ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 5, Issue, 05, pp. 4280-4285, May, 2015

International Journal of
DEVELOPMENT RESEARCH

Full Length Research Article

MICROPROPAGATION AND ISSR-BASED GENETIC FIDELITY ANALYSIS OF *DENDROBIUM BELLATULUM* ROLFE-A RARE ORCHID FROM MANIPUR (INDIA)

Ahongshangbam Kiran Singh, Purnima Dubey, Jadumani Singh Ahongshangbam, Abbareddy Nageswara Rao and *Dhanaraj Singh Thokchom

Centre for Orchid Gene Conservation of the Eastern Himalayan Region, (Orchid Research and Development Centre), KVK-Sylvan, Hengbung-795129, Senapati District, Manipur, India

ARTICLE INFO

Article History:
Received 06th February, 2015
Received in revised form
16th March, 2015
Accepted 19th April, 2015
Published online 25th May, 2015

Key words:
Miniature orchid,
Complex additives,
Dendrobium bellatulum,
ISSR,
Micropropagation

ABSTRACT

An efficient protocol has been devised for the mass *in vitro* propagation of *Dendrobium bellatulum* Rolfe, a rare miniature orchid, through immature embryos obtained from 120-d-old capsules after pollination. *In vitro* seed germination trials were conducted using three different basal media *viz*., half strength Murashige and Skoog (½ MS), and Knudson C (KC) and Vacin & Went (VW), of which ½ MS medium proved to be ideal for getting maximum percentage of seed germination, 83.97 ± 0.83% within 8 weeks. Multiple shoots were induced when the mature protocorms with shoot primordia were cultured on ½ MS medium supplemented with different combinations of NAA, BAP, Kinetin and complex additives. However, the best response in growth of seedlings was observed in ½ MS basal medium supplemented with 15% banana pulp + 15% potato extract without addition of any exogenous hormones. Significant root induction in the multiplied shoots (10.34 ± 0.57) was observed in ½ MS medium fortified with 2 mg/l of NAA. Further, the ISSR (Inter Simple Sequence Repeats) based genetic fidelity test of the *in vitro* raised sub-cultured plantlets aided to the selection of plantlets with genomic consistency to that of the mother plant of which the plantlets were retained. Properly developed seedlings with shoot height 4 cm were hardened in a mixture of charcoal, brick and vermiculite in a ratio of 2:2:1. The *in vitro* raised plantlets showed 75% survivality on field transfer.

INTRODUCTION

The orchid diversity of the world in general and of North-east India in particular has been depleting day by day at an alarming rate due to increased biotic influences and various anthropogenic activities. Large number of wild population of ornamental and medicinally important orchid species like *Dendrobium, Cymbidium, Paphiopedilum,* etc. are being over-exploited by hobbyists as well as illegal traders. Jhum cultivation is an added threat for fast depletion of wild orchids in northeast India leading to their possible extinction in near future. Moreover, as the orchid seeds are non-endospermic, they need specific mycorrhizal association for embryo germination. Non-availability of specific fungal species in the wild leads to poor viability of the seeds (Rao, 1977).

Corresponding author: Dhanaraj Singh Thokchom
E-mail: dsthokchom@gmail.com
Centre for Orchid Gene Conservation of the Eastern Himalayan Region, (Orchid Research and Development Centre), KVK-Sylvan, Hengbung-795129, Senapati District, Manipur, India

Hence, to save the deteriorating orchid wealth in nature, proper conservation and sustainable utilization of this natural treasure is inevitable (Kishor *et al.,* 2006). Large scale multiplication of orchids through micropropagation is in fact, the only means to satisfy the desires of orchid hobbyists which would in turn reduce the pressure in forest ecosystem (Sunitibala and Kishor, 2009). The genus *Dendrobium* Swartz is one of the largest genera of the family orchidaceae comprising of about 1100 species distributed in tropical and sub-tropical habitats of India, China, Japan, Tahiti, Malaysia, New Guinea, Australia and New Zealand (Wood, 2006). In India, it is represented by about 115 species, two sub species and one variety of which about 75 species including 1 variety are found in north-east India and about 50 species alone in Manipur (Kumar and Kumar, 2005; Misra, 2007; Rao, 2007; Zote *et al*., 2009; Nanda *et al.*, 2014). *Dendrobium bellatulum* Rolfe is an attractive, ornamental and rare miniature orchid species which bears beautiful and long lasting flowers. In India, it was reported so far only from Manipur besides other oriental regions *viz.,* Myanmar, China and Thailand.

Looking at the present status of the orchid species, an immediate need for the conservation and mass propagation was felt. Hence, the present study has been carried out to develop a suitable protocol for *ex situ* conservation of *Dendrobium bellatulum* Rolfe through large-scale propagation of the species.

MATERIALS AND METHODS

Plant material

The plants of *Dendrobium bellatulum* were collected from Sadim Pukhri (1556m, 25° 20.50' N & 94° 01.51' E) of Senapati district, Manipur and were further maintained in the orchidarium of the Centre for Orchid Gene Conservation of Eastern Himalayan Region (Orchid Research and Development Centre), Hengbung, Manipur. After its flowering during April-May, the flowers were selfed (hand pollinated) on the second day of anthesis and bagged for a period of one week. The capsules were harvested after 120 days of selfing and seeds were used for *in vitro* propagation.

Sterilization of planting material

The harvested capsules were soaked in aqueous solution of commercial detergent (Labolene, Qualigens, India) for 15 min. Surface sterilization was done using 70% ethanol (v/v) for 30 seconds followed by rinsing thrice in sterile distilled water after every treatment. The capsules were further treated with a combination of 0.1% aqueous $HgCl_2$ solution (w/v) and 2 drops of Tween 20 for a period of 15 min followed by rinsing in sterile double distilled water 3-4 times; the sterilized capsule was transferred to a sterilized Petri dish and subjected for air-drying in a laminar airflow cabinet.

Inoculation of seeds

The green capsules were dissected longitudinally with a sterile surgical blade and the small mass (5 mm × 5 mm) of the immature aggregated seeds were scooped out for inoculation on different culture media in test tubes (30 mm × 200 mm) for *in vitro* germination. *In vitro* seed germination trials were done using three different basal medium *viz.*, ½ MS (Murashige and Skoog, 1962) medium, KC (Knudson, 1946) medium and VW (Vacin and Went, 1946) medium. The pH of different media was adjusted as per the original protocols of the three respective media under trials, prior to the addition of 0.8% agar as gelling agent. The cultures after inoculation were transferred to the culture room and maintained at 25 ± 2 °C under 16 h photoperiod with 3000 lux and 60-70% relative humidity in the culture room. Various parameters were periodically observed and analyzed *viz.*, the time taken for seed germination, germination %, colour and volume of protocorm. The protocorm volume was determined using the formula for an oblate spheroid $4/3\pi a^2 b$, where 'a' and 'b' are the semi- minor and semi-major axis (Kumaria and Tandon, 1991).

Seedling growth

½ MS medium was selected as shoot multiplication medium. The medium was fortified either with three plant growth regulators *viz.*, Kinetin, BAP and NAA ranging from 0.5-4.0 mg/L or with complex additives i.e., 15% banana pulp + 15% potato extracts or 15% coconut water singly. The effect of complex additives without addition of PGRs, and the individual as well as synergistic effect of both auxin (NAA) and cytokinin (Kinetin, BAP) in different combinations on shoot growth were observed. The developed propagules were subcultured every 20 days. After 2-3 subcultures, various growth parameters *viz.*, number of shoots, shoot length (cm), number of leaves, leaf length (cm), number of roots and root length (cm), were recorded for 8 weeks at an interval of 14 days.

Acclimatization

Healthy *in vitro* raised shoots with good number of roots were washed under tap water to remove the adhered agar, treated with 0.5% (w/v) Bavistin (fungicide) for 10 min and were subsequently transferred to community pots with autoclaved potting mixture comprising of brick chips, charcoal pieces and vermiculite in a ratio of 2:2:1 with a layer of moss on top. The seedlings were sprayed daily with ¼ MS medium without sucrose for 30 days, inside the hardening chamber and were further transferred to the field.

Total genomic DNA extraction

DNA was extracted from *Dendrobium bellatulum* by using protocol described earlier (Wang *et al*., 2009) with slight modification. 0.1 g of leaf was homogenised using mortar and pestle and subsequently 500 µl of extraction buffer (2% CTAB, 100 mM Tris HCl, 20 mM EDTA, 1.4 M Nacl, 4% polyvinylpyrrolidone, 10 mM mercaptoethanol) was added. The homogenate was transferred to 2 ml tubes and gently mixed by inverting the tubes. It was then incubated at 65 ˚C for 50 min. 500 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the tubes and mixed. It was then centrifuged at 6000g for 10 min. The supernatant was transferred to fresh 1.5 ml tube followed by addition of equal volume of isopropanol and kept for 2 h for precipitation at room temperature and centrifuged at 10000g for 13 min. The pellet was collected and washed with 70% ethanol twice, followed by washing with absolute alcohol. The pellet was dried and dissolved in 100 µl TE buffer (10 mM tris HCl, pH 8.0, 1mM EDTA).

Touchdown PCR for ISSR-based genetic fidelity test

The PCR reaction mixture was prepared and Inter Simple Sequence Repeats (ISSR) analysis was carried out as described elsewhere with modifications (Wang *et al.* 2009). ISSR primers (microsattelite set 9 University of British Columbia, Vancouver, Canada) was custom designed from Integrated DNA Technologies, India and 17 primers (Table 3) were employed in comparative PCR amplification of ISSR loci in the genome of tissue culture raised plantlets as well as that of the mother plant. The PCR master mix contained 10x buffer (15 mM Tris), $MgCl_2$ (2 mM), dNTP mix (1 mM), Primer (0.2 mM), Taq polymerase (1 unit) and Template DNA (2.5 ng/mL). The PCR reactions were carried out using a (Bio-Rad, My CyclerTM) thermal cycler. The thermo cycling steps for the amplification of ISSR using 3 different primers were

94˚C for 4 min, 10 cycles of denaturation at 94˚C for 60 s, annealing at 53˚C for 60 s (decreasing 0.5˚C per cycle) and extension at 72˚C for 90 s followed by 25 cycles of 94˚C for 60 s, 48˚C for 45 s and 72˚C for 90s and a final extension of 72˚C for 10 min. After amplification, the products were electrophoresed on 1.2% agarose gels containing 0.5 µg/ml ethidium bromide and gel pictures were obtained using a Molecular Imager Gel Doc^{TM} XR+ (Bio-Rad). Plantlets with same genotype to that of the mother plants were selected and sub-cultured and futher analysed for genetic fidelity.

Statistical analysis: Data were subjected to one way analysis of variance (ANOVA) followed by Tukey's post hoc test using SPSS software (version 16.0; SPSS Inc., Chicago). Values are presented as mean ± standard deviation (SD). Values were considered statistically significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Asymbiotic seed germination and early seedling development

Seed germination in asymbiotic way in sterile condition is one of the methods of conservation and propagation of orchids (Rasmussen, 1995). The *in vitro* seed germination trials of *Dendrobium bellatulum* showed that the constituents of the media have an essential role on seed germination. In the present investigation, it was found that the highest percentage of seed germination was in ½ MS medium (83.97 ± 0.83) followed by KC (54.52 ±1.46) and the least response was recorded in VW (25.62 ± 1.35) 8 weeks post-inoculation (Table 1).

Table 1. Effect of different media on seed germination and development of protocorm in *Dendrobium bellatulum* after 8 weeks of inoculation

Sl. No.	Media	Germination (%)	Volume of protocorm (mm^3)	Colour of protocorm
1.	½ MS	83.97 ± 0.83	1.03 ± 0.49	Light green
2.	KC	54.52 ± 1.46	0.10 ± 0.09	Cream
3.	VW	25.62 ± 1.35	0.09 ± 0.05	Light brown

Values are mean ± SD, Means are statistically not different from each other as determined ANOVA followed by Tukey's test of significance at $p \leq 0.05$. ANOVA does not apply between rows. (n = 10)

Hajong *et al*. (2010) reported that differential response of orchid seed in certain media are attributed to the presence or absence of nutrients and vitamins. Both KC and VW media is having ammonium sulphate as one of the constituent of the media which might have accounted for the poor response in seed germination in these media as in the case of B_5 which was reported elsewhere (Hajong *et al.*, 2010). The impact of different media was observed as variation in the protocorm volume. Larger protocorm volume was attained in ½ MS (1.03± 0.49) whereas the least was obtained in the VW medium (0.09 ± 0.05). It was interesting to note that the texture of the protocorm also showed variation, light green protocorm formation was observed in ½ MS medium (Table 1, fig.1a) whereas in KC and VW media, it was cream and light brown respectively. Moreover, protocorm development into seedlings was more pronounced in ½ MS medium (Table 1) and this might be attributed to the availability of micro and macronutrients, vitamins, inositol and glycine in higher amounts as compared to the other media. Higher concentration of Nitrogen, i.e., ammonium nitrate and potassium nitrate in MS medium as compared to other media could have played an important role in the growth and development of protocorms. This was similar to the findings of Gogoi *et al*. (2012) in *Cymbidium eburneum*. The suitability of the MS medium for protocorm development was also reported by Shadang *et al*. (2007).

Effect of growth regulators on growth and development of seedlings

Plant growth regulators have significant impact on the growth of *in vitro* raised seedlings of orchid (Roy *et al.,* 2011). The study for developmental process of protocorms in ½ MS medium was undertaken with two cytokinins (BAP and Kinetin) and one auxin (NAA) (Table 2). The effect of BAP (0.5-4mg/L) alone and in combination with 1mg/L NAA on the development of shoots and roots was observed. Further, the effect of Kinetin in combination with NAA was studied to screen for synergistic effect of the growth regulators on protocorm development. The effect of auxin was studied by supplementing ½ MS medium with varying concentration of NAA (0.5-4mg/L).

Table 2. Effect of PGRs and complex additives on different growth parameters of *in vitro* raised *Dendrobium bellatulum*

PGR	No. of shoots	Shoot height (cm)	No. of leaves	Leaf length (cm)	No. of roots	Root length (cm)
Control	1.00 ± 0.00	1.40 ± 0.10	2.66 ± 0.57	2.20 ± 0.20	7.00 ± 1.00	2.17 ± 0.15
$N_1B_{0.5}$	1.00 ± 0.00	1.30 ± 0.10	2.67 ± 0.59	2.00 ± 0.20	6.66 ± 0.58	2.27 ± 0.25
N_1B_1	1.34 ± 0.58	1.53 ± 0.15	3.33 ± 0.58	2.20 ± 0.21	6.66 ± 0.57	2.20 ± 0.20
N_1B_2	2.00 ± 1.00	2.37 ± 0.17^{a,b}	4.67 ± 0.57^{b}	2.93 ± 0.12	9.66 ± 1.52^{a,b}	3.20 ± 0.20^{a}
N_1B_4	1.67 ± 0.58	1.83 ± 0.15	4.00 ± 1.00	2.17 ± 0.35	8.67 ± 1.53	2.53 ± 0.42
$N_1K_{0.5}$	1.00 ± 0.00	1.23 ± 0.06^{a}	2.33 ± 0.57^{c}	2.00 ± 0.20	6.33 ± 0.58	2.10 ± 0.26
N_1K_1	1.34 ± 0.58	1.43 ± 0.15	2.34 ± 0.55^{c}	2.14 ± 0.31	6.33 ± 0.57	2.14 ± 0.31
N_1K_2	1.66 ± 0.58	2.00 ± 0.20	4.33 ± 0.53	2.93 ± 0.12	9.00 ± 1.00^{a,b}	2.93 ± 0.30
N_1K_4	1.00 ± 0.00	1.70 ± 0.20	4.00 ± 0.57	2.50 ± 0.30	8.00 ± 1.12	2.46 ± 0.31
$B_{0.5}$	1.00 ± 0.00	1.40 ± 0.10	2.66 ± 0.57	2.00 ± 0.20	6.33 ± 0.58	2.00 ± 0.20
B_1	1.33 ± 0.58	1.84 ± 0.05	2.67 ± 0.59	2.03 ± 0.25	6.34 ± 0.57	1.94 ± 0.12^{b,c}
B_2	1.67 ± 0.57	2.36 ± 0.15^{a,b}	4.33 ± 0.58	3.00 ± 0.00^{a}	9.33 ± 1.13	2.60 ± 0.53
B_4	1.33 ± 0.57	1.93 ± 0.31	3.34 ± 0.55	2.53 ± 0.42	8.00 ± 1.53	2.16 ± 0.35
$N_{0.5}$	1.00 ± 0.00	1.30 ± 0.10	2.33 ± 0.58^{c}	1.70 ± 0.10^{c}	7.33 ± 0.58	2.20 ± 0.20
N_1	1.00 ± 0.00	1.40 ± 0.25	2.35 ± 0.57^{c}	1.73 ± 0.23^{c}	8.00 ± 1.00	2.13 ± 0.31
N_2	1.33 ± 0.57	2.00 ± 0.20	2.66 ± 0.54	2.30 ± 0.30	10.34 ± 0.57^{a}	2.56 ± 0.40
N_4	1.00 ± 0.00	1.80 ± 0.21	2.68 ± 0.55	2.20 ± 0.20	8.67 ± 1.16	2.60 ± 0.53
$K_1N_{0.5}$	1.00 ± 0.00	1.33 ± 0.12	2.66 ± 0.59	2.00 ± 0.20	5.66 ± 1.15^{c}	1.93 ± 0.12
K_1N_1	1.33 ± 0.57	1.40 ± 0.20	2.68 ± 0.57	2.00 ± 0.20	5.67 ± 0.58^{c}	1.80 ± 0.20^{b}
K_1N_2	1.67 ± 0.57	2.13 ± 0.12	4.00 ± 1.00	2.77 ± 0.25	8.66 ± 1.15	2.40 ± 0.35
K_1N_4	1.33 ± 0.57	1.83 ± 0.15	3.00 ± 1.12	2.23 ± 0.25	7.65 ± 0.59	2.00 ± 0.20
B+P	2.33 ± 0.57	2.40 ± 0.20^{c}	5.00 ± 1.00^{a}	2.93 ± 0.12	9.67 ± 1.53^{a,b}	3.13 ± 0.31
CW	1.67 ± 0.57	2.23 ± 0.25	4.00 ± 1.02	2.80 ± 0.20	9.00 ± 1.00	2.77 ± 0.25

Values are mean ± SD, Means followed by same letters are statistically not different from each other as determined ANOVA followed by Tukey's test of significance at $p \leq 0.05$. ANOVA does not apply between rows. (n = 10)

Table 3. List of primers, their sequences, annealing temperature (Tm) and number and size of amplified regions generated by ISSR markers

Primer name	Sequence 5´_ 3´	Tm (°C)	No. of scorable bands/ primer	Total number of bands amplified	Range of amplification (bp)
ISSR 12	$(AC)_8$ AT	52.00	10	190	600-2500
ISSR 14	$(AC)_8$ AG	52.90	13	247	400-2500
ISSR 15	$(AC)_8$ TG	52.90	6	114	500-2000
ISSR 125	$(AC)_8$ CA	54.50	9	171	400-2000
ISSR 134	$(AG)_8$ AA	47.50	7	133	200-1500
ISSR 144	$(AC)_8$ GA	54.50	5	95	400-2000
ISSR 155	$(TG)_8$GG	55.50	4	76	600-1500
ISSR 165	$(AG)_8$ CC	51.90	2	38	1500-2000
ISSR 174	$(ACTG)_4$	47.40	8	152	400-2500
ISSR UBC807	$(AG)_8$T	47.00	3	57	600-1000
ISSR UBC812	$(GA)_8$ A	45.70	4	76	200-600
ISSR UBC826	$(AC)_8$ C	52.80	5	95	200-1500
ISSR UBC834	$(AG)_8$ CT	50.60	4	76	400-2000
ISSR UBC835	$(AG)_8$ YC*	50.20	6	114	200-1500
ISSR UBC841	$(GA)_8$ YC	48.50	4	76	600-1000
ISSR UBC842	$(GA)_8$ YG	48.80	2	38	1500-2000
ISSR UBC866	$(CTC)_6$	55.70	5	95	600-2000
Total			97	1843	

** Y = (C,T)*

In the present study, ½ MS supplemented with cytokinin (2mg/L BAP) showed best response in the increase in shoot height (2.36 ± 0.15 cm) and leaf length (3.00±0.00 cm). The beneficiary effect of BAP on the growth and development of *D. bellatulum* is in consistency with earlier reports (Hossain *et al*., 2010; Gogoi *et al*., 2012; Roy *et al.,* 2011). ½ MS supplemented with 2mg/L NAA proved to be ideal for root induction which is similar to earlier finding (Dutta *et al*., 2011). 2mg/L NAA produced an average number of roots (10.34 ± 0.57) but the number of shoot was not considerably high. However, maximum number of shoots were observed in ½ MS supplemented with a combination of 1 mg/L NAA and 2 mg/L BAP. It was observed that further increase in the concentration of auxin and cytokinin beyond the levels stated above, inhibited the growth of the seedlings of *D. bellatulum*.

Nagaraju *et al*. (2003) had also reported that cytokinins at higher concentration have inhibitory effect on the growth of the plant. It was observed that when the media was fortified with cytokinin or auxin alone, the response of the seedlings was poor but both the hormones when used in combination gave enhanced response in the overall development of seedling. When the seedlings were transferred to the ½ MS medium supplemented with NAA + BAP, their growth was greatly enhanced and showed considerably good response as assessed through the parameters studied in ½ MS medium supplemented with 1mg/L NAA + 2mg/L BAP (Table 2, Figure1c). The synergistic effects of cytokinins and auxins when used in combination on orchids is evident from results in earlier publications (Hossain *et al*., 2010; Roy *et al.,* 2011; Gogoi *et al*., 2012). Our findings also supplements to the results reported earlier.

Effect of complex additives on seedling development

Complex additives and undefined organic extracts have been applied for decades in the enhancement and improvement of *in vitro* orchid plant proliferation. A number of organic extracts, such as banana pulp, peptone, honey, and the extracts of beef or taro, are known to be very effective in providing nutrients and growth factors for *in vitro* orchid plantlets. The extracts were reported to be successful in inducing growth and development of several orchid species of genera such as *Phalaenopsis, Doritaenopsis* and *Vanda,* etc. (Arditti *et al*., 1990; Islam *et al*., 2003; Gnasekaran *et al*., 2010; Gnasekaran *et al*., 2012). In the present study, the effect of complex additives on ½ MS medium was studied using 15% coconut water and 15% banana pulp + 15% potato extract without addition of exogenous growth regulators. The later proved to have a prudent impact on seedling development (Table 2, Figure1b) as compared to former. A good index in the parameters considered, in terms of multiple shoot induction, shoot length, leaf length, etc., was observed in ½ MS supplemented with 15% banana pulp + 15% potato extract. This combination of complex additives can be exploited for large-scale production of the species more economically.

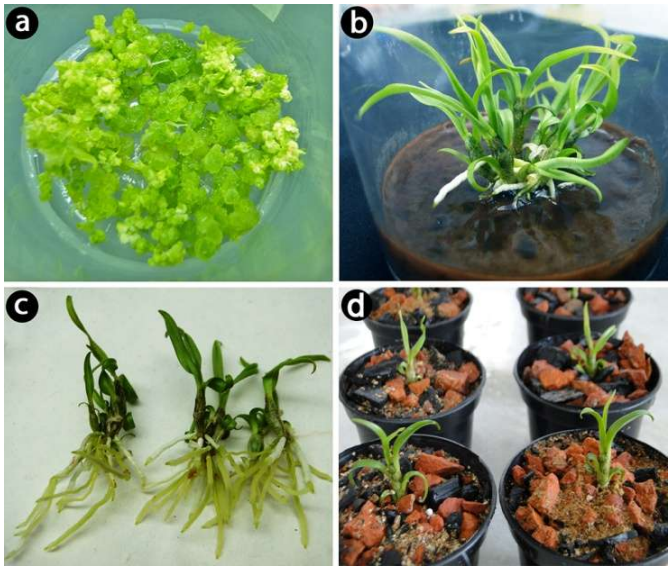

Figure 1. Various stages of micropropagation of *Dendrobium bellatulum* through asymbiotic seed germination. a. Protocorm development in ½ MS medium; b. Multiple shoot induction in ½ MS medium + 15 % banana pulp + 15% potato extract; c. *In vitro* rooting of regenerated plantlets in ½ MS medium + 2 mg/L NAA; and d. Hardening of the *in vitro* raised plantlets

Acclimatization and transfer of *in vitro* raised plantlets to soil

The complete plantlets of *D. bellatulum,* when grown in the compost mixture comprising of brick chips, charcoal pieces and vermiculite in the ratio 2:2:1 (Figure1d), showed highest survivality (75%). This might be due to the fact that the roots were given structural support, and the presence of air spaces between the substratum facilitate the roots to spread out properly. The optimal moisture content could be maintained by the layer of moss on the top (Nongrum *et al*., 2007; Gogoi *et al*., 2012). During the process of hardening, it was observed that the transferred plantlets initially shed their leaves then produced new leaves. According to Preece and Sutter (1991) plantlets when transferred, must produce new leaves to adjust to new conditions in order to enable effective photosynthesis and growth of the *in vitro* raised plants. Similar results were reported in orchids elsewhere (Kauth *et al*. 2006; Dutra et al. 2008: Gogoi *et al*. 2012).

Genetic Fidelity

Inter simple sequence repeat (ISSR) markers are cost-effective, very rapid, reliable and are highly discriminative. Hence, they are suitable to assess genetic fidelity of *in vitro* raised plants (Lakshmanan *et al.,* 2007). Out of the 17 ISSR Primers used for screening of genetic fidelity, 7 primers produced appreciable number of bands. The annealing temperature of the primers ranged from 47 °C to 55.70 °C (Table 3). The primers produced a total of 97 scorable bands of the size between 200-2500 bp. The scorable number of bands varied from 2 (ISSR 165 & ISSR UBC842) to 13 (ISSR 14) with an average of 5.7 bands per primers. The banding patterns of the sub-cultured plantlets and the mother plant were monomorphic (Figure 2). No polymorphism was observed with any of the primers used in the experiment. The experimental findings suggest that the *in vitro* raised plantlets of *D. bellatulum* have the same genotype which is similar to that of the mother plant.

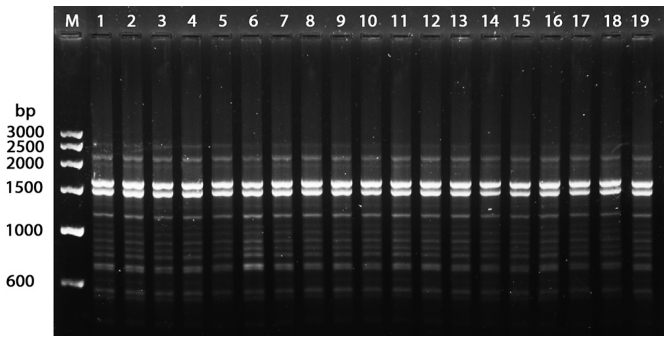

Figure 2. Amplified products obtained with ISSR primer (ISSR 14), *lane* M is 200 bp molecular weight marker, *lane* 1 represents mother plant (*Dendrobium bellatulum*) and *lanes* 2-19 represent selected sub-cultured plants

Conclusion

From the present investigation, it may be concluded that for asymbiotic seed germination, the requirements of nutrients for complete seedling development vary at different stages of growth and development. Complex additives play a vital role in seedling development without any exogeneous hormone supplementation which is a cost effective method for raising orchid through micropropagation. ISSR-based genetic fidelity tests revealed consistency in the genotypic characters of the selected and sub-cultured saplings which is similar to that of the mother plant. The protocol developed through the present study can be used for *in vitro* mass propagation and *ex vitro* conservation of *D. bellatulum*, a rare miniature orchid from Manipur, India.

Acknowledgement

The authors would like to express sincere gratitude to SERB Division of Department of Science and Technology, Government of India, New Delhi for financial support to carry out the study on COCEHR. The authors are also grateful to Mr. H Kipgen, President (FEEDS/ KVK-Sylvan, Hengbung) for facilities and encouragement.

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