ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Characterization of Bioactive Compounds at Seedling Stage and Optimization of Seed Germination, Culture Multiplication of *Dendrobium nobile* Lindl.-A Study *In Vitro*

Saranjeet kaur*, Pamita Bhandari, K.K. Bhutani

Plant Tissue Culture and Biotechnology Laboratory, Department of Natural Products, National Institute of Pharmaceutical and Education Research, S.A.S. Nagar, Mohali-160 062, Punjab, India

Manuscript Info

Manuscript History:

Received: 11 February 2015
Final Accepted: 22 March 2015
Published Online: April 2015

Key words:

Asymbiotic · *Dendrobium nobile* · *In vitro* · Liquid chromatography mass spectrometry · Organic growth supplements · Protocorms

Corresponding Author

Saranjeet kaur

Abstract

An efficient protocol was developed for the characterization of bioactive molecules in *in vitro* raised cultures of medicinally important herb *Dendrobium nobile*. The cultures were initiated *in vitro* through asymbiotic seed germination technique on a defined Mitra medium (M) medium supplemented with plant growth regulators (PGRs) [6-benzyladenine (BA - 4.44 µM), furfuryl aminopurine (Kn-4.65µM) and α-naphthalene acetic acid (NAA- 5.37µM)].The frequency and onset of germination was markedly influenced by the presence of growth regulators in the nutrient medium. Seedlings were formed within 13.25 ± 0.50 weeks in NAA supplemented medium. The efficacy of growth supplements such as banana homogenate (Bh - 10, 20, 30 gl^{-1}) and coconut water (CW -10, 20, 30%) was also tested on the multiplication (*neo*-formations) of the protocorms. Highest percentage of regeneration (80.25 ± 0.20%) and a maximum of 10.00 ± 0.40 protocorm-like bodies (PLBs) / explants formed in CW (20%) treated cultures and PLBs multiplied profusely. Plantlets developed within 14.00 ± 0.40 weeks. Bh (20 g l^{-1}) favoured development of healthy plantlets with a maximum fresh weight of 1.030 ± 0.021 grams.

Simultaneously, a method was developed for the characterization of bioactive molecules in extremely medicinal *D. nobile* herb by using high performance liquid chromatography (HPLC) coupled with electrospray ionization mass spectrometry. The analytical method was developed on X Terra MS C-18 column (250 mm × 4.6 mm, 5µm) by using acetonitrile: water (0.1% formic acid) 10:90 as mobile phase with flow rate of 1 ml min^{-1}. Eleven compounds were identified in the *in vitro* grown seedlings. Therefore, the established quality evaluation method was successfully used for evaluating the quality of *D. nobile* samples of seedlings. Further, the reported methodology can open up avenues for its application in numerous orchidaceous plants of medicinal value prevailing in the Indo-Himalayan region.

INTRODUCTION

Medicinal plants are of great interest to the researchers in the field of biotechnology as most of the drug industries depend on the plants for the production of pharmaceutical compounds. Herbal medicines are the foundation of many common drugs prescribed in the clinical practice in many countries today. Herbs and herbal products are still an important part of the primary health care systems in many parts of the world.

Amongst the important medicinal herbs the genus *Dendrobium* (Orchidaceae), is a highly evolved and diverse group, represented by more than 1100 species. It is distributed throughout Asia, Europe and Australia in tropical to temperate climates. The dried or fresh stems of *Dendrobium* plants, known as Caulis Dendrobii (Shihu or Huang Cao in Chinese) is used in the traditional or folk medication as a Yin tonic to nourish the stomach, promote the production of body fluid and reduce fever. Pharmacological studies have revealed that some of the components and extractives of *Dendrobium* species are anti-tumor (Morita et al., 2005), anti-angiogenic (Gong et al., 2004), anti-platelet aggregation (Fan et al., 2001), anti-inflammatory (Lin et al., 2001) and immune-regulatory in action (Zhao et al., 2001) which are partly responsible for the actions and indications of this herb in traditional remedies. With a view to explore the presence of bioactive compounds in the *in vitro* raised constituent species of this genus *Dendrobium nobile* was selected for the present study.

Dendrobium nobile Lindl. is a semi-evergreen, epiphytic, sympodial orchid species. Biogeographically, the species extends towards south China to Laos and Thailand. In the North-Eastern Indian Himalayas, It is scattered in the tropical to temperate climates at an altitude of 200-2000 meters. *D. nobile*, which is a potential ornamental is a progenitor of several meritorious hybrids of international repute. Besides being a great ornamental due to its appealing floral displays, the species is medicinally valuable. It is one of the 50 fundamental herbs used in the traditional Chinese medicine where it has the name shi hu or shi hu lan. The drug shih- hu made from *D. nobile* has been valued greatly (Lawler, 1984). The drug is used as a tonic and strengthening medicine and reputed to nourish the yin system of body, impart longevity and serve as an aphrodisiac. The stems are useful in alleviating thirst, calm restlessness, accelerating convalescence and reducing dryness of the mouth. (Bensky et al.,1986). Being highly versatile in its ornamental and medicinal characteristics, its wild populations are constantly getting rare swiftly, due to unabated collections, which far exceed its natural regeneration. Several other factors which add up to its current rare, endangered and threatened (RET) status are deforestation, fragmentation of habitat especially in the tropical regions, increased use of fertilizers, excessive exploitation of the soil are equally responsible. The genus *Dendrodium* is included in the Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES 2014). Integrative multidisciplinary approaches are required to save the species from getting extinct in nature. Hence, through this communication we emphasize to conserve, propagate and multiply the species, through tissue culture techniques that assist in saving its wild populations.The traditional vegetative propagation through stem-cuttings in the nursery-beds, is a time consuming process and not even economically consistent. *In vitro* techniques provide an alternative to the large-scale production of the seedlings/plantlets within a short time period. The method of *in vitro* protocorm slices culture is a promising technique through which a large number of plantlets are developed. Even the intact PLBs are also capable of producing daughter PLBs directly through budding or callusing (cf. Kaur and Bhutani 2011, 2012). The technique has been successfully in the orchid species of diverse habit and habitats. The induction of protocorm like-bodies from protocorms and various other explants such as shoot-tips, stem-nodes, root-tips, root-tubers is a reliable method for regeneration and multiplication of orchids (Park et al., 2003; Kosir et al., 2004; Anjum et al., 2006; Kalimuthu et al., 2007; Hong et al., 2008; Medina et al., 2009; Ng and Saleh, 2011; Kaur and Bhutani, 2011; 2012). Those PLBs which develop directly from the meristematic tissues are genetically more stable than those generated from the callus (Lee and Philips, 1988). Owing to the inherent ability of protocorms proliferating into multiple PLBs and retaining the genetic stability, these propagules (PLBs) are the most sought-after tissues being used for the genetic transformation experiments (Sreeramanan et al., 2008) and cryopreservation studies (Yin and Hong, 2009; Mubbarakh et al., 2014; Galdiano et al., 2014). The addition of organic additives to the culture medium, to promote *in vitro* growth and proliferation of *neo*-formations, is a common practice. The addition of organic additives contribute towards the development of a simple and economical plant culture medium furthermore minimizing the utilization of exogenous PGRs, possibly reducing the occurrence of undesired soma-clonal variations (Lee and Phillips, 1988).The effect of organic growth supplements are tested in a large number of orchid species (cf. Kaur and Bhutani, 2012).

Currently, an attempt was made to investigate the chemical constituents of *in vitro* raised seedlings of *D. nobile*. The objective was to set a protocol to make available the chemical compounds of therapeutic use without collecting it from existing populations in nature. This step would definitely assist in the *ex situ* conservation of this species and save them from unabated collection pressures in the wild. By this method, the species would be readily available from the *in vitro* raised plants that could be directly utilized for the isolation of chemical compounds. With such scenario, present studies were conducted in *D nobile*. Voluminous work of characterization of bioactive compounds in many *in vivo* grown species of other orchid species have been accomplished proving their antioxidation, immunity stimulating and anti-tumoral activities.

Previous phytochemical investigations conducted in the *in vivo* grown *D. nobile* plants have revealed the presence of phenolic derivatives such as bibenzyls, phenanthrene, flavones, coumarins as well as alkaloids (Miyazawa et al., 1999; Ye et al., 2002; Jin-Ming et al., 2003; Shu et al., 2004; Zhang et al., 2007a, b; Zhang et al., 2008; Yang et al.,

2007; Yang et al., 2008; Wang et al., 2010). Some of these compounds are reported to have cytotoxic, immuno-regulatory, anti-mutagenic, and anti-inflammatory activities (cf. Yang et al., 2007). However, no work has been so far accomplished on the *D. nobile* species in assessing the bioactive compounds in the *in vitro* raised seedlings. We, herein, report a study showing the presence of bioactive compounds in *Dendrobium nobile* Lindl. orchid herb raised asymbiotically *in vitro*.

High-performance liquid chromatography (HPLC) coupled to diode-array detection (DAD) and mass spectrometry (MS) is a powerful tool for the analysis and quality evaluation of pharmaceuticals and natural products (Escarpa et al., 2000; Cuyckens et al., 2004; Cuyckens et al., 2005; Kachlicki et al., 2005; Gray, 2006). This hyphenated technique greatly facilitates the characterization of chemical markers in complex matrices and in structurally similar natural product mixtures. The high performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry methods (LC-MS) are developed for the quantification and characterization of bioactive molecules in different *Dendrobium* species. So far, no such study has been conducted to investigate the chemical constituents of *in vitro* raised seedlings of *D. nobile* and knowledge, this study presents the first ever report characterizing the bioactive compounds in the *in vitro* raised seedlings of *D. nobile*.

The present study was conducted with an aim to develop a reliable methodology to initiate, multiply the cultures *in vitro* and mainly to identify the presence of bioactive molecules in the extracts of *in vitro* grown *D. nobile* seedlings through HPLC-DAD/electrospray ionization mass spectrometry (ESI-MS).

Materials and methods

Plant collection and greenhouse maintenance

Dendrobium nobile plants and their capsules were collected from a commercial grower of Darjeeling district, West Bengal, India (latitude range: 26°31′ - 27°13′ N; longitude range: 87°59′ - 88°53′ E). The healthy plants were replanted in pots (27.5 cm × 22.4 cm diameter) containing substrate charcoal pieces, brick pieces, bark pieces as a substrate in the ratio of 1:1:1.The sphagnum moss covered the top surface of potting mix. The plants were maintained in the greenhouse under natural light conditions with a 70% relative humidity and 25°C/20°C day/night temperature. The capsules were collected, brought to the laboratory and prepared for inoculations into the medium without and with growth adjuncts.

Culture medium for *in vitro* seed germination

The required quantity of M (Mitra et al., 1976) medium was weighed and dissolved in 200 ml of double-distilled water. To raise the volume of medium 1 litre, requisite amount of double- distilled water was added. The growth regulators such as N^6-benzyladenine (6-BA; 4.44µM, Hi-media), furfuryl aminopurine (Kn; 4.65µM, Hi-media), α-naphthalene acetic acid (NAA; 5.37µM, Hi-media) were used individually in the medium. The viability of the seeds was estimated by staining in 1% solution of 2, 3, 5- triphenyl-tetrazolium chloride (TTC; pH 6.5) (Hi-media) for 3 days at 32 ± 1°C (Lauzer et al., 2004).The embryos which stained red were considered viable. Almost all the seeds were viable.

Capsule sterilization procedure

The capsules were first scrubbed with a soft brush in running tap water to remove any debris. These were rinsed with dish-washing liquid to remove any debris from the surface of the capsules then rinsed thoroughly in water. The capsules were swabbed with ethyl alcohol under a sterile (laminar airflow) hood and surface sterilized with 0.1% (w/v) mercuric chloride ($HgCl_2$; Qualigens, Pvt. Ltd., Mumbai, India) in an aqueous solution containing 1-2 drops of 'teepol' as a wetting agent for 2 minutes. They were rinsed 2-3 times with sterilized double-distilled water to remove any traces of $HgCl_2$ left on the surface of capsules. Thereafter, capsules were flamed on a burner and seeds scooped out into a petri-dish by making a longitudinal slit. The seeds were inoculated onto Mitra medium and its combinations with growth adjuncts.

Culture medium for PLB multiplication

Dendrobium nobile protocorms (2.0 mm long) were procured from 26-weeks old *in vitro* cultures. The protocorms were maintained for 2 weeks on the basal M medium (control), supplemented with sucrose 2.0% (*w/v*) (Daurala sugar works, Daurala, India) was used as source of nutrition and gelled with 0.8% (*w/v*) agar (Hi-Media, Mumbai, India) was used as control.

The complex growth supplements such as banana homogenate (25, 50 and 75 gl^{-1} *w/v*) and coconut water (10, 20 and 30% *v/v*) were used individually in M medium. Banana homogenate was prepared from the ripened fruits purchased from the market. They were peeled-off and cut into small slices. The required quantities were homogenised in a blender and the resultant pulp added to the medium. Coconut water, from fresh green coconuts was drawn-off and collected in a flask. It was filtered through a sieve and used as such in the medium.

The pH of the medium was adjusted to 5.8 after adding the growth regulators and organic growth supplements. The medium was dispensed in the test tubes of size (25 mm × 150 mm) and autoclaved at 121^{o}C at pressure of 1.06 kg cm^{-2} for 15 minutes. The autoclaved medium was kept at 37^{o}C to check any further contamination.

Inoculations and incubation conditions

The inoculations were done under aseptic conditions in a laminar air-flow cabinet. The cultures vessels were incubated at 25 ± 2^{o}C under 12 hr photoperiod of 40 µ mol m^{-2}s^{-1} light intensity provided by white fluorescent tubes (Fluorescent tubes; Philips India Ltd, Mumbai, India). Eight replicates were used for each experiment and to check the reproducibility of the protocol, the experiment was repeated twice.

Percent germination

Nearly after three to four weeks of the inoculations, some of the seeds were scooped out of the test tube with the help of a spatula. These were dispersed in a drop of water on a glass-slide and observed under light microscope.

The percentage of seed germination was calculated by employing the following formula:

Germination (%) = (Number of enlarged seeds showing swelling of the embryo × 100) / Total number of seeds.

Once the spherules were developed, pertinent observations were recorded at the intervals of one week to trace the different stages of development of the cultures. These were observed using a stereozoom microscope (Nikon, H600L, Japan). Sub-culturings were carried out as and when required.

Observations and Statistical Analysis

The cultures were observed regularly under binocular microscope (Olympus SZX10, Japan) and data recorded accordingly. The results were analysed using one-way ANOVA test and were analyzed using Tukey's Multiple Comparison at $p \leq 0.05$ using SPSS (Version 17) software package (SPSS Inc. Chicago, US).

Histological studies

To make histological observations, the PLBs samples were collected from the culture vessels. Free hand sections were cut by placing them in the potato pith. Thin sections floating at the surface of water were selected and placed in a drop of water and observed under sterozoom microscope (Nikon, H600L, Japan). The photographs were captured using a digital camera (Nikon Digital Sight, DS, Ri1 Nikon Corporation, Japan).

For characterization studies preparation of the sample solution

The air-dried plant material (500 mg) of *in vitro* grown *D. nobile* seedlings (1.0-1.5 cm long) was perculated with ethanol – water (80:20) for three times. The obtained extracts were concentrated to dryness under reduced pressure at temperatures 45 ± 5^{o}C. The stock solutions of *D. nobile* (41 mg ml^{-1} each) was dissolved in HPLC grade acetonitrile - water and filtered through syringe filter. An aliquot of 25 µl of the filtered samples were used for further LC-MS analysis.

Liquid chromatography-mass spectrometry conditions

Liquid chromatography-mass spectrometry (LC-MS) system was equipped with Waters 2767 Sample Manager, 2525 Binary Gradient Pump and is coupled to a single quadrupole ZQ mass spectrometer (Micromass 4000), operating in the ESI /APCI modes. The MS experiment setup and data acquisition were conducted using the MassLynx software V 4.0. The mass spectrometer was operated in the positive electrospray mode (+ ve ESI). The optimized values of MS analysis were as follows: capillary voltage, 3.5 kV; cone voltage, 46.5 V; dissolvation temperature, 367 ^{o}C; source temperature, 120 ^{o}C; extractor, 2.5 V; RF lens, 0.2 V; nebulizing gas, 510 l h^{-1}; cone gas 91l h^{-1}. High-purity nitrogen was used as nebulizer and cone gas. MS analysis was performed in the mass range of 100–700 *m*/*z*. The analytical method was developed on X Terra MS C-18 column (250 mm × 4.6 mm, 5µm). The mobile phase used for the analysis was acetonitrile : water (0.1% formic acid) 10 : 90 in isocratic elution with flow rate of 1 ml min^{-1}.

Results and discussion

Presently, the seeds of *D. nobile* germinated successfully in M medium. Earlier, different media were used such as MS, P-668 for testing the germination potential of immature/mature seeds (Vasudevan and van Staden, 2010).The result indicates wide nutritional amplitude and simple nutritional requirements of the seeds of this species.

In vitro asymbiotic seed germination has a great potential as a propagation method to produce and conserve rare, critically endangered and commercially important orchids and other plant species. The technique is widely used in rescuing hybrid embryos, propagating desired genotypes, cloning apomictic taxa, facilitating genetic transformations (Men et al., 2003). Presently, the germination competence was markedly influenced by the chemical stimulus in the nutrient pool (Table1; Fig.1a-e). Almost all the seeds were embryonate (Fig.1a). Germination of the seeds began within 4.82 weeks of culture in the basal medium. The orchid seeds are distinct due to the presence of only a few-celled unorganized (reduced) embryo devoid of functional endosperm.The supply of exogenous water and

nutrients are obligatory for their germination. Upon germination, the swollen seeds emerged out of the seed coat as globular spherules, which were slightly green in colour. They grew in size while still attached to the seed coat (Fig. 1b). After 7 wk, they developed into spherical chlorophyllous protocorms with the appearance of rhizoids at their basal portion (Fig.1c) and a shoot-tip at the opposite end of the chlorophyllous protocorm. Later, these differentiated 1st leaf primordia and later 2nd leaf primordia. The roots initiated soon after at the base of leaf primordia and developed into seedlings after 19.35 weeks.

Addition of NAA into the medium proved beneficial for the early onset of germination. It also favoured highest germination percentage and advanced seedling development. Almost 98.50 per cent seeds germinated and developed into chlorophyllous protocorms (Fig.1d) which further differentiated into leaf and root primordia within 7 and 10 weeks of culture respectively. The seedlings were developed within 13.25 weeks (Fig. 1e). Our findings are in confirmation with similar earlier reports of the benign effect of NAA in *Aerides odorata* (Pant and Gurung, 2005), *Coelogyne suaveolens* (Sungkumlong and Deb, 2008), *Dendrobium chrysotoxum* (Kaur and Bhutani, 2012). According to Hayes (1969), the auxins are known to stimulate the transport of exogenous nutrients during germinations. In our cultures, the use of cytokinins (BA/Kn) lowered germination frequency and delayed morphogenetic processes but favoured healthy shoot growth similarly to those reported earlier in *Encyclia off. oncidioides* (Znaniecka et al., 2005), *Dendrobium chrysotoxum* (Kaur and Bhutani ,2012).From the results in this experiment, it is clear that there is adequate amount of endogenous cytokinin concentration in the seed itself. Literature study also explains that orchid seeds that do not require exogenous cytokinin for germination, they are cytokinin autonomous since they contain sufficient endogenous level of cytokinin (De Pauw et al., 1995).

The technique of protocorm slices to regenerate *in vitro*, giving a large number of plantlets within a short span of time through repeated slicing and culture of daughter PLBs, is an important and high frequency multiplication methodology for orchids (cf. Kaur and Bhutani, 2011). The utility of PLB segment culture in achieving multi-fold increase in the rate of multiplication is due probably to the release of morpho-genetically competent cells from the controls imposed by neighbouring cells and increased availability of nutrients and growth promoting substances to the site of regeneration.

The explants regenerated in the basal M medium (control) with a very low percentage. The regeneration frequency was found significantly higher in organic growth supplement-enriched medium than control. In the basal medium, only 12.5 ± 1.2% explants regenerated into a PLB and developed into a single plantlet per explant after 23 weeks. Organic growth supplements favoured multiplication of the regenerants and early plantlet development (Table 2, 3). CW (20%) was found to be most effective in initiating maximum regeneration percentage in the explants i.e. 80.25 ± 0.20. The PLBs multiplied rapidly and formed dense clumps of PLBs in the cultures (Fig. 2a). Beneficial effect of CW in inducing cell divisions in the non-dividing cells resulting into protocorm differentiation (Intuwong and Sagawa, 1973) is correlated to the presence of growth hormone i.e. kinetin (a cytokinin) in its composition (cf. Kaur and Bhutani, 2012). Literature studies reveal that the addition of CW in the basal medium increased growth of the cultures and shoots vigorously rooted in the epiphytic orchids (McIntyre et al., 1974). CW successfully initiated differentiation of the shoots from PLBs of *Vanda teres* (Sinha and Roy, 2004) and helped in achieving high-frequency multiplication of PLBs in *Phalaenopsis gigantea* (Murdad et al., 2006), *Cymbidium pendulum* (Kaur and Bhutani, 2012). Among all the concentrations of banana homogenate used in the culture medium, a concentration of 20 gl^{-1} proved beneficial in promoting healthy growth of plantlets. The promontory effect of Bh could be attributed to the presence of higher content of sucrose in its pulp composition. Earlier in *Oncidium* species, Banana homogenate (10-15%) promoted growth of the plantlets (Kusumoto and Takeda, 1997) and significantly increased the number of leaves in *Dendrobium nobile* cultures (Sudeep et al., 1997). Literature survey also indicates the beneficial effects of Bh in promoting highest shoot production in *Dendrobium* hybrid. In *Vanda* species Bh (10%) increased the shoot length. Beneficial effects of Bh (10%) on leaf size of *Spathoglottis kimballianai* is also reported (Minea et al., 2004).The positive effects of Bh (50 gl^{-1}) in eliciting moderate regeneration in the explants and healthy growth of plantlets is also on records for *Dendrobium chrysotoxum* (Kaur and Bhutani, 2011) and *Cymbidium pendulum* (Kaur and Bhutani, 2012). A free-hand sections of the PLB cluster showed that the *neo*-formations developed directly without any intervening callus stage from the surface layer of the PLB. The *neo*-formations (PLBs) had a conical and oblong shape characterised by a well-demarcated protoderm layer (Fig. 2b).

Dendrobium nobile is a medicinally important herb and has become rare species due to unscientific over exploitation. In our study it is grown *in vitro* and there is urgency to understand the chemical complexity of the species. The chemical profile of *D. nobile* was developed on HPLC system with mobile phase ACN : Water (0.1% formic acid) 10:90 in isocratic elution. The best resolution was achieved within 25 mintues with the flow rate of 1ml/min.

The HPLC-ESI-MS chromatogram of *D. nobile* displayed more than 20 peaks at λ_{max} 280 nm and were tentatively characterized on the basis of UV spectra and the mass spectrometry (Fig. 3a). HPLC -MS study of ethanolic extract

Table 1 *In vitro* asymbiotic seed germination of immature seeds of *Dendrobium nobile* on M medium and its combinations with growth adjuncts.

of *D. nobile* indicated the presence of phenolic, lignan and alkaloids (Fig.4a-g) As UV spectrum allowed the partial determination of the structure, the conclusive structural informations could be obtained by the LC-MS/MS studies. In order to characterize the targeted peaks in the chromatogram, ESI-MS was coupled to high performance liquid chromatography. HPLC-MS study of ethanolic extract of *D. nobile* and showed the presence of nitrogen containing compounds, and lignans and displayed pseudomolecular (protonated $[M+H]^+$ / sodiated $[M + Na]^+$ ion peaks in positive ion mode. The LC-MS based analysis of *D. nobile* exhibited major characteristics pseudo molecular ion peak at *m / z* 337 $[M+H]^+$ at RT 3.07 min and was uncharacterized. The three chromatographic peaks observed at RT 3.82, 7.86 and 11.2 min. displayed ion peaks at 381$[M+Na]^+$, 373 $[M+H]^+$ and 389 $[M+H]^+$ and were assigned as pinoresinol, pinoresinol monomethylether and medioresinol respectively. The peak observed at RT 12.2 min. displayed pseudomolecular ion peak at *m/z* 289 $[M+H]^+$ corresponded to phenolics and was tentatively assigned as 3-O- Methylgigantol. The seven peaks observed at RT, 13.8 to 21.3 min belongs to dendrobine type alkaloids. The chromatographic peak at RT= 13.8 had protonated molecular ion peak at 278 $[M+H]^+$ and was tentatively characterized as 11-Oxodendrobine. The other alkaloid peaks observed were 264 $[M+H]^+$ (RT=14.6 min) characterized as dendrobine, 280 $[M+H]^+$ (RT=16.2 min) assigned as 2 or 6-Hydroxy dendrobine, 294 $[M+H]^+$(RT=16.9 min) tentatively characterized as nobilonine, 285 $[M+H]^+$ (RT= 19.0 min) characterized as denbinobin and 314 $[M+Na]^+$ (RT= 20.8 min) characterized as dendroxine, 279 $[M+H]^+$ (RT=21.3 min) was characterized as dendrobine-N-methyl. The results of identified and uncharacterized peaks are tabulated (Table 4).The *in vitro* grown *D. nobile* plant was also studied at mature stage and the chemical examination showed that the plant shared the same chemical composition as in early stages.

……..…

Fig.1a-e Asymbiotic seed germination and seedling development of *Dendrobium nobile* on M medium and its combinations with PGR(s). (a) Embryonate seed at the time of inoculation (4 X); (b) Spherule (20 X); (c) Appearance of rhizoids at the spherule surface; (d) Cultures at protocorm stage; (e) Development of healthy shoots in BA-enriched medium

Fig. 2a-b *In vitro* culture multiplication through protocorm segments of *Dendrobium nobile* on M medium and its supplementation with organic growth supplements. (a) PLB development in basal half of protocorm in CW 20% enriched medium, (b) Conical-shaped PLBs developing directly without any intervening callus stage with well demarcated protoderm layer

Fig. 3 HPLC chromatogram. (a) Chromatogram of ethanolic extract of *D. nobile* at λ = 280 nm

.....

Table 1.*In vitro* asymbiotic seed germination of *Dendrobium nobile* in M medium and its various combinations with growth adjuncts.

Growth regulators	Germination frequency (%)	Initiation of germination (weeks)	Protocorm Development (weeks)	Development of Leaf (weeks)	Root	Seedlings (weeks)
Basal	70.12±0.42^{d}	4.82±0.07^{bcd}	7.07±0.09^{d}	12.27±0.15 ^{bcd}	15.2±0.^{16bcd}	19.35±0.12 ^{abcd}
BA	72.75±1.2^{d}	4.00±0.05 ^{ad}	7.27±0.15^{d}	11.0±0.00^{ad}	14.05±0.09^{acd}	17.75±0.28^{ad}
KN	71.25±0.8^{d}	4.5±0.00 ^{bd}	6.72±0.60^{d}	10.85±0.54^{abd}	13.0±0.00^{abd}	17.05±0.38^{ad}
NAA	98.50±1.3^{abc}	2.05±0.05 ^{abc}	4.40±0.14^{abc}	7.0±0.00^{abc}	10.0±0.00^{abc}	13.25±0.50^{abc}

Concentration of growth regulators used=1mgl^{-1}.Values in a column with similar superscripts are not significantly different at $p \leq 0.05$ according to Tukey's test.

Table 2. The effect of different quantity of banana homogenate (mg l^{-1}) on *in vitro* multiplication of *Dendrobium nobile* protocorms in M medium.

Additive	Regeneration response (%)	Number of PLBs/explants	Number of shoots/explants	Development of plantlets (wks)
M	12.5 ± 1.20^{bcd}	1.00 ± 0.40^{c}	1.00 ± 0.40^{bcd}	18.05 ± 1.50^{bd}
M + Bh_{10}	20.05 ± 1.16^{ac}	1.75 ± 0.20^{c}	2.05 ± 0.20^{acd}	21.25 ± 0.70^{acd}
M + Bh_{20}	25.00 ± 2.10^{ab}	3.00 ± 0.28^{abd}	5.00 ± 0.20^{abd}	17.50 ± 1.12^{bd}
M + Bh_{30}	24.75 ± 2.00^{ab}	1.50 ± 0.11^{c}	0.00^{abc}	0.00^{abc}

M – Mitra medium; PLBs – protocorm-like bodies; Bh – banana homogenate [concentration subscript = mg l^{-1} (w/v)]; Values in a column with similar superscripts are not significantly different at $p \leq 0.05$ according to Tukey's test

Table 3. The effect of different concentrations of coconut water (%) on *in vitro* multiplication of *Dendrobium nobile* protocorms in M medium.

Additive	Regeneration response (%)	Number of PLBs/explant	Number of shoots/explant	Development of plantlets (wks)
M	12.12 ± 1.20^{bcd}	1.00 ± 0.41^{c}	1.00 ± 0.40^{bcd}	18.05 ± 1.50^{bcd}
M + $CW_{10\%}$	72.75 ± 1.20^{acd}	2.20 ± 0.25^{c}	2.50 ± 0.28^{ac}	21.25 ± 0.70^{ac}
M + $CW_{20\%}$	80.25 ±0.20^{abd}	10.00 ± 0.40^{abd}	14.00 ± 0.00^{abd}	14.00 ± 0.40^{abd}
M + $CW_{30\%}$	68.25 ± 1.16^{abc}	2.10 ± 0.17^{c}	2.20 ± 0.20^{ac}	19.00 ± 0.40^{bc}

M, PLBs – see Table 2; CW – coconut water [concentration subscript = % (v/v)]; Values in a column with similar superscripts are not significantly different at $p \leq 0.05$ according to Tukey's test.

Table 4 Tentative characterization of chemical constituents in *Dendrobium nobile* by HPLC-ESI-MS.

S. No.	Retention time	Positive ion mode $[M+H]^{+}$/Na	Tentatively Identified compounds
1	3.32	337	Uncharacterized

2	3.6	/381	Pinoresinol
3	4.53	276	Uncharacterized
4	7.86	373	Pinoresinol monomethylether
5	9.72	510	Uncharacterized
6	11.2	389	Medioresinol
7	12.2	289	3-O-Methylgigantol
8	13.8	278	11-Oxodendrobine
9	14.6	264	Dendrobine
10	15.3	287	uncharacterized
11	16.2	280	2/6-Hydroxydendrobine
12	16.9	294	Nobilonine
13	19.0	285	Denbinobin
14	19.4	397	Uncharacterized
15	20.8	/314	Dendroxine
16	21.3	279	Dendrobine-N-methyl

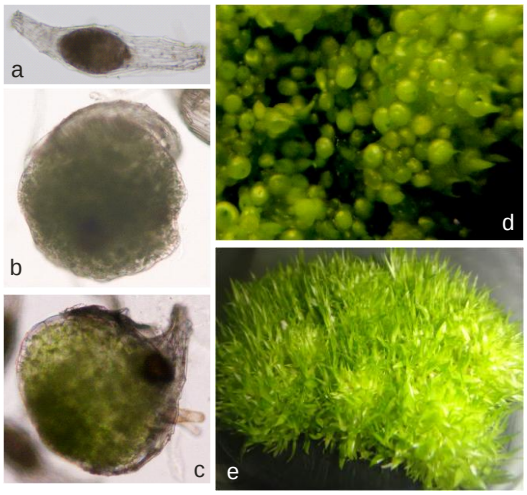

Fig 1

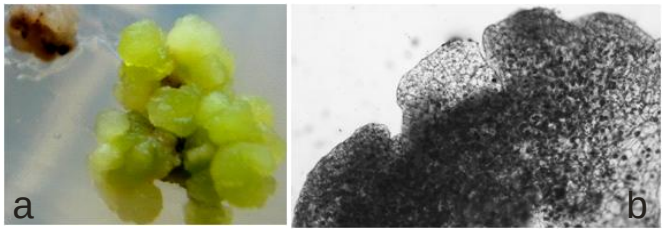

Fig 2

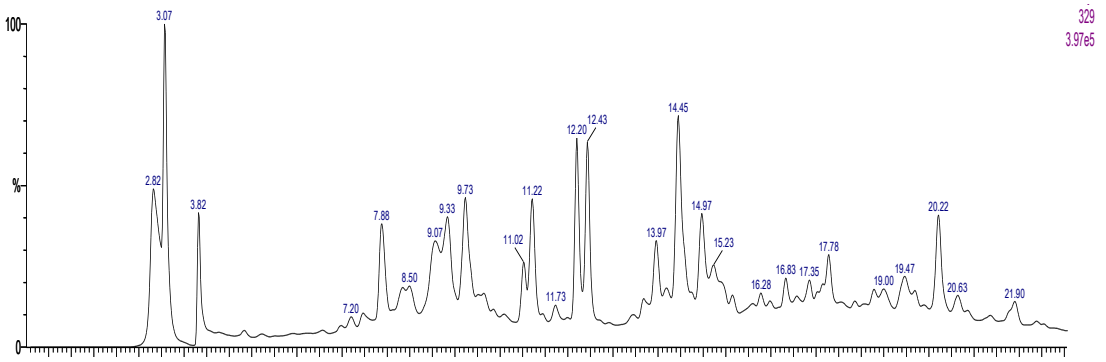

Fig. 3 – HPLC chromatogram of ethanolic extract of *D. nobile* at λ = 280 nm.

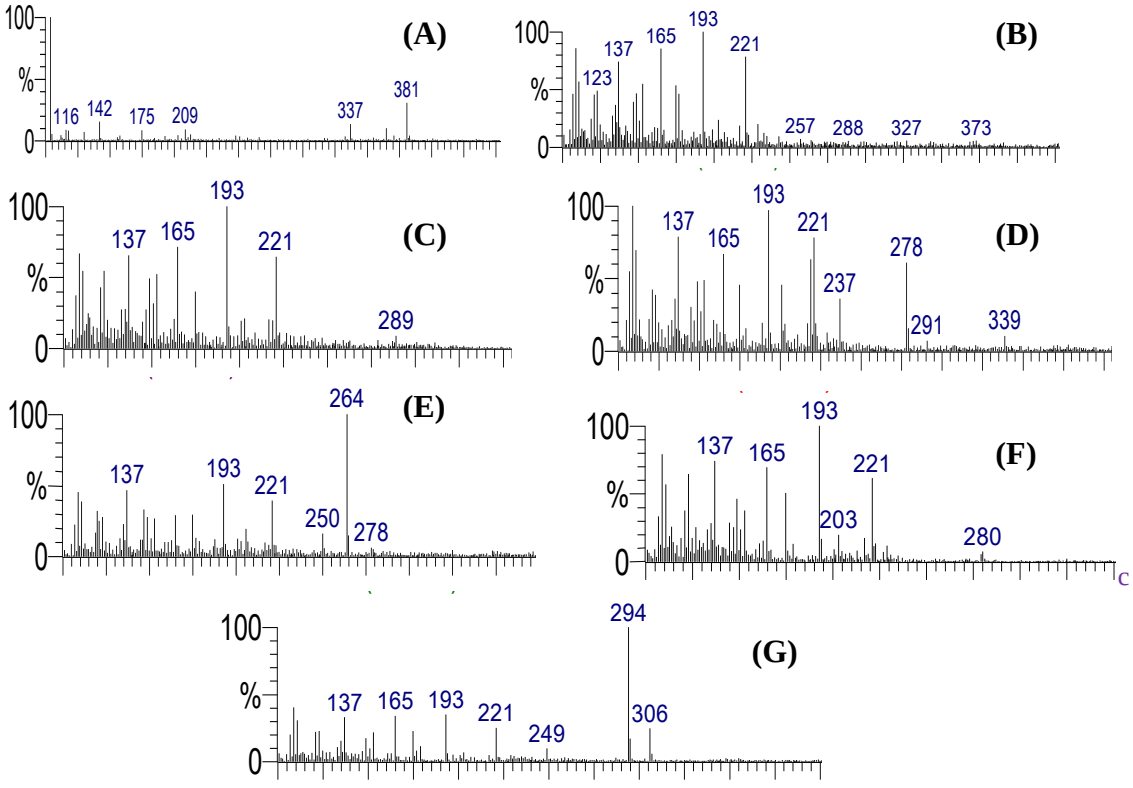

Fig.4. ESI (+) MS spectra of characterized compounds (A) piniresinol, (B) pinoresinol monomethylether (C) 3-O-Methylgigantol, (D) 11-Oxodendrobine, (E) dendrobine, (F) 6/2-Hydroxydendrobine and (G) nobilonine, from ethanolic extract of *D. nobile*.

Conclusion

In this first documentation report, a HPLC-MS method was developed and successfully applied for the analysis of bioactive molecules in the *in vitro* grown *D. nobile* medicinal plant. The compounds identified, were characterised by UV and mass spectral data. The results demonstrated that this method could also provide a reliable methodology

in the quality evaluation of other *Dendrobium* species also. Further studies are required to check the activity of the compounds characterized from *in vitro* grown *D. nobile* seedlings.

In vitro culture techniques assist in the germplasm conservation of medicinally and floriculturally important orchids and other plant species. This simple one-step potential protocol helped to mass propagate and conserve this rare orchid of medicinal and ornamental importance. The seeds of *D. nobile* are capable of germinating with maximum germination percentage in the simple well defined M medium supplemented with NAA. Through the exploration of the effects of different organic growth supplements on the multiplication of cultures, CW (20 %) in the medium efficiently initiated maximum PLB regeneration/explant and early plantlet development. Organic growth supplements proved beneficial for multiplication of the cultures besides enhancing growth of the plantlets as compared to control.

References

Anjum, S., Zia, M., and Chaudhary, M.F. (2006) Investigations of different strategies for high frequency regeneration of *Dendrobium* malones 'Victory'. Afr. J. Biotechnol., 5: 1738-1743.

Bensky, D., Gamble, X., Kaptchuk, T. (1986) Chinese herbal medicine. Eastland Press, Seatle - Washington

Bhasker, J. (1996) Micropropagation of *Phalaenopsis*. [Ph.D. Thesis.] Kerala Agricultural University Thrissur – India.

CITES (2014) Convention on international trade in Endangered Species of wild flora and fauna. Appendices I, II and III.

Cuyckens, F. and Claeys, M. (2004) Mass spectrometry in the structural analysis of flavonoids. J. Mass Spectrom., 39: 1-15.

Cuyckens, F., and Claeys, M. (2005) Determination of the glycosylation site in flavonoid mono-O-glycosides by collision-induced dissociation of electrospray-generated deprotonated and sodiated molecules. J. Mass Spectrom., 40**:** 364-372.

De Pauw, M.A., Remphrey, W.R. and Palmer, C.E. (1995) The cytokinin preference for *in vitro* germination and protocorm growth of *Cypripedium candidum.* Ann. Bot., 75: 267-275.

Escarpa, A. and Gonzalex, M.C. (2000) Optimization strategy and validation of one chromatographic method as approach to determine the phenolic compounds from different sources. J. Chromat., 897: 161-170.

Fan, C.Q., Wang, W., Wang, Y.P., Qin, G.W. and Zhao, W.M. (2001) Chemical constituents from *Dendrobium densiflorum*. Phytochem., 57: 1255-1258.

Galdiano Jr, R.F., de Lemos, E.G.M., de Faria, R.T. and Vendrame, W.A. (2014) Seedling Development and Evaluation of Genetic Stability of Cryopreserved *Dendrobium* Hybrid Mature Seeds**.** App. Biochem Biotechnol., 172: 2521-2529.

Gong, Y.Q., Fan, Y., Wu, D.Z., Yang, H., Hu, Z.B. and Wang, Z.T. (2004) *In vivo* and *in vitro* evaluation of erianin, a novel anti-angiogenic agent. Euro. J. Cancer., 40: 1554-1565.

Gray, D.E., Upton, R., Chandra, A.**,** Porter, A. and Harris, R.K. (2006) Quantitative analysis of flavonol glycosides in *Ginkgo biloba*: a comparison of two analytical methods. Phytochem. Anal., 17: 56-62.

Hayes, A.B. (1969) Mycorrhizal fungi and plant growth hormones. Amer. Orchid Soc. Bull., 38: 597-600.

Hong, P.I.**,** Chen, J.T. and Chang, W.C. (2008) Plant regeneration via protocorm-like body formation and shoot multiplication from seed-derived callus of maudiae type slipper orchid. Acta Physiol. Plant., 30:755–759.

Intuwong, O. and Sagawa, Y. (1973) Clonal propagation of sarcanthine orchids by aseptic culture of inflorescences. Amer. Orchid Soc. Bull., 42: 264–270.

Jin-Ming, K., Nogoh-Khang, G., Chialian-Sai and Chinatet-Fatt. (2003) Recent Advances in Traditional plant Drugs and orchids. Acta Pharma. Sinica., 24: 7-21.

Kalimuthu, K., Senthilkumar, R. and Vijayakumar, S. (2007) *In vitro* micropropagation of orchid, *Oncidium* sp. (Dancing Dolls). Afr. J. Biotechnol., 6: 1171—1174.

Kosir, P., Skof, S. and Luthar, Z. **(**2004**)** Direct shoot regenerated from nodes of *Phalaenopsis* orchids. Acta Agri. Sloven., 83: 233–242.

Kachlicki, P., Marczak, L., Kerhoas, L., Einhorn, J. and Stobiecki, M. J. (2005) Profiling isoflavone conjugates in root extracts of Lupine species with LC/ESI/MSn systems. Mass Spectrom., 40: 1088-1103.

Kaur, S. and Bhutani, K. K. (2011) *In vitro* propagation of *Dendrobium chrysotoxum* (Lindl.). Floricult. Orna. Biotechnol., 5: 50-56.

Kaur, S. and Bhutani, K.K. (2012) Organic growth supplement stimulants for *in vitro* multiplication of *Cymbidium pendulum* (Roxb.) Sw. Hort. Sci., 39: 47-52.

Kusumoto, M. and Takeda, Y. (1997) The effects of basal medium, and the addition of sugar and banana on the growth of *Oncidium* plantlets cultured *in vitro*. In: Combined Proceeding of International Plant Propagators Society, Japan, 47: 678-680.

Lauzer, D.M. and St-arnaud**,** Barabe´ D. (1994) Tetrazolium staining and *in vitro* germination of mature seeds of *Cypripedium acaule* (Orchidaceae). Lindleyana., 9:197–204.

Lawler, L.J. (1984) Ethnobotany of the orchidaceae. In: Arditti J (ed) Orchid Biology - Reviews and Perspectives, (Vol 3), Cornell University Press, Ithaca-New York, pp. 27-145.

Lee, M. and Phillips, R.L. (1988) The chromosomal basis of somaclonal variation – a review. J. Plant Physiol. Plant Mol. Biol., 39: 413- 437.

Lin, T.H., Chang, S.J., Chen, C.C., Wang, J.P. and Tsao, L.T. (2001) Two phenanthraquinones from *Dendrobium moniliforme*. J. Nat. Prod., 64:1084-1086.

Medina, R.D., Flachsland, E.A., Gonzalez, A.M., Terada, G., Faloci, M.M. and Mroginski, L.A. **(**2009) *In vitro* tuberization and plant regeneration from multimodal segment culture of *Habenaria bractescens* Lindl., an Argentinean wetland orchid. Plant Cell Tissue Organ Cult., 97: 91–101.

Men, S., Ming. X., Wang, Y., Liu, R., Wei, C. and Li, Y. (2003) Genetic transformation of two species of orchid by biolistic bombardment. Plant Cell Rep., 21: 592-598.

Minea, M., Piluek, C., Menakani, T.A. and Tantiwiwat, S. (2004) A study on seed germination and seedling development of *Spathoglottis kimballiani*. J. Nat. Acad. Sci., 38: 141–156.

Mitra, G.C., Prasad, R.N. and Chowdhary, A.R. (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.

Miyazawa, M., Shimamura, H., Nakamura, S., Sugiura, W., Kosaka, H. and Kameoka, H. (1999) Moscatilin from *Dendrobium nobile*, a naturally occurring bibenzyl compound with potential antimutagenic activity. J. Agri. Food Chem., 47: 2163-2167.

Murdad, R., Hwa, S.K., Seng, C.K., Latip, A.M., Aziz, A.Z. and Ripin, R. (2006) High frequency multiplication of *Phalaenopsis gigantea* using trimmed bases protocorms technique. Sci. Hort., 111: 73–79.

McIntyre, D.K., Veitch, G.J. and Wrigley, J.W. (1974) Australian orchids from seeds II. Improvement in techniques and further successes. Amer. Orchid Soc. Bull., 43: 52–53.

Morita, H., Fujiwara, M., Yoshida, N., Kobayashi. (2000) New picrotoxinin-type and dendrobine-type sesquiterpenoids from *Dendrobium snowflake* Red Star. Tetrahed., 56: 5801-5805.

Mubbarakh, S.A., Rahmah, S., Rahman, A.Z., Sah, N.N.M. and Subramaniam, S. (2014) Cryopreservation of *Brassidium* Shooting Star Orchid Using the PVS3 Method Supported with Preliminary Histological Analysis**.** Appl. Biochem. Biotech., 172: 1131-1145.

Pant, B. and Gurung, R. (2005) *In vitro* germination and seedling development in *Aerides odorata* Lour. J. Orchid Soc. India., 19: 51-55.

Park, S.Y., Murthy, H.N. and Paek, K.Y. (2003) Protocorm-like body induction and subsequent plant regeneration from root tip cultures of *Doritaenopsis*. Plant Sci., 164: 919–923.

Ng, Chyuam-Y. and Saleh, N.M. (2011) *In vitro* propagation of *Paphiopedilum* orchid through formation of protocorm-like bodies. Plant Cell Tissue Organ Cult., 105: 193-202.

Sinha, P. and Roy, S.K. (2004) Regeneration of an indigenous orchid, *Vanda teres* (Roxb.) Lindl. through *in vitro* culture. Plant Tissue Cult., 14:55–61.

Sungkum, L. and Deb, C.R. (2008) Effects of different factors on immature embryo culture, PLBs differentiation and rapid mass multiplication of *Coelogyne suaveolens* (Lindl.) Hook. Indian J. Exp. Biol., 46: 243-248.

Shu, Y., Guo, S.X., Chen, X.M., Wang, C.L. and Yang, J.S. (2004) On the chemical constituents of *Dendrobium nobile*. Chinese Pharma. J., 6: 421-422.

Sreeramanan, S., Vinod, B., Sashi, S. and Xavier, R. (2008) Optimization of the transient Gus gene transfer of *Phalaenopsis violacea* orchid via *Agrobacterium tumefaciens*: an assessment of factors influencing the efficiency of gene transfer Mechanisms. Adv. Nat. Appl. Sci., 2:77–88.

Sudeep, R., Rajeevan, P.K., Valasalakumari, P. and Geetha, C.K. (1997) Influence of organic supplements on shoot proliferation in *Dendrobium*. J. Horti., 3:38-44.

Vasudevan, R. and van Staden, J. (2010) Fruit harvesting time and corresponding morphological changes of seed integuments influence *in vitro* seed germination of *Dendrobium nobile* Lindl. Plant Growth Reg., 60: 237-246.

Wang, J.H., Luo, J.P. and Zha, X.Q. (2010) Structural features of a pectic polysaccharide from the stems of *Dendrobium nobile* Lindl. Carb. Polymers., 81:1-7.

Yang, H.Y.**,** Sung, S.H. and Kim, Y.C. (2007) Antifibrotic Phenanthrenes of *Dendrobium nobile* Stems. J. Nat. Prod., 70: 1925-1929.

Ye, Q.**,** Qin, G. and Zhao, W. (2002) Immunomodulatory sesquiterpene glycosides from *Dendrobium nobile*. Phytochemistry., 61: 885-890.

Yin, M. and Hong, S. (2009) Cryopreservation of *Dendrobium candidum* Wall. ex Lindl. protocorm-like bodies by encapsulation-vitrification. Plant Cell Tissue Organ Cult., 98:179–185.

Yang, H., Yang, Y.J. and Sung, S.H. (2008) Anti-proliferative constituents isolated from *Dendrobium nobile* stem on hepatic stellate cells. Planta Med., 74:1055-1059.

Zhao, W,M., Ye, Q.H., Tan, X.J., Jiang, H.L., Li, X.Y., Chen, K.X. and Kinghorn, A.D. (2001) Three new sesquiterpene glycosides from *Dendrobium nobile* with immunomodulatory activity. J. Nat. Prod., 64: 1196-1200.

Zhang, X., Liu, H.W. and Gao, H. (2007a) Nine new sesquiterpenes from *Dendrobium nobile*. Acta Chem. Helv., 90: 2386-2394.

Zhang, X., Xu, J.K. and Wang, J. (2007b) Bioactive bibenzyl derivatives and fluorenones from *Dendrobium nobile*. J. Nat. Prod., 70: 24- 28.

Zhang, X., Tu, F.J., Yu, H.Y., Wang, N.L. and Wang, Z. (2008) Copacamphane, picrotoxane and cyclocopacamphane sesquiterpenes from *Dendrobium nobile*. Chem. Pharm. Bull., 56: 854-857.

Znaniecka. J., Królocka, A., Sidwa-Gorycka, M., Rybczynski, J.J., Szlachetko, L.D. and Lojkowska, E. (2001) Asymbiotic germination and seedling development and plantlet propagation of *Encyclia* aff. *oncidioides* – an endangered orchid. Acta Soci. Bot. Pol., 74:193-198.