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RAJINDER SINGH CHAUHAN

See next page for additional authors

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In vitro protocorm development and mass multiplication of an endangered orchid, *Dactylorhiza hatagirea*

Ashish Rambhau WARGHAT¹, Prabodh Kumar BAJPAI¹, Ravi Bihari SRIVASTAVA¹, Om Prakash CHAURASIA¹, Rajinder Singh CHAUHAN², Hemant SOOD*²

¹Defence Institute of High Altitude Research, Defence Research and Development Organisation, Leh-Ladakh, Jammu and Kashmir, India ²Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh, India

Abstract: Immature seeds were cultured on 10 different media for germination. Maximum germination was achieved on Lindeman orchid medium (37.12%) within 17 days of culturing. Protocorms with leaf primordia were cultured on BM-2 and 7 different modifications of Murashige and Skoog (MS) medium with various hormone combinations [0–3 mg/L indole butyric acid (IBA) and 0–3 mg/L kinetin (Kin)] for plantlet regeneration and mass multiplication. Maximum number of shoots (18.12 ± 0.3), highest shoot length (17.80 cm ± 2.16), maximum root number (8.25 ± 0.69), and highest root length (8.02 cm ± 1.45) were found on MS medium with 3 mg/L IBA and 1 mg/L Kin. Plantlets with 2–3 shoots were transferred to different potting mixtures for acclimatization to field conditions and further multiplication. One hundred percent survival was obtained in C-8 potting mixture consisting of cocopeat + vermiculite + perlite (1:1:1), which produced 75 shoots (25 plantlets) after 1 month of transplantation in the greenhouse. The current study describes for the first time a rapid in vitro protocorm development and mass multiplication protocol for *Dactylorhiza hatagirea* (D.Don) Soo that holds robust potential for large-scale propagation and metabolite production.

Key words: Conservation, development, mass multiplication, potting mixture, protocorm formation

1. Introduction
Among the flowering plants, orchids represent one of the largest groups, with highly colorful, attractive flowers exhibiting a long shelf-life with varied shapes and sizes. They are of great value to the floriculture industry as cut flowers and potted plants. Orchid seeds are microscopic and nonendospermous with undifferentiated embryos. They are produced in large numbers and their germination in nature depends upon a suitable association with mycorrhizal fungus, thus making it difficult to observe their further development after they are released from the seed capsule soil (Temjensangba and Deb, 2006). Currently, orchids are the second most popular potted floriculture crop and the production and sales of native orchids are on the rise. The small market for some native terrestrial orchids is due to the difficulties in propagation methods and the long time period required to obtain flowering (USDA, 2004).

The orchid *Dactylorhiza hatagirea* (D.Don) Soo is indigenous to the Himalayas and is exclusively found in the Ladakh region of Jammu and Kashmir, India. It has been identified as a critically endangered species (CAMP status) and is listed under Appendix II of Convention of International Trade in Endangered Species (Uniyal et al., 2002). *Dactylorhiza hatagirea* is also a high-value medicinal orchid used in the Indian system of medicine, particularly in Ayurveda, Siddha, and Unani medicine (Pant and Rinchen, 2012). It is widely used to cure various diseases including dysentery, diarrhea, chronic fever, cough, stomachache, wounds, cuts, burns, fractures, and general weakness. The tubers of *Dactylorhiza hatagirea* contain glucoside, a bitter substance, and starch, mucilage, albumen, a trace of volatile oil, and ash (Dutta and Karn, 2007). Chemically, dactylorhins A to E, dactyloses A and B, lipids, and so on are found as major constituents. The tubers of this species yield a high quality salep, which is used as a tonic to increase immunity (immunomodulator) and as a vajikaran drug (aphrodisiac); it increases sexual performance as well as serving as a nerve tonic (Baral and Kurmi, 2006). The annual consumption of salep obtained from the species in India is about 7.38 t (valued at about 5,000,000 rupees or US$83,333) and has great demand in national and international markets (Badola and Pal, 2002).
The orchid species are rapidly declining because of their low rate of propagation, poor seed germination, and ongoing collection from nature. Careless collection of these species has led to serious genetic and ecological erosion; many have already been listed as endangered species (Machaka-Houri et al., 2012). The rate of vegetative propagation in Dactylorhiza hatagirea is very slow and seed germination in nature is very poor, i.e. 0.2%–0.3% (Vij, 2002). In vitro germination of seeds can be an important mode for orchid multiplication and conservation programs, since the dust seeds are tiny and contain few food reserves. Both in situ and ex situ approaches are important for the protection of rare and endangered orchid species. Tissue culture is one of the most important measures in ex situ conservation of terrestrial orchids (Jakobsone et al., 2007). Knowledge of the physiological and morphological aspects of the germination and development of particular orchid species is of critical importance for the establishment of tissue culture. The current study reports for the first time on the optimization of increased in vitro immature seed germination, seedling development, and plantlet formation, as well as multiplication of Dactylorhiza hatagirea plants grown under in vitro and in vivo conditions, for rescuing their declining population by reintroducing them to their native habitats.

2. Materials and methods

2.1. Seed source

Seeds of Dactylorhiza hatagirea were obtained from immature capsules collected from naturally pollinated plants growing in a farm at Tirith village (altitude 3183 ± 8.2 m, 34°32′09.977″N, 77°38′52.386″E), in the Nubra valley of the Ladakh region of India. The seeds were air-dried for 2 weeks at room temperature and stored in small bottles at 4 °C until use.

2.2. Seed viability test

2.2.1. TTC staining

Immature seeds were soaked in filtered triphenyl tetrazolium chloride (TTC) solution (1 g in 100 mL of phosphate buffer, pH 6.5–7.0) for 48 h in darkness at 20 ± 2 °C and rinsed 5 times in sterile distilled water. Seeds were agitated between cover slides to remove testa and viewed using a Nikon microscope. The embryos that stained pink to red were considered viable, while seeds with partially colored, white, or brown embryos were assumed to be nonviable (Van Waes and Deberg, 1986).

2.2.2. FDA staining

The immature seeds were soaked in fluorescein diacetate (FDA) solution composed of equal volumes of distilled water and FDA stain (0.5 g in 100 mL of absolute acetone) for 15 min and viewed with an Olympus BH-2 (UV light) fluorescence microscope. Seeds with embryos completely stained (fluorescent) were considered viable (Rasmussen, 1995). The same test was repeated for viability of seeds collected from stored seed samples at 4 °C at 1-week intervals for 1 month.

2.3. Nutrient media

Ten different basal media were tested for immature seed germination. Five of the media were commercial preparations with modifications from HiMedia Laboratories Pvt. Limited, Mumbai, India: terrestrial orchid medium (BM-1); Vacin and Went modified (VW) medium (Vacin and Went, 1949); Malmgreen modified terrestrial orchid (MM) medium (Malmgren, 1996); Mitra (PT139) orchid medium (Mitra et al., 1976), and Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). Three of the media were modified by Titan Biotech Limited, Rajasthan, India: Knudson C (KC) orchid medium (Knudson, 1946); Heller (TP039) medium (Heller, 1953), and Lindemann (LD) orchid medium (Lindemann et al., 1970). Two of the media were procured from Phyto Technology Laboratories, LCC (Shawnee Mission, KS, USA): Fast (F522) terrestrial orchid medium (Fast, 1976) and orchid seed sowing medium (P723). The pH of the media was adjusted to 5.7 with 0.1 N HCl and 0.1 N NaOH, after the addition of sucrose and agar; 15 mL of medium was dispensed in each test tube (25 × 150 mm), and tubes were plugged and autoclaved for 20 min at 121 °C and 1.05 kg cm⁻² pressure.

2.4. Seed surface sterilization and inoculation

Immature seeds were surface-sterilized for 2 min in 5 mL of ethanol (100%) and for 15–20 min in 5 mL of 0.1% NaOCl, followed by rinsing in 90 mL of sterile distilled deionized water for 15–20 min. Solutions were removed from the surface sterilization vials using disposable 1000-µL sterile pipette tips that were replaced after each use. Seeds were then suspended in sterile deionized distilled water and a sterile inoculating loop was used for inoculating culture tubes. The inoculating loop was immersed once into the seed suspension and seeds were placed in the culture tubes. One replicate consisted of 1 test tube containing 100–150 seeds.

2.5. Culture conditions

Cultures were incubated in a growth chamber maintained at 25 ± 1 °C under a 16/8-h photoperiod with illumination of 3000 lux intensity of white light.

2.6. In vitro seed germination and development of protocorms

The seeds were germinated and differentiated into protocorm-like bodies (PLBs) on the same germination medium. The cultures were monitored regularly and the data were scored at weekly intervals. After 2 and 3 weeks of germination, the protocorm development was assessed using a dissection stereoscope. Germination and protocorm
development were scored on a scale of 0–5 (Zettler and McInnis, 1994): stage 0, no germination; stage 1, rupture of testa due to swelling of embryo (i.e. germination); stage 2, protocorm formation and emergence of rhizoids; stage 3, emergence of leaf primordium (shoot); stage 4, appearance of first leaf; stage 5, elongation of leaf and root differentiation. The percentage germination was calculated by dividing the number of seeds in each individual germination and development stage by the total number of viable seeds in the sample. The remaining seeds were dried in petri dishes with anhydrous CaCl₂, for 2 weeks at 5 °C. Dried seeds were transferred into Eppendorf microtubes and stored in hermetically closed containers at −20 °C. All experiments were repeated 3 times. The experimental design was completely randomized.

2.7. Plantlet regeneration and mass multiplication
The PLBs developed from cultured immature embryos were maintained on optimum basal germination medium for further differentiation to form leaf primordia. The PLBs with leaf primordia were separated from the germination medium and cultured on regeneration media. For regeneration of plantlets and their multiplication, BM-2 and MS media supplemented with combinations of growth regulators [0–3 mg/L indole butyric acid (IBA) and 0–3 mg/L kinetin (Kin)] were tested. The resulting microshoots were separated from the regeneration medium for further mass multiplication. During plantlet regeneration, data were recorded for shoot number, shoot length, root number, root length, and number of days required for shoot/root formation. The well-developed plantlets were maintained on the same regeneration medium before transferring for hardening.

2.8. Acclimatization and in vivo multiplication of plantlets
Fully grown plantlets were taken from the regeneration medium and traces of agar were removed with a soft brush. Plantlets of 5–10 cm in shoot length, with 5–7 roots, were successfully transplanted to controlled greenhouse conditions into 8 combinations of potting mixtures: C-1 (sand + soil, 1:1), C-2 (sand + soil + farmyard manure, 1:1:1), C-3 (sand + soil + cocopeat, 1:1:1), C-4 (sand + soil + vermiculite, 1:1:1), C-5 (sand + soil + perlite, 1:1:1), C-6 (cocopeat + vermiculite, 1:1), C-7 (vermiculite + perlite, 1:1), and C-8 (cocopeat + vermiculite + perlite, 1:1:1). Initially, for 10–15 days the plantlets were covered with glass jars to provide sufficient humidity and avoid desiccation until the plantlets showed new growth. During the hardening process, glass jars were taken off every day for 1–2 h so as to acclimatize the plantlets to the external environment, and data were recorded for percent survival, number of shoots and roots, and length of shoots and roots.

2.9. Data analysis
Univariate statistical procedures were used to analyze the data. Descriptive analysis of the data was performed using SPSS 19. Analysis of variance (ANOVA) with comparative Duncan’s multiple range tests at 5% was used to determine the significance of differences between treatments.

3. Results

3.1. Seed viability
The interpretation of seed viability using TTC was very difficult. Seeds of Dactylorhiza hatagirea are shown in Figure 1A. Viewing the immature embryos was only possible after removing the testa, a tedious and delicate process, since terrestrial orchid seeds are very minute (200 to 1700 µm). Staining colors that distinguished viability were very difficult to determine and the method was abandoned. By contrast, FDA was an excellent stain for observing viable seeds (Figures 1B and 1C). Fluorescence was easily recognized and stained seeds were easily counted without removing the testa, which was transparent. With the FDA method the highest percentage (24%) of viable embryos was observed in the first week of sampling, followed by a significant reduction (13%) in the fourth week of sampling, whereas the TTC method showed high viability (22%) in the first week and 11% in the fourth week of sampling (Table 1).

3.2. Asymbiotic seed germination
Seeds began swelling within 2 weeks after inoculation and germinated to form protocorms within the third week of inoculation, as is shown in stage 1 and Figures 2A–2C. Of the 10 media tested, seed germination was highest (37.12%) in LD medium, followed by 35.48%, 34.48%, and 31.68% in TP039, BM-1, and 0.5 MS media, respectively. Maximum protocorm formation (23.40%) occurred in LD medium, followed by 21.40%, 20.4%, and 18.6% on TP039, BM-1, and 0.5 MS media, respectively (Table 2). Seed germination was minimal in MM, KC, VW, F522, and orchid seed media, even after 4 weeks of inoculation. Protocorm formation occurred on the rest of the media after 7 weeks of incubation. The germinating seeds converting into PLBs were maintained on the same germination medium for further differentiation into leaf primordia. Formation of first leaf from PLBs was developed in all media except PT139, as is shown in stage 3 and Figures 2D and 2E.

3.3. Regeneration and mass multiplication
The protocorms with first leaf primordia were cultured on BM-2 and 7 different MS media supplemented with different concentrations and combinations of IBA and Kin for shoot formation. The protocorm with first leaf primordia developed into multiple shoots within 15 to 20 days of incubation in shoot regeneration media and further developed into plantlets with shoots and roots within 28 to
Figure 1. Immature seeds (3 week after pollination) of Dactylorhiza hatagirea. A- 10×; B- viable seeds stained with TTC (60×); C- viable seeds stained with FDA (200×). Scale bars = 1 mm.

Table 1. Viability of Dactylorhiza hatagirea stored seeds (4 °C) at 1-week intervals.

<table>
<thead>
<tr>
<th>Days of storage</th>
<th>Percent seed viability in TTC stain</th>
<th>Percent seed viability in FDA stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>22 ± 5</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>14</td>
<td>14 ± 2</td>
<td>18 ± 7</td>
</tr>
<tr>
<td>21</td>
<td>11 ± 8</td>
<td>15 ± 3</td>
</tr>
<tr>
<td>28</td>
<td>11 ± 1</td>
<td>13 ± 9</td>
</tr>
</tbody>
</table>

Values are mean ± SE.

Table 2. Immature seed germination and protocorm formation in Dactylorhiza hatagirea on different nutrient media.

<table>
<thead>
<tr>
<th>Nutrient medium</th>
<th>Immature seed germination (%)</th>
<th>Immature seeds converting to PLBs (%)</th>
<th>No. of days required for protocorm formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM1</td>
<td>34.48 ± 0.69&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>20.4 ± 0.30&lt;sup&gt;d&lt;/sup&gt;</td>
<td>17</td>
</tr>
<tr>
<td>VW</td>
<td>1.52 ± 0.36&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.92 ± 0.86&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>25</td>
</tr>
<tr>
<td>MM</td>
<td>2.48 ± 0.35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.64 ± 0.82&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>22</td>
</tr>
<tr>
<td>Orchid seed</td>
<td>0.60 ± 0.26&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.68 ± 0.70&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>23</td>
</tr>
<tr>
<td>F522</td>
<td>0.92 ± 0.31&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.46 ± 0.77&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>21</td>
</tr>
<tr>
<td>KC</td>
<td>1.58 ± 0.37&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.08 ± 0.88&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>22</td>
</tr>
<tr>
<td>PT139</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LD</td>
<td>37.12 ± 0.91&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>23.40 ± 0.30&lt;sup&gt;d&lt;/sup&gt;</td>
<td>17</td>
</tr>
<tr>
<td>TP039</td>
<td>35.48 ± 0.70&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>21.40 ± 0.31&lt;sup&gt;d&lt;/sup&gt;</td>
<td>23</td>
</tr>
<tr>
<td>0.5 MS</td>
<td>31.68 ± 0.72&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>18.6 ± 0.43&lt;sup&gt;d&lt;/sup&gt;</td>
<td>20</td>
</tr>
</tbody>
</table>

PLBs: Protocorm-like bodies.
ANOV A test results are highly significant according to the Duncan multiple range test at 5% (P = 0.05). Values with different letters in the same column are statistically different.
<sup>*</sup>: Data shown are the mean of 3 replicates of 25 cultures in each treatment ± SE. Data were recorded at 1-week intervals.
42 days of incubation (Table 3). Growth and development of plantlets with maximum number of shoots/protocorm (18.12 ± 0.3), shoot height (17.80 cm ± 2.16), number of roots (8.25 ± 0.69), and maximum root length (8.02 cm ± 1.45) occurred on MS medium supplemented with IBA (3 mg/L) + Kin (1 mg/L) within 28 to 30 days of incubation. MS medium containing Kin at 3 mg/L showed relatively fewer numbers of shoots (16.00 ± 1.26) and other respective parameters of growth and development until 34 to 40 days of culturing (Figures 3A–3C).

**Figure 2.** Classification of in vitro seed germination and seedling development into different stages in *Dactylorhiza hatagirea*. A- Stage 0, no germination; B- stage 1, swelling of embryos and bursting of testa; C- stage 2, protocorm formation and emergence of rhizoids; D- stage 3, emergence of leaf primordia; E- stages 4 and 5, appearance of first leaf and root development. Scale bars: A, B, and C = 1 mm; D and E = 1 cm.
3.4. Acclimatization and in vivo multiplication of plantlets

The plantlets grown in vitro with 2–3 shoots were transferred to different potting mixtures for their acclimatization to field conditions and for further growth and multiplication. The highest plantlet survival (100%) was in the C-8 potting mixture consisting of cocopeat + vermiculite + perlite (1:1:1), with a significantly higher number of shoots (75), maximum number of plantlets (25), highest shoot length (18.8 cm), maximum number of roots

Table 3. In vitro shoot multiplication in *Dactylorhiza hatagirea* on different media.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Shoots/protocorm</th>
<th>Shoot length</th>
<th>Root number</th>
<th>Root length</th>
<th>No. of days required for shoot/root formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM-2</td>
<td>7.32 ± 1.3a</td>
<td>7.80 ± 1.16a</td>
<td>6.01 ± 0.15abc</td>
<td>6.73 ± 0.68abc</td>
<td>37–42</td>
</tr>
<tr>
<td>MS + 3 mg/L Kin</td>
<td>16.00 ± 1.26a</td>
<td>17.31 ± 0.51a</td>
<td>8.00 ± 2.04abc</td>
<td>7.04 ± 0.16abc</td>
<td>34–40</td>
</tr>
<tr>
<td>MS + 1 mg/L IBA + 2 mg/L Kin</td>
<td>14.02 ± 0.32abc</td>
<td>16.00 ± 1.26a</td>
<td>7.00 ± 0.51bcd</td>
<td>6.73 ± 0.23abc</td>
<td>31–35</td>
</tr>
<tr>
<td>MS + 1 mg/L IBA + 3 mg/L Kin</td>
<td>13.34 ± 0.28bc</td>
<td>13.96 ± 1.27bc</td>
<td>7.11 ± 1.67bcd</td>
<td>6.70 ± 0.27bcd</td>
<td>30–35</td>
</tr>
<tr>
<td>MS + 2 mg/L IBA + 1 mg/L Kin</td>
<td>12.42 ± 0.43abc</td>
<td>16.92 ± 3.78b</td>
<td>6.11 ± 2.04cd</td>
<td>5.94 ± 0.28ab</td>
<td>29–35</td>
</tr>
<tr>
<td>MS + 2 mg/L IBA + 2 mg/L Kin</td>
<td>10.67 ± 0.67b</td>
<td>16.67 ± 1.67bc</td>
<td>6.22 ± 2.40cd</td>
<td>5.38 ± 0.39ab</td>
<td>30–31</td>
</tr>
<tr>
<td>MS + 3 mg/L IBA</td>
<td>9.11 ± 0.44b</td>
<td>15.00 ± 1.15ab</td>
<td>6.11 ± 2.04ab</td>
<td>5.34 ± 0.23ab</td>
<td>35–40</td>
</tr>
<tr>
<td>MS + 3 mg/L IBA + 1 mg/L Kin</td>
<td>18.12 ± 0.3b</td>
<td>17.80 ± 2.16b</td>
<td>8.25 ± 0.69bc</td>
<td>8.02 ± 1.45b</td>
<td>28–30</td>
</tr>
</tbody>
</table>

BM-2: Basal medium-2; MS- Murashige and Skoog medium; IBA- indole butyric acid; Kin- kinetin.

ANOVA test results are highly significant according to Duncan's multiple range test at 5% (P = 0.05). Values with different letters in the same column are statistically different.

*: Data shown are the mean of 10 replicates ± SE.

Figure 3. In vitro shoot multiplication, hardening, and in vivo mass multiplication of *Dactylorhiza hatagirea*. A- Initiation of shooting; B- in vitro shoot proliferation; C- fully developed plantlets in vitro; D- hardening of plantlets; E- in vivo growth, development, and mass multiplication of plant (15 days); F- after 30 days. Scale bars = 5 cm.
(23), and highest root length (44.7 cm) after 1 month of transplantation in the greenhouse. Satisfactory percentage survival of plantlets was observed on the potting mixtures, whereas plantlets’ multiplication parameters were lowest in C-1 potting mixture with a survival percentage of 52.5%, 29 shoots, 9.66 plantlets, 13.2 cm shoot length, 15 roots, and 33.2 cm root length (Table 4; Figures 3 and 4).

4. Discussion
Habitat destruction, fragmentation, overexploitation, and the effects of global climate change are the main threats to the survival of orchids (Bubb et al., 2004). *Dactylorhiza hatagirea* is indigenous to the temperate regions of Sikkim, Uttaranchal, and parts of Jammu and Kashmir. Very little information is published on the growth and

<table>
<thead>
<tr>
<th>Potting mixture</th>
<th>Survival (%)</th>
<th>Shoots' (no.) (1 month)</th>
<th>Plantlets' (no.)</th>
<th>Average shoot length' (cm)</th>
<th>Roots' (no.) (1 month)</th>
<th>Root length' (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1</td>
<td>52.5</td>
<td>29 ± 0.2bc</td>
<td>9.66 ± 1.0d</td>
<td>13.2 ± 0.5c</td>
<td>15 ± 0.1ab</td>
<td>33.2 ± 0.2bc</td>
</tr>
<tr>
<td>C-2</td>
<td>60.3</td>
<td>34 ± 0.1b</td>
<td>11.33 ± 0.3d</td>
<td>13.5 ± 0.5bc</td>
<td>16 ± 0.4bc</td>
<td>34.7 ± 0.3b</td>
</tr>
<tr>
<td>C-3</td>
<td>75.2</td>
<td>55 ± 0.2ab</td>
<td>18.33 ± 0.4ab</td>
<td>13.0 ± 0.1bc</td>
<td>19 ± 0.6ab</td>
<td>36.8 ± 0.2bc</td>
</tr>
<tr>
<td>C-4</td>
<td>70.9</td>
<td>52 ± 0.3b</td>
<td>17.33 ± 0.2a</td>
<td>15.0 ± 0.3bc</td>
<td>18 ± 0.5bc</td>
<td>40.5 ± 0.4a</td>
</tr>
<tr>
<td>C-5</td>
<td>70.2</td>
<td>51 ± 0.9d</td>
<td>17 ± 0.0b</td>
<td>15.8 ± 0.7c</td>
<td>16 ± 0.2a</td>
<td>41.0 ± 0.2c</td>
</tr>
<tr>
<td>C-6</td>
<td>90.7</td>
<td>63 ± 0.8bc</td>
<td>21 ± 0.0b</td>
<td>16.2 ± 0.2b</td>
<td>21 ± 0.1b</td>
<td>42.9 ± 0.0b</td>
</tr>
<tr>
<td>C-7</td>
<td>84.9</td>
<td>59 ± 0.9bc</td>
<td>19.66 ± 0.2bc</td>
<td>17.5 ± 0.2bc</td>
<td>19 ± 0.2b</td>
<td>43.5 ± 0.1a</td>
</tr>
<tr>
<td>C-8</td>
<td>100</td>
<td>75 ± 1.0abc</td>
<td>25 ± 0.1a</td>
<td>18.8 ± 0.8ab</td>
<td>23 ± 0.9bc</td>
<td>44.7 ± 0.9bc</td>
</tr>
</tbody>
</table>


ANOVA test results are highly significant according to Duncan’s multiple range test at 5% (P = 0.05). Values with different letters in the same column are statistically different.

*: Data shown are the mean of 3 replicates of 10 cultures in each treatment ± SE.

Figure 4. Mass-multiplied plantlets of *Dactylorhiza hatagirea* ready for transfer to field conditions. Scale bar = 15 cm.
seedling development of Dactylorhiza hatagirea in its natural habitat and as of today no information is available on in vitro seed germination and mass multiplication of plantlets. Ramsay and Stewart (1998) suggested that the period of time when immature seeds germinate efficiently in axenic conditions is short, approximately 7–10 days, and should be experimentally estimated for each species. Previous studies revealed that no single nutrient medium is universally suitable for asymbiotic seed germination for most of the orchid taxa; PT139 medium was found suitable for Cymbidium macrorhizon Lindl. (Vij et al., 1998) and Goodyera biflora Lindl. (Pathak et al., 1992), KC medium for Cymbidium elegans (Lindl.) and Coelogyne punctulata Lindl. (Sharma and Tandon, 1990), VW medium for Vanda coerulea Lindl. (Devi et al., 1998), Nitsch medium for Cymbidium iridioides D.Don (Jamir et al., 2002), and KC, VW, and MS media for Aerides rosea ex Lindl. (Sinha et al., 1998).

In vitro germination of terrestrial orchid species seeds is often a long and slow process due to considerably longer required periods for germination and specific mycorrhiza as reported by De Pauw and Remphery (1993), and as in Dactylorhiza majalis (Rchb.) seeds that started to germinate after 6 weeks of culture on F522 medium (Znaniecka and Łojkowska, 2004). Dactylorhiza rutheï (M.Schulze) and Dactylorhiza praetermissa (Druce) seeds started to germinate after 4 months of culture on Norstog medium (Vaasa and Rosenberg, 2004). Germination of Dactylorhiza rutheï (M.Schulze) and Dactylorhiza praetermissa (Druce) seeds depended on the concentration of MnSO₄ in the media (Vaasa and Rosenberg, 2004). Seeds of Dactylorhiza maculata (Lindl.) started to germinate after 3.5 months of incubation in soil (Kinderen, 1995). Vassa and Rosenberg (2004) found that a high percentage of seeds of Dactylorhiza baltica (Klinge), Dactylorhiza praetermissa (Druce), and Dactylorhiza rutheï (M.Schulze) germinated within 3 months of culture, and seed germination decreased afterwards. Bektaş et al. (2013) found the highest germination rate (44.2%) of Orchis coriophora Lindl. on Orchimax medium. Aggarwal and Zettler (2010) found 100% germination in Dactylorhiza hatagirea within 10 days after sowing and plants developed seedlings after 3 months. Giri and Tamta (2012) used 4 media for the germination of Dactylorhiza hatagirea and found a better response on MS medium supplemented with peptone (1.0 g/L), morpholino ethanesulfonic acid (1.0 g/L), and activated charcoal (0.1%). However, in our study, seeds of Dactylorhiza hatagirea germinated within 1 week on LD and TP039 media, which has MnSO₄ as an important component. The percentage of in vitro protocorm formation varied on different media, along with differences in duration, such as MS and KC media. Protocorm formation was 30%–40% in 49 days of culture in Cleisostoma racemiferum Lindl. (Temjensangba and Deb, 2006), while in Malaxis khasiana (Hook.f.) Kuntze, 20%–30% of protocorm formation occurred on MS medium with 500 mg/L casein hydrolysat + 1 µM BA in 107 days (Deb and Temjensangba, 2006). Similarly, in Ophrys species, 23% of protocorms were formed after 107 days of culture on Malmgreen medium (Kitsaki et al., 2004). We observed 22%–23% of protocorm formation within 17 days of culture on LD medium. There are reports of multiple shoot formation under in vitro conditions, such as in epiphytic orchid Cleisostoma racemiferum Lindl. within 30 days on MS medium supplemented with 10.0 µm naphthalene acetic acid (NAA) + 8.0 µm 6-benzylaminopurine (BAP) (Temjensangba and Deb, 2006), in Cymbidium eburneum Lindl. (MS + 15 µm NAA + 15 µm BAP) in 28 days (Gogoi et al., 2012), and in Coelogyne nervosa Lindl. (MS + 3 mg/L BAP + 1 mg/L NAA) in 90 days (Abraham et al., 2012). Multiple shoots were formed in MS + 6 µm IAA + 18 µm BAP + 18 µm Kin in 49–56 days in Malaxis khasiana (Hook.f.) Kuntze (Deb and Temjensangba, 2006). Significant differences in multiple shoot formation on MS medium supplemented with 3 mg/L IBA and 1 mg/L Kin in a shorter duration of 28 to 30 days of incubation occurred in our study. Differences in the survival of plantlets in potting mixtures have been reported, such as 65% survival of transplants of Malaxis khasiana (Hook.f.) Kuntze in charcoal pieces + coconut husk + sterilized forest litter (1:1:1) (Deb and Temjensangba, 2006), and 90% survival of transplants of Pholidota pallida Lindl. in charcoal chips + coconut husk + and broken tiles (2:2:1) (Mulgund et al., 2012). We observed 100% survival in the C-8 potting mixture; other potting mixtures (C-3 and C-6) also gave good shoot and root growth because of the presence of cocopeat and vermiculite, which provided enough moisture, aeration, and micronutrients for the profuse growth of plants. This observation is supported by Hartmann et al. (2007), who found that vermiculite, when combined with perlite and cocopeat, promotes faster shoot/root growth and gives quick anchorage to young plants.

In the present study, a successful attempt has been made to culture immature seed embryos of Dactylorhiza hatagirea for developing protocorms, shoot regeneration, and mass multiplication. The growth and multiplication
of plants further continued after their transplantation to the greenhouse in potting mixtures, thereby resulting in the development of a micropropagation technology to meet the growing demand for *Dactylorhiza hatagirea*. This technology will help not only in multiplying the plantlets but can also play a major role in its conservation and metabolite production through tissue/cell culture systems.

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References


