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Research Article
Plumbagin, a Plant-derived Naphthoquinone Production in Tissue Cultures of *Drosera spatulata* Labill

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Abstract

Background and Objective: *Drosera spatulata* 'Sundew' is a carnivorous plant valued for its pharmaceutical properties. Indiscriminate use of *D. spatulata* by herbal industries has made the species highly vulnerable and resulted in the dwindling wild population. Therefore, the aim of the present study was to develop a simple micro propagation system and to carry out HPLC analysis of plumbagin in tissue cultured plants and *in vitro* shoots. **Materials and Methods:** Leaf explants from previously grown aseptic shoots were cultured on ½X Murashige and Skoog's (MS) salts and vitamins (MS basal medium) supplemented with 6-benzyladenine (0.1-2.0 mg L^{-1}) or kinetin (0.1-2.0 mg L^{-1}), 3% sucrose and 0.35% gelrite. For rooting, four different basal media, i.e., B_5, N_6, WPM and Murashige and Skoog were tested. Finally, HPLC analysis of plumbagin in tissue cultured plants and commercial crude drug samples was carried out. **Results:** The maximum adventitious shoots (6.4/leaf explant) could be induced on ½X MS basal medium supplemented with kinetin (0.1 mg L^{-1}), 3% sucrose and 0.35% gelrite. Induction of rooting in cent percent *in vitro* shoots with an average number of 48.9 roots was achieved on ½X MS basal medium without any growth regulator. Out of 10 potting mix tested, a mixture of sphagnum: scrap of cyatheaceae (1:1, v/v) was found to be the most suitable with a plant survival rate of 90%. HPLC analysis results showed that *in vitro* derived greenhouse plants contained plumbagin comparable to a commercial crude drug. **Conclusion:** The study demonstrates the production of precious plumbagin, a plant derived naphthoquinone in tissue cultures of *Drosera spatulata* species.

Key words: *Drosera spatulata*, medicinal plant, micro-propagation, naphthoquinone, plumbagin, terpenoid

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Carnivorous plants have been known to grow in nutrient-impoverished environments where readily available nutrients (especially nitrogen) are restricted[1,2]. The genus *Drosera* commonly known as 'Sundews' belonging to family Droceraceae is one of the most wide-ranging genera of carnivorous plants with about 130 species found around the world[3-6]. The genus *Drosera* is distributed in Japan, tropical Asia, Australia and wet places and soggy soils in Taiwan. Out of 130 *Drosera* species nearly 50% of them are found in Australia[7,8]. One of the species, *D. spatulata,* occurs in mesic habitats and usually grows on hummocks and lawns surrounding marshes and ponds and on sandstone soils[9]. A typical Drosera plant uses filaments formed by the specialized leaves to secrete dew-like mucus and stick those insects which enter it. Once captured, insects are digested on leaves covered by stalked glands called tentacles. The glandular head of such tentacle is usually reddish and covered with droplets of mucilage. *Drosera* species are highly valued for their medicinal properties and ornamental traits. All species of *Drosera* contain plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinones) or ramentaceone (7-methyljuglone) or both[10,11] useful in curing whooping cough and bronchial infection. Investigations on extracts of aerial parts of *D. spatulata* Smith showed significant antimicrobial properties against oral bacteria indicating its potential use in treatment of dental caries and periodontitis[3]. Also, extracts of *D. spatulata* plants are used in herbal preparations for anti-cancer, anti-spasmodic, anti-microbial and immunomodulatory remedies[1]. Plumbagin is a useful and very expensive medicinal compound. Because of its pharmacological benefits, production of plants with high-plumbagin content is of immense interest. Many species of *Drosera* are threatened because of their indiscriminate usage by herbal industries. According to a report, exporters in Asia are collecting the wild plants of *Drosera* plants in an unsustainable manner[12]. Plant tissue culture technique has been an important tool in the micro propagation of medicinal plants, including *Drosera* species[6,12,13]. The technique allows for an increase in the propagation rate of valuable plant materials. Micro propagation can be a means of multiplication of ornamental plants, rare and endangered species as well as plants that are a source of secondary metabolites, plumbagin in case of *Drosera* species. Production of plumbagin by plant tissue culture is important because the compound can be obtained irrespective of season and with an assured quality under controlled conditions of growth. Previous studies have reported negligible quantities of plumbagin in *in vitro* and *in vivo* grown plants of *Drosera* species[14-15]. While in another report[16], plumbagin content in *Drosera* cultures was not analyzed and Zeatin (an expensive chemical) was found to be the most effective cytokinin for shoot proliferation. In Another study[17], plumbagin content in two *in vivo* grown *Drosera* species was recorded as 0.0025 and 0.0048% of fresh weight, while *in vitro* grown plants had comparatively lower quantity of plumbagin (0.001% of fresh weight). Therefore, the study aimed to develop a simple *in vitro* regeneration system of *D. spatulata* without use of Zeatin and to carry out HPLC analysis to investigate if tissue cultured derived plant materials of *D. spatulata* including *in vitro* shoots contain plumbagin. So that this compound can be obtained from tissue cultured *D. spatulata* whole plants or *in vitro* shoots grown in controlled laboratory conditions around the year. Also, this could boost an *in situ* conservation of a threatened wild population of the species since one need not depend on the collection of plant material from the natural habitats.

MATERIALS AND METHODS

Mature plants of *D. spatulata* were collected from 'Paoma Historic Trail', Jiaoxi Township, Yilan County, Taiwan (altitude about 500 m) (Fig. 1a). These plants were replanted in 18 cm diameter pots containing a mixture of soil: peat moss (1:1 v/v) and then kept in a growth chamber (Hotech Instruments Corp., Model 624 HD, Taipei, Taiwan) having light intensity of 100 mol m^{-2} sec^{-1}, 16 h photo period and 25/20 day/night temperature. Plants were irrigated once a week with tap water.

Establishment of aseptic cultures: The plants were taken out of pots and washed in running tap water for 30 min then older leaves were removed. Shoot apices of about 1.0 cm were excised from plants and were surface disinfected by washing several times with sterile distilled water. After that, these were immersed for 5 min in a sodium hypochlorite solution 0.5% (v/v) containing one drop of Tween-20. Final washing consisted of 3 rinses of 5 min each with sterile distilled water was carried out in a laminar hood. After that, the disinfected shoot apices were cultured on the full strength of Murashige and Skoog's salts and vitamins[17], from now on referred as MS basal medium. The pH of the culture medium was adjusted to 5.7±0.1 then 0.35% gelrite (Sigma-Aldrich, Inc., St. Louis, MO) was added before autoclaving. After autoclaving, 100 mL medium was poured into glass bottles (650 mL capacity). These bottles with medium were stored at 25±1°C until use. To obtain aseptic cultures for further experiments, surface sterilized shoot apices were inoculated onto the medium in

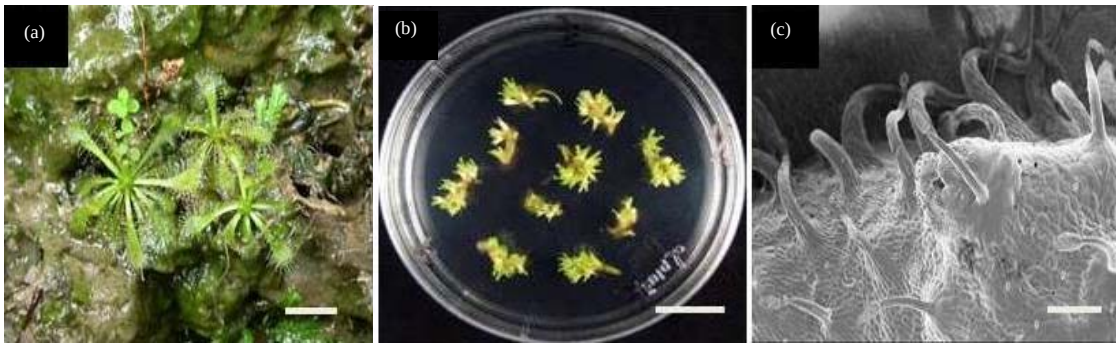

Fig. 1(a-c): *In vitro* propagation of *D. spatulata*, (a) Field specimen (bar = 2.0 cm), (b) Induction of adventitious shoots in leaf explants of *D. spatulata* cultured in dark with 0.1 mg L^{-1} kinetin (5 weeks) (bar = 1.7 cm) and (c) Leaf explant with induced shoots as observed using a scanning electron microscope (after 10 days of culture) (bar = 291 µm)

these bottles and were incubated at 25±1°C under the 16/8 h light and dark cycle, with the light intensity of 38 µmol m^{-2} sec^{-1} provided by cool fluorescent tubes.

Adventitious shoot induction: For induction of adventitious shoots in *D. spatulata*, initial experiments were performed to find out a suitable culture medium. Leaf explants were excised from the previously grown aseptic shoots and inoculated in sterilized Petri dishes (90 mm), each containing 20 mL of ½X MS basal medium supplemented with a range of concentrations (0.1-2.0 mg L^{-1}) of 6-benzyladenine (BA) or kinetin (Kin) (0.1-2.0 mg L^{-1}). Each medium was supplemented and 3% sucrose and 0.35% gelrite. Cultures were incubated at 25±1°C under the 16/8 h light and dark cycle, with the light intensity of 38 µmol m^{-2} sec^{-1} provided by cool fluorescent tubes. After optimization of the culture medium, another experiment was carried out with different colored lights. Here, leaf explants were inoculated in Petri dishes containing ½XMS basal medium supplemented with Kin (0.1 mg L^{-1}), 3% sucrose and 0.35% gelrite. Cultures in Petri dishes were incubated in a LED-light plant growth chamber (900 FLED, Taiwan Hipoint Corporation, Taiwan) at 25±1°C, with a 16 h photoperiod. The LEDs are semiconductor diodes that typically emit a single wavelength of light when charged with electricity. In this experiment, it examined the influence of 3 LED lights viz. red (660 nm), far-red (730 nm) and blue (450 nm) on the induction of adventitious shoots. Observations were recorded after 5 weeks of incubation.

Rooting of *in vitro* shoots: For further growth of shoots and induction of rooting, induced shoot clusters along with original leaf explants were transferred to glass bottles (650 mL capacity) each containing 100 mL medium. Four different basal media, i.e., B_5[18], N_6[19], WPM[20], Murashige and Skoog[21] and different strengths of MS basal medium (¼X, ½X, 1X, 2X) were tested. Each medium was supplemented with 3% sucrose and 0.35% gelrite. Cultures were incubated in a growth room at 25±1°C under the 16 h light and 8 h dark cycle, with the light intensity of 38 µmol m^{-2} sec^{-1} provided by cool fluorescent tubes. The final observations were recorded after 5 months of incubation.

Acclimation and survival of tissue culture plants: Regenerated plants with well-developed roots were removed from the culture vessels, rinsed gently with the tap water to remove adhering medium and then were transferred to plastic pots (18 cm diameter) containing 10 different mixtures, i.e., (1) peat moss (Pm), (2) Pm: vermiculite (1:1 v/v); (3) Pm: vermiculite: hydrocorns (1:1:1 v/v), (4) sand, (5) sand: vermiculite (1:1 v/v); (6) sand: vermiculite: hydrocorns (1:1:1 v/v); (7) Sphagnum moss (Sm), (8) Sm: vermiculite (1:1 v/v), (9) Sm: vermiculite: hydrocorns (1:1:1 v/v); (10) Sm: scrap of cyatheaceae (1:1 v/v). All potting mixtures were procured from Yu Kuan, Taichung, Taiwan. These pots were kept in the university greenhouse. The plants were water edan alternate day. The survival rate of the plants was recorded after 6 weeks of transplantation.

HPLC analysis of tissue culture plants and commercial crude drug

Preparation of samples: Samples of the whole *D. spatulata* plants (dried) sold as a commercial crude drug were purchased from the drug market in Taichung, Taiwan. *In vitro* shoots (collected from ½ MS basal medium cultured for 5 weeks) and tissue culture derived green house plants (3 months old) were analyzed. These samples were uniformly ground separately and soaked overnight with methanol. After ultrasonic vibration for 60 min, the filtrate was collected and

the residue with the solvent was extracted three times with the above procedure, the filtrate was then mixed and concentrated in a vacuum decompression concentrator with the temperature of 40°C.

Standard, reagents, materials and conditions: HPLC analysis was carried out following our previous reports[22,23]. The mobile phase for plumbagin was a gradient eluting with water/methanol (4:6) at 1 mL min^{-1} at 42°C. The plumbagin was monitored at 245 nm. Plumbagin (Sigma-Aldrich, Inc., St. Louis, MO) of *D. spatulata* were taken and prepared on the standard solution concentration of 100, 50, 25, 12.5, 6.25, 3.125 and 1.5625 μg mL^{-1}, etc, by the serial half-dilution method and then filter it with a 0.45 μm microporous membrane. The tested sample was placed in an auto injector with an injection volume of 10 μL for HPLC analysis. Each concentration treatment was repeated three times of injections for analysis. Afterward, three of them which were averaged to obtain a regression line were combined. According to the sample preparation method described above, the sample of the small felted moss was extracted with methanol, the sample was placed in an automatic injector and the injection amount is 10 μL.

Microphotographs were taken with a microscope (Nikon ECLIPSE E400, Japan) using a digital camera (Nikon Coolpix 4500, Japan). Adventitious shoots were also frozen in liquid nitrogen and observed using a scanning electron microscope (JEOL-JSM-6330F, Japan).

Statistical analysis: Software SAS 9.1 was used for statistical analysis. Data were subjected to the least significant difference (LSD) tested at 5% probability level ($p>0.05$) wherever possible. Each treatment had a minimum of 40 replicates.

RESULTS

Adventitious shoot induction: The induction of adventitious shoots in leaf explants varied with cytokine in type and concentration (Table 1). The response in medium supplemented with Kin (0.1 mg L^{-1}) was higher compared to the medium with BA or devoid of growth regulators. The maximum average number of shoots (1.6) in 65% explants was obtained on ½X MS basal medium supplemented with Kin (0.1 mg L^{-1}), 0.35% gelrite and 3% sucrose after 5 weeks of inoculation (Table 1). Adventitious shoots developed directly from terminal broadly spatulate part of leaf with glandular hairs (Fig. 1b-c). The results on medium supplemented with BA (0.1, 0.3, 0.5, 1.0 and 2.0 mg L^{-1}) had no significant responses on shoots induction. The medium without plant growth regulators supported a lower induction rate and a lower number of shoots per explant compared to 0.1 mg L^{-1} Kin. Concentrations of Kin 0.3-2.0 mg L^{-1} showed lower responses compared to 0.1 mg L^{-1} Kin.

Table 1: Influence of BA and Kinetin (Kin) on the induction of adventitious shoots in leaf explants of *D. spatulata*

Cytokinin (mg L^{-1})*	Explants producing shoots (%)**	Average number of shoots per explant**
0	60^{a}	1.2^{ab}
BA 0.1	9^{cd}	0.3^{ef}
BA 0.3	0^{d}	0.0^{f}
BA 0.5	0^{d}	0.0^{f}
BA 1.0	0^{d}	0.0^{f}
BA 2.0	0^{d}	0.0^{f}
Kin 0.1	65^{a}	1.6^{a}
Kin 0.3	32^{b}	0.9^{bcd}
Kin 0.5	27^{bc}	0.6^{de}
Kin 1.0	22^{bc}	0.7^{cde}
Kin 2.0	30^{b}	1.0^{bc}

*Basal medium: 1/2 MS basal salts supplemented with 3% sucrose, 0.35% gelrite and pH: 5.7±0.1. Data were recorded after 5 weeks of culture. Experiments with 40 replicates in each treatment. **Means followed by the same letter were not significantly different at 5% level by LSD test

Table 2: Influence of different light spectra on induction of adventitious shoots in leaf explants of *D. spatulata*

Light sources*	Explants producing shoots (%)**	Average number of shoots per explant**
Fluorescent	62^{b}	2.5^{d}
Dark	93^{a}	6.4^{a}
Red light (660 nm)	93^{a}	3.5^{c}
Far-red light (730 nm)	93^{a}	4.5^{b}
Blue light (450 nm)	44^{c}	1.6^{e}
White light	28^{d}	1.2^{f}

*Basal medium:1/2 MS basal salts supplemented with 3% sucrose, 0.35% gelrite, 0.1 mg L^{-1} kinetin and pH: 5.7±0.1. Data were recorded after 5 weeks of culture. Experiments with 40 replicates in each treatment. **Means followed by the same letter were not significantly different at 5% level by LSD test

The study showed that incubation under dark, far-red light and red light had the highest induction rate (93%) and induced the maximum number of shoots in *D. spatulata* with an average number of 6.4, 4.5 and 3.5/explant, respectively (Table 2).

Rooting of *in vitro* shoots: Induction of root initials was observed in some treatments after 5 weeks of culture. ½X MS basal medium was found to be more suitable for rooting induction in *D. spatulata* compared to other basal media. The number of adventitious shoots increased with the lower salt concentrations in the medium (Table 3). The superiority of ½X MS basal medium was shown, although ¼X MS and 1X MS basal media also provided satisfactory results. However, 2X MS suppressed the induction of rooting. The

Fig. 2(a-d): *In vitro* growth and rooting of adventitious shoots and acclimation of *D. spatulata*, (a-b) *In vitro* multiple shoots on ½ MS basal medium after five months of culture (bars = 1.3 cm), (c) Induction of *in vitro* flowering in shoots in WPM medium after five months of culture (bars = 1.3 cm) and (d) Potted plants in greenhouse after three months of transfer (Bar = 3.0 cm), (Inset showing *D. spatulata* flower)

Table 3: Influence of different basal media salts on rooting of *in vitro* shoots of *D. spatulata*

Basal salts*	Root of rooting (%)**	Average number of roots per plant cluster**	Average length of roots (cm)**
B_5	98^{a}	19.8^{c}	4.6^{d}
N_6	100^{a}	14.9^{c}	2.5^{e}
WPM	98^{a}	35.6^{b}	2.9^{e}
1/4 MS	100^{a}	31.8^{b}	6.1^{b}
1/2 MS	100^{a}	48.9^{a}	6.7^{a}
1 MS	100^{a}	20.9^{c}	5.2^{c}
2 MS	0^{b}	0.0^{d}	0.0^{f}

*Basal medium: Basal salts supplemented with 3% sucrose, 0.35% gelrite and pH: 5.7±0.1. Data were recorded after 5months of culture. Experiments with 40 replicates in each treatment. **Means followed by the same letter were not significantly different at 5% level by LSD test

maximum average number of roots (48.9) and the average length of roots (6.7 cm) in 100% explants was obtained on ½X MS medium after 5 months of culture (Table 3). Healthy plantlets with a large number of roots were obtained after 5 months of culture (Fig. 2a-b). Among different basal media tested, it was observed that shoots cultured on WPM medium, showed the color of tentacles changed into bright red and *in vitro* plantlets induced precocious flowering after three months of culture (Fig. 2c).

Table 4: Influence of the different potting mixtures on *ex vitro* survival rate of tissue culture plants of *D. spatulata*

Potting mixtures (Ratios)	Survival rate (%)*
Peat moss (Pm)	75^{bcd}
Pm: vermiculite (1:1)	45^{e}
Pm vermiculite hydrocorns (1:1:1)	25^{r}
Sand	85^{ab}
Sand vermiculite (1:1)	75^{bcd}
Sand vermiculite 1 hydrocorns (1:1:1)	65^{d}
Sphagnum moss (Sm)	80^{abc}
Sm vermiculite (1:1)	85^{ab}
Sm vermiculite hydrocorns (1:1:1)	70^{cd}
Sm scrap of cyatheaceae (1:1)	90^{a}

*Means followed by the same letter were not significantly different at 5% level by LSD test

Acclimation and survival of tissue culture plants: Ten different potting mixtures showed acclimation rates in the range of 25-90% (Table 4). A potting mix of sphagnum moss: a scrap of cyatheaceae (1:1) resulted in the highest survival rate of 90%. The plants did not show any morphological variation. Normal flowering (Fig. 2d) was observed after 3 months of transfer to pots.

HPLC analysis of tissue culture plants and commercial crude drug: In the present study, commercial crude drugs, *in vitro*

Table 5: HPLC analysis for plumbagin contents in commercially available crude drug and tissue culture derived materials of *D. spatulata*

Plant samples	Plumbagin (μg g^{-1} dry wt.)
Commercial crude drug (market)	56.94
In vitro shoots (5 weeks old)	11.29
Tissue culture derived greenhouse plants (3 months old)	42.64

plants and tissue culture plant in the greenhouse were used to analyze plumbagin content in *D. spatulata*. The results showed that the content of plumbagin in commercial crude drugs was the highest (56.94 μg g^{-1} DW), followed by tissue culture derived greenhouse plants (42.64 μg g^{-1} DW) and *in vitro* shoots (11.29 μg g^{-1} DW), respectively (Table 5).

DISCUSSION

In the present study, use of a low concentration of Kin and induction of a higher number of *de novo* shoots in *D. spatulata* is an improvement over the earlier studies. The previous report on *Drosera intermedia*[16] showed that a supplement of BA and Kin in the medium caused suppression of growth and resulted in hyperhydricity of *in vitro* cultures. In their study, zeatin was found to be the most suitable cytokinin for multiplication of *D. intermedia*. However, zeatin is a very expensive compound, hence BA and Kin are often used due to its comparatively lower price and effectiveness for an *in vitro* propagation protocol.

Light quantity, quality and duration are known to control morphogenesis, growth and differentiation of plant cells, tissues and organ cultures[24]. Blue and red LED lamps as light sources were mixed for cultivating *Doritaenopsis*[25]. Similarly, researchers studied a series of light sources affecting the plant growth, pointing out that blue light contributes to the induction of shoots in stem culture, while red light helps to generate adventitious roots and increases the fresh weight of leaves, roots, carbon and the synthesis of compounds and leaf pigments[26]. Some of these light sources emit non-essential wavelengths of low quality affecting plant growth and development[27]. Hence, dark and three LED light sources were tested for increasing the induction rate of adventitious shoots. In the present study, incubation in the dark, far-red and red light conditions induced the maximum number of shoots.

Rooting induction results conformed with earlier findings and understandable since carnivorous plants normally grow in nutrient-poor soils[1]. Several other reports on the genus *Drosera* demonstrated that these plants readily and even more successfully grow on diluted culture media[28-31]. Also, similar to the present study, multiplication of *Drosera* species was recommended on a lower strength (¼X) of MS basal medium[32]. In contrary to our results, different nutrient concentrations had no influence on shoot proliferation in *D. spatulata*[33]. The effect of color change of tentacles and induction of precocious flowering in shoots in the present study may be due to WPM medium. When we compare the composition of WPM and MS media, it found that WPM medium is a low salt medium (about ¼ then MS medium). Also, WPM contains Ca $(NO_3)^2 \cdot 4H_2O$ which is absent in MS medium. Therefore, it is possible that WPM may have affected the color change in tentacles. However, further research is needed to confirm this statement. However, a change in tentacles color and flowering under *in vitro* conditions could be a desirable trait for ornamental purpose.

Similar to the findings in the present study, previous researches have shown survival rates in the range of 60-98% in different *Drosera* species such as in *D. indica*[12], *D. burmanii*[34] and *D. intermedia*[18] and the survival rates depended on different potting mixtures and acclimation conditions. A reasonable survival rate is an important step in an in vitro propagation protocol.

Plumbagin, a secondary metabolite contained in *D. spatulata*, is a precious terpenoid that inhibits proliferation of breast cancer cells, initiates autophagy and inhibits AKT activation. Compared to an earlier report[35] the content of plumbagin in *D. spatulata* as 19.6±0.6 μg g^{-1} DW, tissue culture derived greenhouse plants in the present study showed plumbagin content of 42.64 μg g^{-1} DW. Also, a report of plumbagin content in *in vitro* shoots (11.29 μg g^{-1} DW) is a significant finding. One need not wait for rooting and plant establishment steps and can just multiply *in vitro* shoots in large scale to obtain the compound. Though the quantity of plumbagin in *in vitro* shoots is less compared to greenhouse grown plants but that can be compensated by eliminating the crucial rooting and plant establishment steps and thus saving in time and resources. Thus, the present study demonstrates that plumbagin can be obtained by tissue culture round the year under laboratory conditions. Also, it will help in conservation of dwindling resources of the species in the wild.

CONCLUSION

In the present study, micro-propagation method of *D. spatulata* has been standardized. HPLC analysis confirmed the presence of plumbagin in *in vitro* shoots and tissue culture plants in the greenhouse. The developed method will be useful in mass propagation of *D. spatulata* for a continuous supply of plant biomass for a drug industry interested in the production of plumbagin. Also, it will help in conservation of dwindling wild population of *D. spatulata*.

SIGNIFICANCE STATEMENT

Developed micro-propagation method of *Drosera spatulata* is a significant contribution to research community. It will be beneficial for the drug industry and will help in conservation of the species.

REFERENCES

1. Juniper, B.E., R.J. Robins and D.M. Joel, 1989. Phytochemical Aspects. In: The Carnivorous Plants, Juniper, B.E., R.J. Robins and D.M. Joel (Eds.). Academic Press, London, ISBN: 9780123921703, pp: 229-240.
2. Adamec, L., 1997. Mineral nutrition of carnivorous plants: A review. Bot. Rev., 63: 273-299.
3. Didry, N., L. Dubreuil, F. Trotin and M. Pinkas, 1998. Antimicrobial activity of aerial parts of *Drosera peltata* Smith on oral bacteria. J. Ethnopharmacol., 60: 91-96.
4. Bekesiova, I., J.P. Nap and L. Mlynarova, 1999. Isolation of high quality DNA and RNA from leaves of the carnivorous plant *Drosera rotundifolia*. Plant Mol. Biol. Rep., 17: 269-277.
5. Hook, I.L.I., 2001. Naphthoquinone contents of *in vitro* cultured plants and cell suspensions of *Dionaea muscipula* and *Drosera* species. Plant Cell Tissue Organ Cult., 67: 281-285.
6. Kawiak, A., A. Krolicka and E. Lojkowka, 2003. Direct regeneration of *Drosera* from leaf explants and shoot tips. Plant Cell Tissue Organ Cult., 75: 175-178.
7. Conn, B.J., E.A. Brown and A.T. Fairley, 2004. *Utricularia sandersonii* (Lentibulariaceae), a new record for Australia. Telopea, 10: 811-814.
8. Jobson, R.W. and B. Conn, 2012. *Drosera capensis* (Droseraceae), a new naturalised record for Australia. Telopea, 14: 89-92.
9. Nakano, M., E. Kinoshita and K. Ueda, 2004. Life history traits and coexistence of an amphidiploid, *Drosera tokaiensis* and its parental species, *D. rotundifolia* and *D. spatulata* (Droseraceae). Plant Species Biol., 19: 59-72.
10. Zenk, M.H., M. Furbringer and W. Steglich, 1969. Occurrence and distribution of 7-methyljuglone and plumbagin in the droseraceae. Phytochemistry, 8: 2199-2200.
11. Langer, R., I. Pein and B. Kopp, 1995. Glandular hairs in the genus *Drosera* (Droseraceae). Plant Syst. Evol., 194: 163-172.
12. Jayaram, K. and M.N.V. Prasad, 2007. Rapid *in vitro* multiplication of *Drosera indica* L.: A vulnerable, medicinally important insectivorous plant. Plant Biotechnol. Rep., 1: 79-84.
13. Kim, K.S. and G.W. Jang, 2004. Micropropagation of *Drosera peltata*, a tuberous sundew, by shoot tip culture. Plant Cell Tissue Organ Cult., 77: 211-214.
14. Finnie, J.F. and J. van Staden, 1993. *Drosera* spp. (Sundew): Micropropagation and the *in vitro* Production of Plumbagin. In: Medical and Aromatic Plants, Bajaj, Y.P.S. (Ed.). Springer, Berlin, Heidelberg, pp: 164-177.
15. Samaj, J., A. Blehova, M. Repcak, M. Ovecka and M. Bobak, 1999. *Drosera* species (Sundew): *In vitro* Culture and the Production of Plumbagin and other Secondary Metabolites. In: Biotechnology in Agriculture and Forestry, Vol. 43, Medicinal and Aromatic Plants XI, Bajaj, Y.P.S. (Ed.). Springer-Verlag, Berlin, Heidelberg, ISBN: 978-3-642-08363-1, pp: 105-135.
16. Rejthar, J., I. Viehmannova, P.H. Cepkova, E. Fernandez and L. Milella, 2014. *In vitro* propagation of *Drosera intermedia* as influenced by cytokinins, pH, sucrose and nutrient concentration. Emirates J. Food Agric., 26: 558-564.
17. Crouch, I.J., J.F. Finnie and J. van Staden, 1990. Studies on the isolation of plumbagin from *in vitro* and *in vivo* grown *Drosera* species. Plant Cell Tissue Organ Cult., 21: 79-82.
18. Gamborg, O.L., R.A. Miller and K. Ojima, 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res., 50: 151-158.
19. Chu, C.C., C.C. Wang, C.S. Sun, K.C. Hsu, K.C. Yin, C.Y. Chu and F.Y. Bi, 1975. Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Scient. Sin., 18: 659-668.
20. Lloyd, G. and B. McCown, 1980. Commercially-feasible micropropagation of mountain laurel, Kalmia latifolia, by use of shoot-tip culture. Comb. Proc. Int. Plant Prop. Soc., 30: 421-427.
21. Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Planta., 15: 473-497.
22. Chang, H.C., D.C. Agrawal, C.L. Kuo, J.L. Wen, C.C. Chen and H.S. Tsay, 2007. *In vitro* culture of *Drynaria fortunei*, a fern species source of Chinese medicine "Gu-Sui-Bu". *In vitro* Cell. Dev. Biol.-Plant, 43: 133-139.
23. Chen, C.C., H.C. Chang, C.L. Kuo, D.C. Agrawal, C.R. Wu and H.S. Tsay, 2014. *In vitro* propagation and analysis of secondary metabolites in *Glossogyne tenuifolia* (Hsiang-Ju)-a medicinal plant native to Taiwan. Bot. Stud., Vol. 55. 10.1186/s40529-014-0045-7.
24. Reuveni, M. and D. Evenor, 2007. On the effect of light on shoot regeneration in petunia. Plant Cell Tissue Organ Cult., 89: 49-54.
25. Shin, K.S., H.N. Murthy, J.W. Heo, E.J. Hahn and K.Y. Paek, 2008. The effect of light quality on the growth and development of *in vitro* cultured *Doritaenopsis* plants. Acta Physiol. Plant., 30: 339-343.

26. Chee, R. and R. Pool, 1989. Effects of light quality on tissue culture. J. Am. Soc. Hort. Sci., 6: 114-350.
27. Kim, S.J., E.J. Hahn, J.W. Heo and K.Y. Paek, 2004. Effects of LEDs on net photosynthetic rate, growth and leaf stomata of chrysanthemum plantlets *in vitro*. Scient. Hortic., 101: 143-151.
28. Anthony, J.L., 1992. *In vitro* propagation of *Drosera* spp. HortScience, 27: 850-850.
29. Crouch, D.J. and J. van Staden, 1988. *In vitro* propagation of *Drosera natalensis*. S. Afr. J. Bot., 54: 94-95.
30. Kukulczanka, K. and B. Czastka, 1987. Propagation of *Drosera* L. *in vitro* condition. Wiad. Bot. Biul. Ogrodow Botanicznych, 31: 61-64.
31. Simola, L.K., 1978. Dipeptides as nitrogen sources for *Drosera rotundifolia* in aseptic culture. Physiol. Plant., 44: 315-318.
32. Grevenstuk, T., N. Coelho, S. Goncalves and A. Romano, 2010. *In vitro* propagation of *Drosera intermedia* in a single step. Biol. Planta., 54: 391-394.
33. Perica, M.C. and J. Berljak, 1996. *In vitro* growth and regeneration of *Drosera spatulata* Labill. on various media. HortScience, 31: 1033-1034.
34. Jayaram, K. and M.N.V. Prasad, 2008. Rapid *in vitro* multiplication of *Drosera burmanii* Vahl.: A vulnerable and medicinally important insectivorous plant. Indian J. Biotechnol., 7: 260-265.
35. Babula, P., R. Mikelova, V. Adam, R. Kizek, L. Havel and Z. Sladky, 2006. Using of liquid chromatography coupled with diode array detector for determination of naphthoquinones in plants and for investigation of influence of pH of cultivation medium on content of plumbagin in *Dionaea muscipula*. J. Chromatogr. B, 842: 28-35.