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Original Paper

In vitro propagation and chemical profiling of *Herminium lanceum* (Thunb. ex Sw.) Vuijk, a medicinally important orchid, for therapeutically important phenolic acids

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Abstract The paper describes an efficient and reproducible protocol for in vitro propagation of *Herminium lanceum*, a medicinal orchid, natural polulations of which are adversely affected because of its indiscriminate collections and other anthropogenic activities. The germination of seeds at different stages of embryo development was compared on four media: Mitra's (M), M +0.1% peptone (M+P), 1/2 Murashige and Skoog's+0.1% peptone (MS+P) and Knudson C+0.1% peptone (KC+P). The seeds that contained multicellular oval embryos and cultured on M+P exhibited the highest percentage (82.94%) of germination. Protocorm-like bodies (PLBs), developed from the seeds, could be multiplied by repeated sub-cultures on M+P (hereafter referred to as basal medium, BM). PLBs sub-cultured on BM, supplemented with different concentrations of 6-benzylaminopurine (BAP), kinetin (KN) or 1-phenyl-3-(1, 2, 3-thiadiazol-5-yl)-urea (TDZ) differentiated shoots. The maximum number of shoots per culture developed on BM+4 μM TDZ. Shoot elongation was the best on BM+0.1% AC+1 μM GA_3. Among various media tested for rooting of the shoots, the best rhizogenic response was obtained on BM+4 μM TDZ+0.1 μM IBA. About 82% of in vitro regenerated plantlets survived on transfer to the potting mixture of sand : vermiculite (1 : 1). High performance liquid chromatographic (HPLC) analyses revealed the presence of medicinally important phenolic acids in leaves and tubers of both in vitro and in vivo plants. The developed protocol could be used for the large-scale production of propagules required for the cultivation of this plant as a regular source of herbal, and also for eco-restoration of its native habitats.

Key words: asymbiotic seed germination, HPLC, in vitro multiplication, medicinal plant, phenolic acids, protocorm-like bodies.

For centuries, orchids have been used in traditional systems of medicine for curing a number of ailments (Hossain 2011). In the absence of agro-technologies for their cultivation, majority of the medicinal orchids are collected from the wild. Traditional cultivation through seeds, as practiced for majority of crops, cannot be applied to orchids because of their reproductive peculiarities. Orchids produce millions of microscopic seeds, which lack endosperm and contain immature embryo. Only a tiny fraction of these germinate, because of the mandatory requirement of mycorrhizal association (Robert and Dixon 2008). However, in in vitro cultures all the seeds can be made to germinate asymbiotically, as highlighted for the first time by Knudson (1922). The protocorms developed from the germinating seeds can be further multiplied to have large number of propagules. In vitro differentiation from the somatic tissues can also produce large number of plants (Paek et al. 2011). Consequently, tissue culture has found immense application in multiplication of orchids.

Phenolics because of their antioxidant, anti-carcinogenic, anti-mutagenic and anti-inflammatory activities, constitute important constituents of medicinal herbs (Huang et al. 2009). Some of the important phenolic acids are gallic acid (GA), *p*-hydroxybenzoic acid (HBA), syringic acid (SA) and caffeic acid (CA). GA has been shown to have anti-melanogenic, antioxidant and anti-carcinogenic properties (Faried et al. 2007; Kim 2007). HBA has antioxidant (Natella et al. 1999), antifungal, antimicrobial (Chong et al. 2009) and oestrogenic properties (Pugazhendhi et al. 2005). SA exhibited antibacterial (Kong et al. 2008), hepatoprotective (Itoh et al. 2009, 2010) and antioxidant properties (Khadem and Marles 2010). Likewise, CA is well known to have antioxidant (Moon and Terao 1998; Yumrutas et al. 2011) and antifungal activities (Silici et al. 2007), besides having cytotoxic effects on tumor cells (Chen et al. 1996; Grunberger et al. 1988).

Herminium lanceum, locally known as 'Jalya,' is a terrestrial tuberous herb (Jalal et al. 2008). The extract of the plant is prescribed to cure urinary disorders, diabetes, fever and bleeding (Joshi et al. 2009; Mall et al. 2015).

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The populations of this medicinally important orchid are adversely affected owing to anthropogenic activities and extensive collections from the wild. Currently, no information on its in vitro propagation and biochemical profiling is available. The present study describes an efficient and reproducible in vitro propagation protocol for its mass multiplication. Besides, the presence and contents of four medicinally important phenolic acids in the leaves and tubers of in vitro raised plants and those collected from their native habitats are also reported.

Materials and methods

Plant material

The inflorescences of *Herminium lanceum* with capsules at various stages of development (Figure 1A) were collected from Dhanaulti, Uttarakhand, India (Latitude: 78°12′02.82″E, Longitude: 30°25′42.91″N, Altitude: 2378 m), during the first week of September 2011 and the last week of August 2012. The botanical identity of the plants was authenticated at Botanical Survey of India, Dehradun (Uttarakhand) and the herbarium specimen of one of the collected plants was deposited in the Delhi University herbarium (DUH, Voucher No. 13740).

In vitro studies

Freshly collected capsules of different sizes were washed under running tap water for 30 min and were then submerged in a mixture of Tween-20 and teepol, a commercial detergent (2 drops of each/50 ml) for 3 min each. Thereafter, under aseptic conditions, capsules were treated with 0.25% mercuric chloride (w/v)) for 8 min and 70% ethyl alcohol for 30 s. These were then flamed. Finally, capsules were rinsed 5–6 times with sterilized distilled water. Seeds having embryos at different stages of development (Figure 1C–D), were scooped out from the surface-sterilized capsules of three different sizes (ca. 0.5, 0.6–0.7 and 0.8 cm, Figure 1B, C), and were cultured on four nutrient media: Mitra's (M, Mitra et al. 1976), M+0.1% peptone (M+P)), Knudson C (Knudson 1946)+P (KC+P) and 1/2 strength Murashige and Skoog's (MS, Murashige and Skoog 1962)+P (MS+P), supplemented with 2% sucrose and gelled with 0.8% agar (w/v). To ascertain the stage of embryo development in seeds of capsules of different sizes, seeds scooped out from each were stained with 1% acetocarmine and were examined and photographed under a Zeiss photomicroscope. Prior to autoclaving at 121°C and 105 kPa for 15 min, pH was adjusted to 5.6 with 0.1 N NaOH or 0.1 N HCl. About 20 ml of culture medium was dispensed in each culture tube (Borosil, India, size: 25×150 mm), which were plugged with a cotton plug (non-adsorbent cotton wrapped in a layer of cheese cloth). For further experiments, M+P medium, hereafter referred to as basal medium (BM), was used. The protocorms and protocorm-like bodies (PLBs) developed after 60 days of culture of seeds on BM, were either sub-cultured on BM alone for multiplication and maintenance of PLBs or on BM supplemented with 1, 2, 4 or 8 μM of BAP, KN or TDZ. The shoots differentiated from the protocorms were multiplied by sub-culturing on the same regeneration medium for one more passage in 250 ml flasks, each containing 100 ml of culture medium. The shoots were sub-cultured on BM containing PVP (0.1%) or AC (0.1%) supplemented with 0.2, 1, 4 or 8 μM of GA_3 For elongation. Individual shoots, each having two expanded leaves, were separated from the shoot clusters and transferred to BM containing 4 μM TDZ along with 0.1, 0.5, 1 or 2 μM of either IAA or IBA. After 8 weeks of incubation on the rooting medium, the rooted shoots (plantlets), after thorough rinsing with lukewarm water (40–50°C) to remove traces of agar and residual nutrients, were transplanted to perforated plastic pots (12 cm diameter) filled with potting mixture of sterile sand and vermiculite (1:1). The plants were covered with porous transparent polybags for initial four weeks. During the first two weeks, the plantlets were mist irrigated with liquid BM without sucrose but supplemented with 0.1% Bavastin (w/v) on alternate days. Third week onwards, these were irrigated with tap water. All cultures and the transferred plants were maintained at 25±2°C in 16 h photoperiod (63 μmol $m^{-2} s^{-1}$) provided by cool white 40 W fluorescent tubes (Philips, India). Survival rates of the transferred plants were recorded after 8 weeks.

Scanning electron microscopy

For scanning electron microscopic study, samples (capsules, seeds and protocorms) were fixed in Karnovsky's fluid (Karnovsky 1965), made in 0.2 M Phosphate buffer (pH 7.4), for 12 h at 4°C. Thereafter, specimens were dehydrated in graded acetone series (10–100%), with 5 min exposure in each concentration. Critical Point Drying (CPD) of the samples was carried out at 1100 psi and 35°C using CO_2. Gold sputter coating was done under reduced pressure of 10^{-1} Pa in an inert argon gas atmosphere (Auto Fine Coater JFC-1600) at 25°C. The coated samples were examined and photographed under Jeol Scanning Electron Microscope (JSM 6610LV) operated at 3 kVA.

Biochemical profiling for phenolic acids

The oven dried (48 h at 50°C) leaves and tubers from the in vitro plants (length of the plants 2.5–5.0 cm, leaves' length 0.7–2.5 cm, 0.5–2.6 cm long tubers with diameter being 0.2–0.8 cm) and those from the wild (33.5–40 cm height of the plants, 5.3–12.5 cm long leaves and tubers having lengths of 2.1–2.9 cm and diameters 0.7–1.5 cm) were pulverized in liquid nitrogen. The powdered 100 mg of each sample was incubated for 12 h in 5 ml solution of acetonitrile (Merck Specialist Pvt. Ltd., India) and water (HPLC grade, HiMedia) (1:1) contained in 50 ml Falcon tubes on a rotary shaker (200 rpm, 25°C). After centrifugation at 10,000 *g* for 20 min, supernatant was filtered through Millipore filters (0.22 μm). The filtrates were stored at 4°C till further use. The profile of phenolic acids in each sample was analyzed and the concentrations of individual phenolic acids was estimated by 'Waters' High Performance Liquid chromatographic system consisting of dual 515 pump, C18

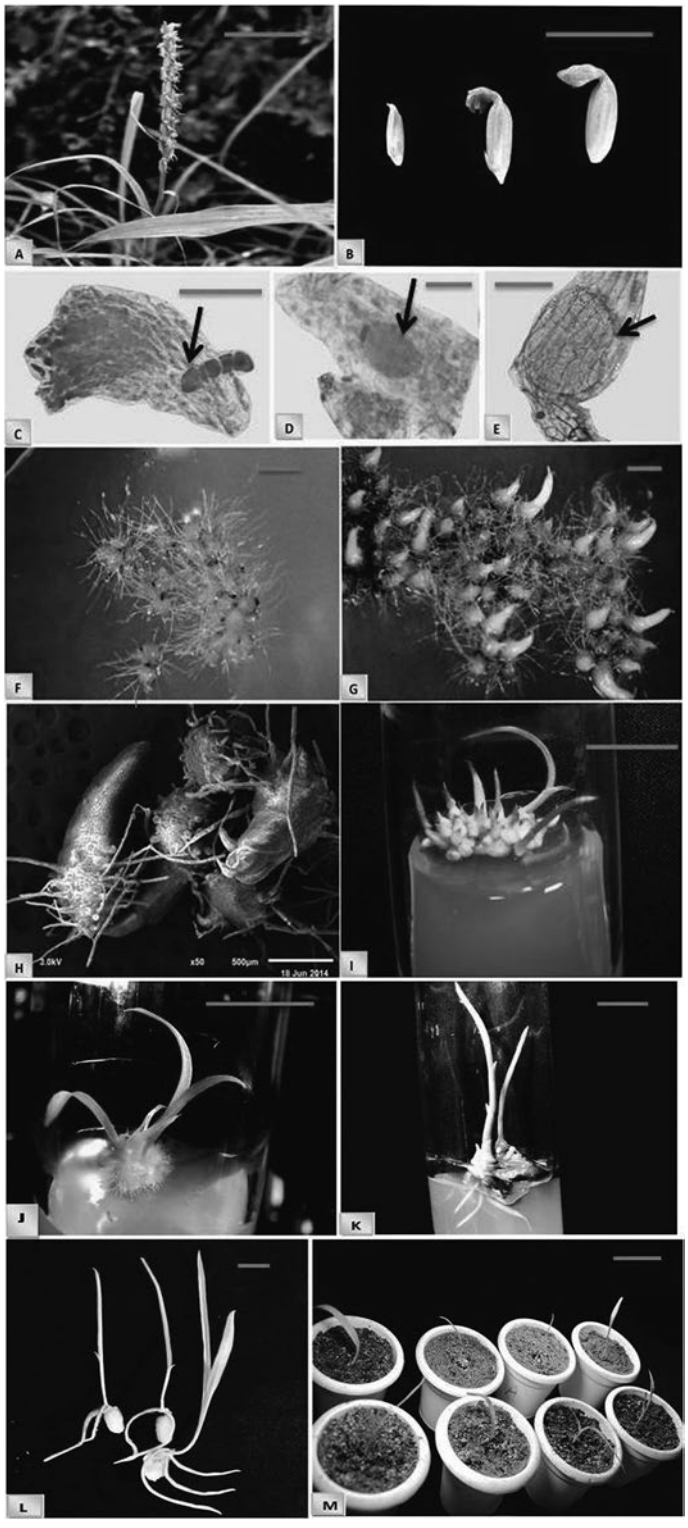

Figure 1. *Herminium lanceum*, seeds to plants: (A) A mature plant with capsules at different stages of development in its natural habitat (Bar=1 cm); (B) Green capsules of three different sizes (Bar=1 cm); (C–E) Acetocarmine stained representative seeds from the capsules of different sizes (smaller to larger, respectively) to show the stages of embryo (arrow) in each (Bars=0.1 mm); (F) PLBs developed from seeds germinated on M+P medium after 40 days of culture (Bar=1 mm); (G) Initiation of leaves on PLBs on BM after 60 days of culture (Bar=1 mm); (H) Scanning electron micrograph of PLBs with rhizoids; (I) Multiple shoots from sub-cultured PLBs after 60 days of culture on BM+4 μM TDZ (Bar=1 cm); (J) Elongated shoots 60 days after transfer to BM+1 μM GA_3 (Bar=1 cm); (K) Rooted shoots (plantlets), 60 days after transfer to M+4 μM TDZ+0.1 μM IBA (Bar=1 cm); (L) Plantlets ready for transfer to pots (Bar=1 cm); (M) Plantlets transferred to potting mix (sand and vermiculite (1 : 1) for hardening (Bar=5 cm).

Colum (250 mm×4.6 mm, 5 μm) and 2998 photodiode array detector. Estimations were done using gradient elution system. The column was washed with both mobile phases, comprising 50 mM ammonium acetate (pH 3.5 adjusted with acetic acid), sonicated for 2 min, and acetonitrile, till a stable base was obtained. The initial and final conditions for ammonium acetate were set at 90% for 0–20 min, so that both mobile phases returned to the original condition in the last 5 min. A flow rate of 1 ml/min was maintained during the course of estimation. The eluent was monitored at 270 nm. The calibration curve was plotted by using 50 to 500 μg/ml of each standard, prepared in acetonitrile : water (1 : 1). Twenty microliters of each sample was injected to the column for analyses. 'Empower' software was used for the identification and estimation of the amount of individual phenolic acids. This was further confirmed by 'spiking' the samples with standard solutions of the phenolic

acids.

Experiment design and data analysis

For the experiment on seed germination, each treatment per replicate comprised ten culture tubes, each containing an average of 9.32±0.12 seeds per test tube. For shoot multiplication, elongation and rooting, the number of cultures per treatment of each replicate was also ten. For shoot multiplication three PLBs per tube were cultured, while for elongation and rooting there were two shoots per culture tube. Each of the four experiments was repeated at least twice. The % seed germination represents the percentage of seeds developing spherules. Shoot multiplication and growth responses are being presented as % cultures responding, average number of shoots per culture and average length of shoots after 60 days of culture. As many a times, it was difficult to ascertain the origin of shoots from individual PLBs in a tube, the culture tube having shoots was counted as the responding culture. The analysis of phenolic acids in various samples was also repeated twice. The data were analyzed using one-way ANOVA and the significance of the differences among treatments of each experiment was evaluated by Duncan's multiple range test at $p=0.05$ using SPSS (version 16.0) software package. The values of one parameter followed by the same superscript(s) in each of the tables presented are not significantly different.

Results and discussion

Orchids require species specific germination media (Arditti and Ernst 1984; Kauth et al. 2008). Several media have been tested for asymbiotic seed germination of orchids. The most widely used media are MS, Mitra's and Knudson C media. On all these media, first sign of seed germination of *Herminium lanceum* was observed after 10–12 days of inoculation as nodular swellings. The embryos swelled to form spherules with rhizoids and later developed into achlorophyllous protocorms (1F, G). Chlorophyll development preceded proliferation of protocorm-like bodies (PLBs), which developed leaf primordia from their apical regions (1H). The highest percentage of seed germination was obtained on Mitra's medium (M) supplemented with peptone (Table 1). This observation is in conformity with findings of Hossain et al. (2010), who observed Mitra's medium to be the most supportive for in vitro seed germination and seedling development of *Cymbidium giganteum*.

Table 1. Comparison of germination of seeds of *Herminium lanceum*, scooped from capsules of different sizes and cultured on different nutrient media, after 40 days of culture.

Capsule size/Media	% Seed germination[#]			
	M ($\bar{X}\pm$SE)	M+P ($\bar{X}\pm$SE)	MS+P ($\bar{X}\pm$SE)	KC+P ($\bar{X}\pm$SE)
Small	4.17 ± 0.53^{fg}	4.90 ± 0.85^{efg}	7.90 ± 1.19^{ef}	1.36 ± 0.24^{g}
Medium	5.17 ± 0.75^{efg}	9.21 ± 1.46^{ef}	11.28 ± 1.67^{e}	3.63 ± 0.56^{fg}
Large	43.21 ± 2.01^{b}	82.94 ± 1.65^{a}	35.81 ± 1.42^{c}	27.01 ± 1.78^{d}

[#]Seeds that developed spherules. Values followed by same superscript(s) in columns are not significantly different at $p=0.05$, $N=36$, ±Standard Error.

The immature seeds germinate readily and much better than mature ones, as has been evidenced for *Dendrobium florum, Cymbidium elagans* (Pradhan and Pant 2009), *Satyrium nepalense* (Mahendran and Bai 2009), *Cypripedium calceolus*, *Dactylorhiza maculata*, *Epipactis helleborine*, *Goodyera repens*, *Gymnadenia conopsea* (Pindel and Pindel 2004). Among three sizes of capsules, seeds from the largest, containing oval-shaped multicellular embryos (Figure 1E), exhibited much higher germination response on all the tested media than those from the smaller capsules, seeds of which contained embryos as small as tetrad to octant stages with few cell suspensors (Figure 1C, Table 1).

Peptone, a water soluble protein with high contents of amino acids, amides and vitamins, stimulates seed germination (Oliva and Arditti 1984). In the present study, addition of peptone to Mitra's medium almost doubled the percentage of seed germination (Table 1). The stimulatory effect of peptone on the germination of orchid seeds has earlier been reported for *Paphiopedilum* and *Vanda* (Curtis 1947), *Dactylorhiza incarnata* ssp. *serotina*, *Dactylorhiza maculata* ssp. *maculata*, *Liparis loeselii* (Vejsadová 2006), *Cymbidium giganteum* (Hossain et al. 2010). In contrast, peptone adversely affected the seed germination of *Habenaria clalvellata* (Kauth 2005).

PLBs of *Hermimium lamceum* multiplied when sub-cultured on the BM. These have now been maintained for one and half years by repeatedly sub-culturing on BM after intervals of two weeks. The choice of cytokinin and its concentration plays a determining role in in vitro shoot multiplication in plants including orchids. Therefore, in the present study, for the development of shoots, 8-week-old PLBs were transferred from BM to the same medium supplemented with 0, 1, 2, 4 or 8 μM of BAP, KN or TDZ. On all these media, PLBs multiplied and developed into shoots (Figure 1H, I). None of the cytokinins at the tested concentrations affected percentage of responding cultures significantly. However, the average number of shoots per culture was enhanced by all. This increase in number of shoots was significantly higher on the media containing BAP (2, 4 or 8 μM), KN (2 or 4 μM) or TDZ (4 μM) than the control (BM). The best response of 3.58 shoots per culture was observed on BM +4 μM TDZ (Table 2). TDZ, a substituted phenylurea, has proved to be better than purine containing cytokinins, such as, BAP and KN, for the regeneration of a number of plants including recalcitrant woody species, legumes (Shulze 2007) and orchids, *Acampe praemorsa* (Ernst 1994; Nayak et al. 1997a, b), *Anoectochilus formosanus* (Ket et al. 2004), *Dendrobium* hybrids (Martin and Madassery 2006), *Dendrobium candidum* (Zhao et al. 2007), *Satyrium*

nepalese (Mahendran and Bai 2009) and *Phalaenopsis gigantea* (Niknejad et al. 2011).

The shoots developed on cytokinin supplemented media failed to elongate further and started turning brown. Therefore, these were transferred to BM fortified with either activated charcoal (0.1% AC), polyvinylpyrrolidone (0.1% PVP), different concentrations of gibberellic acid (GA_3) or the last in combination with either AC or PVP (Table 3). GA_3 is well-known for its effect on elongation of stems, petioles and inflorescences (DeMason 2005). It promoted the elongation of shoots significantly at all the tested concentrations (Table 3, Figure 1J). Activated charcoal (AC), a non-selective adsorbent is known to absorb a number of constituents from the culture medium, including inhibitory substances like phenols, and essential adjuvant, like myo-inositol and phytohormones (Babbar and Gupta 1986; Fridborg et al. 1978). In the present study, AC significantly promoted the elongation of shoots and along with GA_3 (1 μM) supported the best response among all the treatments (Table 3). The elongation of shoots of *Nephelium lappaceium* was also promoted by a combination of GA_3 and AC (Chew et al. 2008). PVP (0.1%) neither alone nor in combination with GA_3 had a similar positive effect on shoot elongation.

In the current investigation, rooting competency of the in vitro generated shoots was tested on BM supplemented with 0.1, 0.5, 1 or 2 μM IAA or IBA. Among all these treatments, stout and highest number of roots per responding shoot was obtained on the medium containing 0.1 μM IBA (Table 4, Figure 1K). Sultana and Handique (2005) too reported IBA to be better than IAA for the rooting of *Wedelia chinensis* shoots. The efficiency of IBA in root induction has also been observed in *Cymbidium pendulum* (Nongdam et al. 2006). For *Vanilla planifolia*, IBA alone at 0.5 mg l^{-1} induced the highest number of roots (Neelannavar et al. 2011). The observed differential effects of various auxins on the rooting of in vitro raised shoots could be due to the differences in their uptake, transport and metabolism (see Barpete et al. 2014).

Effective and successful tissue culture can only be realized if plantlets are successfully transferred from in vitro to ambient conditions (Hazarika 2003). During the current investigation, 82% of 72 plantlets of *Herminium lanceum* transferred to ex vitro conditions survived (Figure 1L, K). The reduced size and density of stomata, limited water supply because of low root conductivity and poor root–stem connection in the in

Table 2. Effect of cytokinins, BAP and KN, supplemented individually to BM at varying concentrations, on the differentiation and development of shoots from in vitro developed PLBs of *Herminium lanceum* compared after 60 days of culture.

Cytokinin (μM)	% Responding cultures[#]	Average No. of shoots/culture[#]	Average length of shoots (cm) ($\bar{X}\pm$SE)
0	73.33^{ab}	0.80±0.16^{f}	3.30±0.25^{a}
BAP (1)	53.33^{b}	1.22±0.41^{def}	3.50±0.38^{a}
BAP (2)	60.00^{ab}	2.56±0.71^{abcd}	3.00±0.29^{ab}
BAP (4)	60.00^{ab}	2.24±0.65^{abcde}	3.30±0.35^{a}
BAP (8)	73.33^{ab}	3.18±0.59^{ab}	1.50±0.12^{bcde}
KN (1)	60.00^{ab}	1.26±0.31^{def}	2.50±0.24^{abcd}
KN (2)	73.33^{ab}	2.87±0.56^{abc}	1.20±0.12^{cde}
KN (4)	66.67^{ab}	2.43±0.58^{abcde}	2.30±0.20^{abcde}
KN (8)	60.00^{ab}	1.20±0.30^{ef}	2.90±0.22^{abc}
TDZ (1)	93.33^{a}	1.93±0.26^{bcdef}	1.80±0.13^{abcde}
BM+TDZ (2)	73.33^{ab}	1.53±0.34^{cdef}	2.00±0.17^{abcde}
BM+TDZ (4)	86.67^{ab}	3.58±0.52^{a}	1.00±0.10^{de}
BM+TDZ (8)	86.67^{ab}	1.87±0.29^{bcdef}	0.60±0.05^{e}

[#]Each culture contained three PLBs. Values followed by same superscript(s) in a column are not significantly different at $p=0.05$, $N=39$, ±Standard Error.

Table 3. Effect of activated charcoal (AC), polyvinylpyrrolidone, (PVP) and gibberellic acid (GA_3), provided individually or in combinations in BM, on the elongation of shoots of *Herminium lanceum* compared after 60 days of transfer.

Treatment	Average Initial height (cm) ($\bar{X}\pm$SE)	Mean length of shoots (cm) ($\bar{X}\pm$SE)	Percent increase in shoot length (%)
0	0.72±0.01	1.40±0.05^{efg}	106.02^{fghi}
GA (0.2 μM)	0.61±0.01	0.75±0.03^{g}	41.30^{i}
GA (1 μM)	1.06±0.05	2.80±0.11^{bc}	227.36^{de}
GA (4 μM)	0.87±0.03	3.20±0.10^{b}	328.46^{bc}
GA (8 μM)	0.75±0.01	2.00±0.08^{de}	261.06^{cd}
AC (0.1%)	0.63±0.01	2.20±0.09^{cd}	368.09^{b}
AC (0.1%)+GA(0.2 μM)	0.61±0.02	0.90±0.05^{g}	82.61^{ghi}
AC (0.1%)+GA(1 μM)	0.64±0.02	4.00±0.08^{a}	565.63^{a}
AC (0.1%)+GA(4 μM)	0.60±0.02	1.60±0.06^{def}	174.74^{defg}
AC (0.1%)+GA (8 μM)	0.64±0.02	1.20±0.09^{fg}	183.85^{def}
PVP (0.1%)	0.69±0.03	0.78±0.05^{g}	89.42^{ghi}
PVP (0.1%)+GA (0.2 μM)	0.67±0.02	1.20±0.08^{fg}	165.50^{defg}
PVP (0.1%)+GA (1 μM)	0.79±0.03	1.10±0.05^{fg}	67.80^{hi}
PVP (0.1%)+GA (4 μM)	0.52±0.02	1.00±0.06^{fg}	144.87^{efgh}
PVP (0.1%)+GA (8 μM)	0.69±0.03	0.79±0.05^{g}	37.98^{i}

Values followed by same superscript(s) in a column are not significantly different at $p=0.05$, $N=45$, ±Standard Error.

Table 4. Rooting of the in vitro regenerated shoots of *Herminium lanceum* as affected by the concentration and type of auxin supplement to BM.

Auxin (μM)	% Responding culture[#]	Average No. roots/responding shoot	Average length of roots (cm) ($\bar{X}\pm$SE)
0	66.67^{a}	2.32±0.08^{cd}	1.06±0.13^{c}
IAA (0.1)	73.33^{a}	2.00±0.06^{cde}	0.85±0.18^{c}
IAA (0.5)	48.33^{a}	1.50±0.07^{e}	0.60±0.11^{c}
IAA (1)	60.00^{a}	1.75±0.07^{de}	0.72±0.09^{c}
IAA (2)	60.00^{a}	1.83±0.08^{cde}	4.32±0.53^{a}
IBA (0.1)	73.33^{a}	4.29±0.15^{a}	1.96±0.29^{b}
IBA (0.5)	60.00^{a}	2.45±0.09^{c}	0.65±0.10^{c}
IBA (1)	46.67^{a}	2.07±0.07^{cde}	1.60±0.24^{b}
IBA (2)	66.67^{a}	3.09±0.12^{b}	1.05±0.16^{c}

[#]Each culture contained two shoots. Values followed by same superscript(s) in a column are not significantly different at $p=0.05$, $N=27$, ±Standard Error.

vitro regenerated are some of the factors which affect the successful transfer of micropropagated plants to field (Fila et al. 1998; Pospóšilová et al. 1999).

Apart from other unidentified peaks, the presence of GA, HBA, SA and CA was detected in leaves and tubers of both in vitro as well as in vivo plants (Figure 2A–D). The concentrations of the first three phenolic acids, GA, HBA and SA, were significantly higher in the leaves of both in vitro and in vivo plants than in their respective tubers. On the contrary, caffiec acid content in all four explants was statistically similar. Among the four, levels of CA were the highest in all four types of tissues, followed by GA, SA and HBA (Table 5). The leaves and tubers of in vitro plants contained higher amount of GA and SA than those of in vivo plants (Table 5). The presence of secondary metabolites in higher concentrations in in vitro raised plants than in wild ones has also been reported earlier. For example, the concentrations of rosmarinic acid, chlorogenic acid, hydrobenzoic acid and pellitorine were higher in in vitro regenerated plants of *Rosmarinus officinalis* (Guo et al. 2007), *Saussurea involucrate* (Yesil-Celiktas et al. 2007), *Habenaria edgeworthii* (Giri et al. 2011) and *Anacyclus pyrethrum* (Singh et al. 2015), respectively, than in the

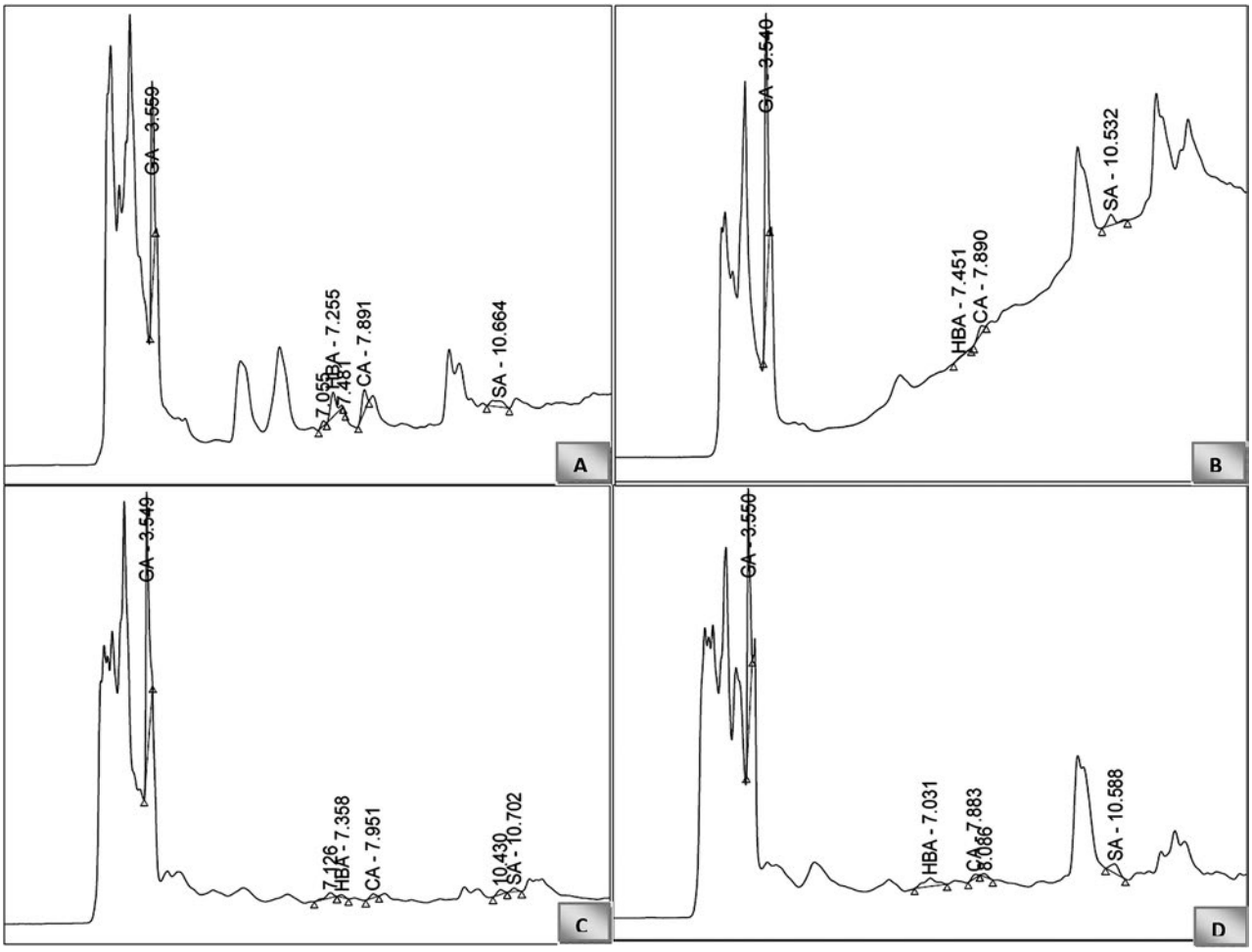

Figure 2. HPLC profiles of the extracts of tubers and leaves of in vivo (A, B, respectively) and in vitro (C, D respectively), analyzed for the presence and contents of gallic acid (GA), *p*-hydrooxybenzoic acid (HBA), syringic acid (SA) and caffeic acid (CA).

Table 5. Contents of four medicinally important phenolic acids, gallic acid (GA), *p*-hydroxybenzoic acid (HBA), syringic acid (SA) and caffeic acid (CA) in leaves and tubers of both in vitro raised plants of *Herminium lanceum* and those from their native habitat (in vivo).

Plant samples	Phenolic acids (μg/g DW)			
	GA	HBA	SA	CA
In vitro leaves	3461.60 ± 78.82^{b}	235.33 ± 56.59^{h}	1676.50 ± 66.23^{d}	4099.17 ± 60.1^{a}
In vitro tubers	2847.77 ± 45.66^{c}	24.67 ± 11.97^{i}	725.73 ± 87.45^{g}	4044.62 ± 82.76^{a}
In vivo leaves	2878.12 ± 66.97^{c}	157.83 ± 47.46^{hi}	1121.55 ± 72.34^{f}	4159.08 ± 37.15^{a}
In vivo tubers	1305.72 ± 43.57^{e}	21.87 ± 10.43^{i}	844.65 ± 60.76^{g}	4163.47 ± 78.36^{a}

Values followed by same superscript in columns are not significantly different at $p=0.05$, $N=12$, $\pm$Standard Error.

corresponding plants from the wild. The observed differences in the phenolic acids' contents in the organs of in vitro and in vivo plants, in the present study as well few earlier investigations on different plants, should not be emphasized as these could be because of the differences in their age and the climatic conditions under which the two types of plants grew.

Conclusions

The paper describes an efficient and reproducible protocol for mass multiplication of a medicinal orchid, *Herminium lanceum*. The application of this protocol could minimize the stress on its populations in wild by meeting the demand of pharmaceutical industries through micropropagation. The regenerated plants could be relocated to the sites where population of this species has dwindled or has completely disappeared. The analysis of phenolic acids in plants from the wild as well as in vitro regenerated plants is first report of any kind of biochemical profiling of *Herminium lanceum* and could be considered as the first step towards providing rational explanation for the observed therapeutic effects of the plant.

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References

Arditti J, Ernst R (1984) Physiology of germinating orchid seeds. In: Arditti J (ed) *Orchid Biology: Reviews and Perspectives*. Cornell University Press, Ithaca, New York, pp 172–222

Babbar SB, Gupta SC (1986) Promotory and inhibitory effects of activated charcoal on microspore embryogenesis in *Datura metel*. *Physiol Plant* 66: 602–604

Barpete S, Khavar KM, Özcan S (2014) Differential competence for in vitro adventitious rooting of grass pea (*Lathyrus sativus* L.). *Plant Cell Tissue Organ Cult* 119: 39–50

Chen JH, Shao Y, Huang MT, Chin CK, Ho CT (1996) Inhibitory effect of caffeic acid phenethyl ester on human leukemia HL-60 cells. *Cancer Lett* 108: 211–214

Chew P, Normah M, Clyde M (2008) Activated charcoal is crucial for successful micropropagation of rambutan (*Nephelium lappaceum* Linn.). *Malaysian Appl Biol* 37: 11–20

Chong KP, Rossall S, Atong M (2009) In vitro antimicrobial activity and fungitoxicity of syringic acid, caffeic acid and 4-hydroxybenzoic acid against *Ganoderma Boninense*. *J Agric Sci* 1: 15–20

Curtis JT (1947) Studies on the nitrogen nutrition of orchid embryos. I. Complex nitrogen sources. *Am Orchid Soc Bull* 16: 654–660

DeMason DA (2005) Auxin–cytokinin and auxin–gibberellin interactions during morphogenesis of the compound leaves of pea (*Pisum sativum*). *Planta* 222: 151–166

Ernst R (1994) Effects of thidiazuron on in vitro propagation of *Phalaenopsis* and *Doritaenopsis* (Orchidaceae). *Plant Cell Tissue Organ Cult* 39: 273–275

Faried A, Kurnia D, Faried L, Usman N, Miyazaki T, Kato H, Kuwano H (2007) Anticancer effects of gallic acid isolated from Indonesian herbal medicine, *Phaleria macrocarpa* (Scheff.) Boerl, on human cancer cell lines. *Int J Oncol* 30: 605–613

Fila G, Ghashghaie J, Hoarau J, Cornic G (1998) Photosynthesis, leaf conductance and water relations of in vitrocultured grapevine rootstock in relation to acclimatisation. *Physiol Plant* 102: 411–418

Fridborg G, Pedersen M, Landstorm LE, Eriksson T (1978) The effect of activated charcoal on tissue culture: Absorption of metabolites inhibiting morphogenesis. *Physiol Plant* 43: 104–106

Giri L, Jugran A, Rawat S, Dhyani P, Andola H, Bhatt ID, Rawal RS, Dhar U (2011) In vitro propagation, genetic and phytochemical assessment of *Habenaria edgeworthii*: An important Astavarga plant. *Acta Physiol Plant* 34: 869–875

Grunberger D, Banerjee R, Eisinger K, Oltz E, Efros L, Caldwell M, Estevez V, Nakanishi K (1988) Preferential cytotoxicity on tumor cells by caffeic acid phenethyl ester isolated from propolis. *Experientia* 44: 230–232

Guo B, Gao M, Liu CZ (2007) In vitro propagation of an endangered medicinal plant *Saussurea involucrata* Kar. et Kir. *Plant Cell Rep* 26: 261–265

Hazarika BN (2003) Acclimatization of tissue-cultured plants. *Curr Sci* 85: 1704–1712

Hossain MM (2011) Therapeutic orchids: Traditional uses and recent advances: An overview. *Fitoterapia* 82: 102–140

Hossain M, Sharma M, Teixeira da Silva JA, Pathak P (2010) Seed germination and tissue culture of *Cymbidium giganteum* Wall. ex Lindl. *Sci Hortic* (Amsterdam) 123: 479–487

Huang HW, Cai YZ, Zhang Y (2009) Natural phenolic compounds from medicinal herbs and dietary plants: Potential use for cancer prevention. *Nutr Cancer* 62: 1–20

Itoh A, Isoda K, Kondoh M, Kawase M, Kobayashi M, Tamesada M, Yagi K (2009) Hepatoprotective effect of syringic acid and vanillic acid on concanavalin a-induced liver injury. *Biol Pharm Bull* 32: 1215–1219

Itoh A, Isoda K, Kondoh M, Kawase M, Watari A, Kobayashi M, Tamesada M, Yagi K (2010) Hepatoprotective effect of syringic acid and vanillic acid on CCl_4-induced liver injury. *Biol Pharm Bull* 33: 983–987

Jalal JS, Kumar P, Pangtey Y (2008) Ethnomedicinal orchids of Uttarakhand, Western Himalaya. *Ethnobot Leafl* 12: 1227–1230

Joshi G, Tewari LM, Lohani N, Upreti K, Jalal JS, Tewari G (2009) Diversity of orchids in Uttarakhand and their conservation strategy with special reference to their medicinal importance. *Report Opinion* 1: 47–52

Karnovsky MJ (1965) A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J Cell Biol* 27: 1A–149A

Kauth P (2005) *In vitro* seed germination and seedling development of *Calopogon tuberosus* and *Sacoila lanceolata* var. *lanceolata*: Two Florida native terrestrial orchids. Master Thesis, University of Florida

Kauth PJ, Johnson TR, Stewart SL, Kane ME (2008) A classroom exercise in hand pollination and in vitro asymbiotic orchid seed

germination. *Plant Cell Tissue Organ Cult* 93: 223–230
Ket N, Hahn E, Park S, Chakrabarty D, Paek K (2004) Micropropagation of an endangered orchid *Anoectochilus formosanus*. *Biol Plant* 48: 339–344
Kim YJ (2007) Antimelanogenic and antioxidant properties of gallic acid. *Biol Pharm Bull* 30: 1052–1055
Khadem S, Marles RJ (2010) Monocyclic phenolic acids; hydroxy- and polyhydroxybenzoic acids: Occurrence and recent bioactivity studies. *Molecules* 15: 7985–8005
Knudson L (1922) *Nonsymbiotic* germination of orchid seeds. *Bot Gaz* 73: 1–25
Knudson L (1946) A new nutrient solution for germination of orchid seed. *Am Orch Soc Bull* 15: 214–217
Kong W, Zhao Y, Shan L, Xiao X, Guo W (2008) Thermochemical studies on the quantity-antibacterial effect relationship of four organic acids from *Radix Isatidis* on *Escherichia coli* growth. *Biol Pharm Bull* 31: 1301–1305
Mahendran G, Bai VN (2009) Mass propagation of *Satyrium nepalense* D. Don.: A medicinal orchid via seed culture. *Sci Hortic* (Amsterdam) 119: 203–207
Mall B, Gauchan DP, Chhetri RB (2015) An ethnobotanical study of medicinal plants used by ethnic people in Parbat district of Western Nepal. *J Ethnopharmacol* 165: 103–117
Martin K, Madassery J (2006) Rapid in vitro propagation of *Dendrobium* hybrids through direct shoot formation from foliar explants, and protocorm-like bodies. *Sci Hortic* (Amsterdam) 108: 95–99
Mitra G, Prasad R, Roychowdhury A (1976) Inorganic salts and differentiation of protocorms in seed callus of an orchid and correlated changes in its free amino acid content. *Indian J Exp Biol* 14: 350–351
Moon HJ, Terao J (1998) Antioxidant activity of caffeic acid and dihydrocaffeic acid in Lardand human low-density lipoprotein. *J Agric Food Chem* 46: 5062–5065
Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 15: 473–497
Natella F, Nardini M, Di Felice M, Scaccini C (1999) Benzoic and cinnamic acid derivatives as antioxidants: Structure–activity relation. *J Agric Food Chem* 47: 1453–1459
Nayak N, Patnaik S, Rath S (1997a) Direct shoot regeneration from foliar explants of an epiphytic orchid, *Acampe praemorsa* (Roxb.) Blatter and Mccann. *Plant Cell Rep* 16: 583–586
Nayak NR, Rath SP, Patnaik S (1997b) In vitro propagation of three epiphytic orchids, *Cymbidium aloifolium* (L.) Sw., *Dendrobium aphyllum* (Roxb.) Fisch. and *Dendrobium moschatum* (Buch-ham) Sw. through thidiazuron-induced high frequency shoot proliferation. *Sci Hortic* (Amsterdam) 71: 243–250
Neelannavar V, Biradar M, Kumar A, Shivamurthy D (2011) In vitro propagation studies in vanilla (*Vanilla planifolia* Andr.). *Plant Arch* 11: 377–378
Niknejad A, Kadir M, Kadzimin S (2011) In vitro plant regeneration from protocorms-like bodies (PLBs) and callus of *Phalaenopsis gigantea* (Epidendroideae: Orchidaceae). *Afr J Biotechnol* 10: 11808–11816
Nongdam P, Nirmala C, Tewari R (2006) In vitro multiplication of *Cymbidium pendulum* orchids via embryo culture. *Plant Cell Biotechnol Mol Biol* 7: 145–150
Oliva AP, Arditti J (1984) Seed germination of North American orchids. II. Native California and related species of *Aplectrum, Cypripedium*, and *Spiranthes*. *Bot Gaz* 145: 495–501
Paek Y-P, Hahn E-J, Park S-Y (2011) Micropropagation of *Phalaenopsis* orchids via protocorms and protocorm-like bodies. In: Thorpe TA, Yeung EC (eds) *Plant Embryo Cuture: Methods and Processes*. Methods in Molecular Biology 710: 293-306
Pindel A, Pindel Z (2004) Initiation of in vitro cultures of chosen endangered European species of orchids. *Folia Hort* 16: 111–117
Pospóšilová J, Tichá I, Kadleček P, Haisel D, Plzáková Š (1999) Acclimatization of micropropagated plants to *ex vitro* conditions. *Biol Plant* 42: 481–497
Pradhan S, Pant B (2009) In vitro seed germination in *Cymbidium elegans* Lindl. and *Dendrobium densiflorum* Lindl. ex Wall. (Orchidaceae). Bot. Orientalis. *J Plant Sci* 6: 100–102
Pugazhendhi D, Pope G, Darbre P (2005) Oestrogenic activity of *p*-hydroxybenzoic acid (common metabolite of *Paraben esters*) and methylparaben in human breast cancer cell lines. *J Appl Toxicol* 25: 301–309
Robert DL, Dixon KW (2008) Orchids. *Curr Biol* 18: R325–R329
Shulze J (2007) Improvements in cereal tissue culture by thidiazuron: A review. Fruit, Vegetable and Cereal. *Sci Biotech* 1: 64–79
Silici S, Ünlü M, Vardar-Ünlü G (2007) Antibacterial activity and phytochemical evidence for the plant origin of *Turkish propolis* from different regions. *World J Microb Biot* 23: 1797–1803
Singh F (1986) Orchids. In: Chadha K, Chaudhury B, Pal BP (eds) *Ornamental Horticulture in India*. ICAR, New Delhi, India. pp 127–153
Singh DK, Nirwan S, Babbar SB (2015) Micropropagation of *Anacyclus pyrethrum* and chemical profiling of the regenerated plants for pellitorine, the active principle. *Plant Cell Tissue Organ Cult* 122: 249–255
Sultana S, Handique PJ (2005) Micropropagation of *Wedelia chinensis* through high frequency shoots multiplication using nodal explants. In: Kumar A (ed) *Environmental Biology*. APH Publishing Corporation, New Delhi, India, pp 677–683
Vejsadová H (2006) Factors affecting seed germination and seedling growth of terrestrial orchids cultured in vitro. *Acta Biol Cracoviensia Botanica* 48: 109–113
Yesil-Celiktas O, Girgin G, Orhan H, Wichers H, Bedir E, Vardar-Sukan F (2007) Screening of free radical scavenging capacity and antioxidant activities of *Rosmarinus officinalis* extracts with focus on location and harvesting times. *Eur Food Res Technol* 224: 443–451
Yumrutas O, Sokmen A, Ozturk N (2011) Determination of *in vitro* antioxidant activities and phenolic compounds of different extracts of *Salvia verticillata* ssp. *verticillata* and ssp. *amasiaca* from Turkey's flora. *J Appl Pharm Sci* 1: 43–46
Zhao P, Wang W, Feng SF, Wu F, Yang JQ, Wang WJ (2007) High-frequency shoot regeneration through transverse thin cell layer culture in *Dendrobium candidum* Wall. ex Lindl. *Plant Cell Tissue Organ Cult* 90: 131–139