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High frequency regeneration protocol for *Dendrobium nobile*: A model tissue culture approach for propagation of medicinally important orchid species

Paromik Bhattacharyya, Suman Kumaria *, Pramod Tandon

Plant Biotechnology Laboratory, Department of Botany, North-Eastern Hill University (NEHU), Shillong 793 022, India

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ABSTRACT

Low rates of shoot multiplication, clonal instability, poor rooting frequency and high cost of production are major impediments challenging the micropropagation of orchids and other ornamental plants. These problems can be solved with the proper usage of plant growth regulators (PGRs) in the medium. The highest shoot proliferation rate of 21 shoots/explant was recorded in Murashige and Skoog (MS) medium containing 1 mg/l meta-topolin and 0.8 mg/l Putrescine. To date, this is the best frequency of shoot induction for *Dendrobium nobile* and one of the highest among other orchids. Higher rooting frequency was also recorded with the highest rooting of 10.1 roots/shoot achieved in plants grown in half strength MS media supplemented with 2 mg/l of indole butyric acid and 0.5 mg/l of phloroglucinol. The plantlets were successfully acclimatized in the greenhouse with a survival rate of 82.3% exhibiting normal developmental patterns. The regenerated plantlets were assessed for genetic stability using the molecular markers namely SCoT and IRAP revealing high degree of genetic stability within the micropropagated plants of *D. nobile*. Being a highly medicinal orchid, the antioxidant potentials of the mother and the micropropagated plants were assessed using DPPH, FRAP and metal chelating assays which revealed a significantly higher antioxidant activity in the micropropagated plants. Rapid multiplication rate, higher genetic stability and significantly higher antioxidant power reported in the present study on *D. nobile* ensure the use of this micropropagation protocol for *ex-situ* conservation and commercial exploitation which could also be extended to other important orchid species.

1. Introduction

The conservation of orchids is becoming a matter of global concern as their natural populations are depleting at an alarming rate. Northeast India, which is also a part of the Indo-Burma mega biodiversity hotspot (http://www.conservation.org) alone harbors around 750–800 species (Chowdhery, 1998). The orchid diversity in Northeast India and the country as a whole is being threatened by various factors such as deforestation, habitat destruction and overexploitation (Singh, 2001). At present all the orchid species are listed in the Red Data Book of the International Union of Conservation of Nature and Natural Resources (IUCN). Also, the family Orchidaceae on the whole is now included in the Appendix-II of CITES (Senthilkumar, 2001).

Dendrobium nobile Lindl is an extremely important medicinal orchid having a widespread usage in various traditional herbal drug preparations housing important biochemical entities like dendrobine, moscatilin, gigantol, and denbinobine which are also having anticancerous properties (Hossain, 2011; Bhattacharyya et al., 2014). Apart from that, the beautiful flowers have made *D. nobile* a major stake holder in the cut flower industry (Bhattacharyya et al., 2014). Being prized highly for its immense commercial importance, *D. nobile* is subjected to increasing anthropogenic pressures threatening its wild natural populations (Mohanty et al., 2013; Bhattacharyya et al., 2014). In order to formulate sustainable conservation of these rare, endangered and threatened (RET) plants especially orchids, *in vitro* technologies play an important role (Bhattacharyya et al., 2015a). Orchids are primarily micropropagated through protocorm like bodies (PLB) proliferation, which is induced by cytokinins. However, recently with the use of topolins (a group of aromatic cytokinins) promising results have been obtained. Tested in a variety of plant systems, topolins have proved to be highly efficient over the conventional cytokinins like BAP, Kn *etc.* (Aremu et al., 2012a).

However, in spite of having so many advantages, the greatest impediment for *in vitro* propagated plants is the development of clonal variations during the course of tissue culture passages altering the true-to-

Abbreviations: BAP, 6-Benzyl Amino Purine; Kn, Kinetin; TDZ, Thidiazuron; IBA, Indole Butyric Acid; NAA, Napthalene Acetic Acid; SCoT, Start Codon Targeted Polymorphism; IRAP, Inter-Retrotransposon Amplified Polymorphism; TPC, Total Phenolic Content; TFC, Total Flavonoid Content; TAC, Total Alkaloid Content; PG, Phloroglucinol; CA, Chlorogenic Acid; SA, Salicylic Acid.

* Corresponding author. Fax: +91 364 272 2000.
E-mail address: sumankhatrikumaria@gmail.com (S. Kumaria).

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type nature of the micropropagated plants (Braun, 1959; Feyissa et al., 2007; Peyvandi et al., 2009). The occurrence of these variations depends on different factors like explant source, media composition and culture conditions (Salvi et al., 2001). Therefore, periodic assessment of the degree of genetic stability within the *in vitro* raised-plants is important for commercial utilization and large-scale production of true-to-type plants (Larkin and Scowcroft, 1981). The clonal variations can be assessed using morphological, cytological, biochemical, and molecular markers. In orchids, although many protocols on the micropropagation exist reports on genetic stability of *in vitro* raised plantlets are very few (Bhattacharyya et al., 2015a). Thus, detection of somaclonal variations at early stages of regeneration is important for any micropropagation protocol (Oh et al., 2007). Molecular markers are efficiently used for analyzing genetic fidelity of *in vitro* raised plantlets. As the molecular tools are not influenced by environmental factors they are reliable and reproducible. Markers such as randomly amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR), and simple sequence repeats (SSR) are used extensively in genetic assessment studies (Rathore et al., 2011; Devi et al., 2013). However as the conventional molecular markers target a particular region of genome, they have certain limitations which have been largely alleviated by the use of retrotransposon targeted and start codon targeted (SCoT) molecular markers (Collard and Mackill, 2009; Kilinç et al., 2015). Larkin and Scowcroft (1981) reported that transposable element movement may be one of the main reasons for the occurrence of somaclonal variations. Thus, in order to detect retrotransposon movement in the genome different marker techniques are used, of which inter-retrotransposon amplified polymorphism (IRAP) is one of the most preferred techniques which amplifies genomic distances between two long terminal repeats (LTR) belonging to the subclass of retrotransposons (Kalendar and Schulman, 2006). Studies show that, it is more advantageous to use more than one DNA amplification technique in evaluating somaclonal variations (Palombi and Damiano, 2002). Therefore, SCoT was used in addition to IRAP in the present study. It is a very recent marker technique having advantages over other traditional marker techniques like RAPD and ISSR and has proved to be extremely efficient in detecting genetic variability within the *in vitro* raised plantlets (Collard and Mackill, 2009; Bhattacharyya et al., 2014; Rathore et al., 2014).

Apart from ensuring clonal fidelity there is an urgent need for the assessment of drug yielding potentials of tissue culture — derived plants of *D. nobile*, being an important medicinal orchid. Few reports are available on secondary metabolite analysis of the phytoconstituents in orchids. In our earlier report, *D. nobile* has been found to possess a high degree of antioxidant activity (Bhattacharyya et al., 2014, 2015b). Antioxidants possess the ability to reduce the oxidative damage associated with many diseases including neurodegenerative diseases, cancer, cardiovascular disease, cataracts and AIDS. Hence, restoration of antioxidant activity in micropropagated clones should always be associated with the evaluation of its antioxidant activity in the micropropagated plants for large scale propagation and commercialization. Thus, taking into consideration the above discussed facts, the present study was aimed at developing a genetically stable, sustainable regeneration protocol for *D. nobile* and to assess how regeneration pathway and plant growth regulators (PGRs) influence the phytochemical yielding capacity of the micropropagated plants.

2. Materials and methods

2.1. Source materials

Explants were excised from ten years old *D. nobile* plants growing in the greenhouse of the Plant Biotechnology Laboratory, Department of Botany, North-Eastern Hill University, Shillong India (Fig. 1A). The nodal explants (0.5–1 cm long) were cleaned with a soft brush and mild detergent, washed under running tap water for 15–20 min and rinsed thoroughly with distilled water. These were surface sterilized with 10% (*v*/*v*) NaOCl (4–6% available chlorine; Merck) for 10 min followed by treatment with $HgCl_2$ (0.1% *w*/*v*; Hi-Media) for 2 min.

2.2. Culture medium and growth conditions

The explants were washed 5–6 times with sterilized distilled water to remove the traces of surface sterilants and finally shortened to 3–4 mm for culture in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 3% sucrose (Hi-Media, India) and 0.8% agar (Hi-Media, India). The medium was supplemented with various PGRs, including BAP, thidiazuron (TDZ), and meta-topolin (mT) either separately or in combination with α-naphthaleneacetic acid (NAA) (Cheruvathur et al., 2010). The pH of the medium was adjusted to 5.8 with 0.1 N NaOH or HCl prior to autoclaving. All medium containing culture tubes were autoclaved at 104 kPa and 121 °C for 15 min.

For each treatment, 10 replicates were taken and the experiments were repeated three times. Each culture period lasted for 8 weeks. All cultures were maintained at 25 ± 2 °C, 80% RH, and 12 h photoperiod of 35–50 μmol/m^2/s irradiance provided by cool-white fluorescent tubes (Philips, Kolkata, India). Data on number of explants responding and number of developing shoots per explant were recorded.

2.3. Effect of various polyamines on shoot proliferation

The adventitious shoots regenerated from the medium supplemented with BAP, TDZ, mT singly and in combination with NAA were randomly selected and transferred to MS medium supplemented with polyamines including spermine, spermidine and putrescine at concentrations ranging from 0.2–1.0 mM. Number of explants, replications and culture conditions were as described above. The average shoot number was calculated at the time of culture as well as at the 4^{th} and 8^{th} weeks after culture.

2.4. Rooting and acclimatization

2.4.1. Effect of auxins on rooting

Each shoot with two or three expanded leaves was transferred to half-strength MS medium supplemented with 1 to 4 mg/l of indole butyric acid (IBA) or NAA (1–4 mg/l). Cultures were incubated in light; total number of roots produced and length of the roots were measured after 4 weeks.

2.4.2. Effect of phenolic compounds on root induction

Using the conventional auxins, the highest rooting frequency was observed in medium supplemented with 2 mg/l of IBA (Table S1). Thus, in a separate experiment, 0.2, 0.5, 1.0 and 2.0 mg/l of phloroglucinol (PG), chlorogenic acid (CA) and salicylic acid (SA) were added to half strength MS medium containing 2 mg/l IBA to examine the effect of phenolic compounds and auxins on *in vitro* root induction in *D. nobile*. Cultures were incubated in light; total number of roots produced and length of the roots were measured after 4 weeks.

2.5. Hardening and acclimatization

Survival rate (%) was recorded after 60 days from transfer to greenhouse conditions. Plantlets were initially covered with a polythene sheet to maintain relative humidity (90%). The hardened plants were regularly sprayed with $1/10^{th}$ liquid MS medium. The number of surviving plants was recorded after 12 weeks of transfer. All of the surviving acclimatized plants were transferred to a greenhouse. The recorded data were subjected to analysis of variance using SAS and means were compared using Duncan's multiple range test (Duncan, 1955).