8 weeks culture on MS medium

MS medium with cytokinins (mg L^{-1})	No. of shoots/explant Mean ± SE	No. of shoots from the axils of bracts Mean ± SE	% of response of shoots induced from axil of bract
MS + 0.1Kn	1.9 ± 1.5	4.0 ± 2.2	40.0
MS + 0.5 Kn	4.6 ± 3.3	4.8 ± 3.0	53.3
MS + 1.0 Kn	7.5 ± 4.3	3.1 ± 2.6	63.3
MS + 2.0 Kn	8.6 ± 3.4	3.3 ± 1.6	46.6
MS + 0.1 BAP	2.5 ± 1.4	2.7 ± 1.2	36.6
MS + 0.5 BAP	6.3 ± 3.5	2.0 ± 1.4	33.3
MS + 1.0 BAP	8.7 ± 3.7	1.7 ± 0.8	23.3
MS + 2.0 BAP	7.2 ± 2.0	2.2 ± 1.1	16.6

of leaves, the above phenomena was more pronounced in BAP than in Kn. Similar results were also observed in *D. indica*[39,43], *D. rotundifolia* and *D. peltata*[19,24,29].

When growth suppressed plantlets of *D. burmanii* were transferred to MS medium plantlets regained normal growth. When stunted inflorescences were cut and cultured on MS basal medium or lower concentrations of BAP or Kn (0.1 and 0.5 mg L^{-1}) multiple shoots developed from the axils of the bracts. Occasionally at lower concentrations of BAP (0.5 and 1.0 mg L^{-1}) plantlet regeneration was observed from the base of the flower stalks.

Direct plantlet regeneration from somatic tissue is a rare phenomenon in *Drosera* but has been described in *D. rotundifola, D. natalensis, D. capensis, D. anglica* and *D. cuneifolia*[16,18,19,25,28-30]. The growth retarded plants of *D. burmanii* when transferred to MS medium or lower concentration of Kn or BAP (<0.5 mg L^{-1}) regained normal growth and immediately started developing numerous shoots on leaves. However, these were difficult to count after 8 weeks.

In vitro Flowering

Induction of *in vitro* flowering is mainly due to stimulation of endogenous cytokinins[44]. In *D. burmanii*, induction of *in vitro* flowering was observed in all the tested strengths of MS medium, different concentrations of sucrose and at various *p*H values, which can be attributed to the endogenous cytokinin stimulation (Fig. 1C). *In vitro* flowering was observed by supplementation with low concentration of cytokinins likes Kn and BAP (0.1 and 0.5 mg L^{-1}); however, at higher concentrations of Kn and BAP (1.0 and 2.0 mg L^{-1}), growth of plantlets and inflorescence was retarded albeit an increase in the number of multiple shoots was observed. This indicates that exogenous supplementation with high concentrations of cytokinins influenced multiple shoot development but with compromised *in vitro* flowering. Explants were collected from almost mature, one month old plants. This also adds to the list of factors influencing *in vitro* flowering. Hence, age of the tissue is also an endogenous factor that controls *in vitro* flowering, which in turn is the physiological state of the explant. In *D. burmanii*, the *in vitro* flowers failed to produce seeds though the flowers opened. In nature, the genus is insect or self-pollinated. When compared to other species of the genus, the morphology of *D. burmanii* shows more of insect/wind-pollinated characters. Hence, the absence of seed set is probably due to the lack of pollinating insects or wind in the culture vessels. Similarly, *in vitro* flowering was also observed in *D. indica*[39], *D. rotundifolia, D. capensis* and *D. binata*[19,29-31].

In vitro Rooting

The best medium for rooting of *D. burmanii* is MS basal medium without any plant growth regulators. In all the tested experiments rooting was observed except at higher concentrations of Kn (>1.0 mg L^{-1}) and BAP (>0.5 mg L^{-1}). Black, thick unbranched roots (4-5) with dense root hairs developed from each shoot within 15-20 d of culture (Fig. 1C). Similar results were also observed in *D. indica*[39] and *D. rotundifolia*[15,30]. Anthony[29] reported that all subcultured *D. rotundifolia* and *D. capensis* plantlets produced extensive root system after 6-8 weeks, in contrast to the generally week rooted plants found in the wild[45].

Acclimatization of *in vitro* Raised Plants through Simulated Habitat for Field Transfer

During acclimatization, in growth chambers at 23±2°C with light intensity of 50 μmol m^{-2} s^{-1} on a 16/8 h photoperiod, plant survival rate was more than 60%. *In vitro* produced root systems continued to grow, indicating their normal viability and function. To our knowledge there are no reports on field transfer of *in vitro* raised *Drosera* species. Acclimatized plants of *D. burmanii* when transferred through simulated habitat (transit between *in vitro* and field conditions) in the greenhouse, the survival rate was not satisfactory. Within 1 h, plants started wilting and did not survive further. This indicates that *D. burmanii* requires low temperature and high humidity for their survival. Moreover *in vitro* raised insectivorous plants have to be transferred to their natural habitat where the soil is nutrient poor, acidic in nature and open marshy areas (restricted habitat), if the plants have to survive. Since tissue cultured plants of *D. burmanii* are sensitive, field transfer to marshy environment is extremely difficult. Further work is required to achieve successful acclimatization and field transfer for the purpose of restoration and translocation of all the *Drosera* species.

This is the first report of a detailed and reproducible protocol for the rapid multiplication of *D. burmanii* from shoot tips without using expensive plant growth regulators. From a small amount of plant tissue large biomass can be produced within 60 d (Fig. 1D). Procurement of large quantities of fresh material in short seasons from natural habitat is difficult, laborious, time-consuming and expensive. Hence, *in vitro* multiplied plants could serve as a major source for pharmacologically important quercetin, plumbagin and other secondary metabolites for bioprospection[46]. However, we have to compare the quantity of secondary metabolites content obtained from wild and *in vitro* raised plants.

In vitro produced biomass is a reliable source for commercial purposes and is of export value. China and European countries demand huge quantities of the material for the preparation of "Herba Droserae" and indigenous demand in Ayurveda (Swarnabhasma) and herbal industries is also quite high. Since *D. burmanii* is a vulnerable medicinal species, *in situ* conservation, successful acclimatization and field transfer of *in vitro* plants would help in the optimization of existing restoration and conservation strategies in future.

Acknowledgement

The authors gratefully acknowledge the Department of Biotechnology, Government of India, New Delhi (Ref: BT/PR 2273/PBD/ 17/117/2000 dt.7-9-01) for financial support and infrastructural facilities for this project.

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