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In vitro seed germination, protocorm and seedling development of Dendrobium jenkinsii Wall. Ex Lindl. - an ornamental, medicinal and threatened orchid

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Abstract

Dendrobium jenkinsii is a threatened ornamental, medicinal epiphytic orchid. The plant is used to cure fever, dried eyes, thrush and gastritis. Due to over exploitation, habitat destruction and inefficient seed germination, the species is becoming extinct. The seeds of *Dendrobium jenkinsii* are non-endospermous and require a particular fungus for germination. In natural condition propagation of the orchid is very slow and problematic. Therefore, it is an urgent requirement to establish a micro propagation protocol for its conservation as well as to fulfil commercial demand. The objective of the present study was to establish an effective protocol for *in vitro* seed germination, protocorm and plantlet development of *Dendrobium jenkinsii*. Murashige and Skoog (MS), Half MS, Knudson 'C' (KC), Vacin and Went (VW), Gamborg et al. (B5) media were used to study their effects in seed germination and protocorm development. Effects of different plant growth regulators were also studied. Maximum percentage (93.7%) of seed germination was observed in MS medium and minimum percentage (59.2%) was in VW medium. Time taken for germination of seeds in MS medium is also less as compared to other medium used in the experiment. The best suitable medium for seed germination i.e MS media was supplemented with various combinations of Cytokinins (BAP, Kinetin) and Auxin (IAA, NAA) and used for further growth and development of the protocorms. MS medium fortified with BAP (2µM) and NAA (0.5 µM) gives maximum number of shoot and leaves. On the other hand maximum number of root was obtained with combination (i) BAP (2 µM) + NAA (0.5µM) and (ii) BAP (0.5 µM) + IAA (0.4 µM).

Keywords

Acclimatization, *Dendrobium jenkinsii*, plant growth regulators, protocorm, seed germination, shooting and rooting

Abbreviation

IAA	Indole -3- acetic acid
BAP	6- Benzylaminopurine
KIN	Kinetin
NAA	α- Napthaleneacetic acid
PGR	Plant growth regulator

1. Introduction

Dendrobium jenkinsii Wall. Ex Lindl. (Captain Jenkin's Dendrobium) is an important ornamental as well as medicinal orchid. It is mainly found in North-East India, Bhutan, South China, Myanmar, Thailand Laos and North Vietnam [18]. According to Edward's Botanical register the plant was collected by Captain Jenkins from Goalpara Assam and was submitted to Dr. Wallich in November, 1836 [9]. The dried stem of the plant is used to cure gastritis, fever, thrush and dried eyes [23]. This orchid species is added in Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES) (www.cites.org/eng/cop/12/doc/E12–64.pdf). Due to extensive collection from the wild, deforestation and various types of anthropogenic activities, natural population of the species is rapidly decreasing. Clonal propagation i.e. separation of adventitious root bearing growth from the mother plant is very slow and is not appropriate for large scale propagation. Germination of seed in natural condition required specific fungus hence germination rate is very low in environment

[24]. Therefore there is an urgent need for the development of a suitable mass propagation technique for conservation as well as commercial purpose of the plant species.

Although a number of seed germination and *in vitro* mass propagation protocol of different *Dendrobium* orchid species, including those of *Dendrobium aphyllum* [6, 8, 12], *Dendrobium nobile* [31], *Dendrobium fimbriatum* [14, 19, 34), *Dendrobium chrysotoxum* [17, 22], *Dendrobium hookerianum* [24], *Dendrobium aggregatum* [32, 13], *Dendrobium Devonianum* [4] were developed in the past by many authors, in vitro seed germination and mass propagation of *Dendrobium jenkinsii* has not been reported yet as per best of our knowledge.

In this study we assessed the effect of MS, Half strength MS, KC, VW and B5 media on germination of seeds and supplementation of different concentration of cytokinin (BAP, Kin) and auxin (IAA, NAA) on MS medium for plantlet development. This protocol will help to conserve this threatened ornamental and medicinal orchid.

2. Materials and methods

2.1. Plant material, sterilization and culture media

Green capsule of *Dendrobium jenkinsii* were collected from Botanical Garden of Goalpara College, Goalpara, Assam. Collected explant was washed properly under running tap water and rinsed with distilled water. The explant was then surface disinfected with 0.1% HgCl2 (Himedia) for 10 min and finally rinsed with sterile distilled water 4 to 5 times followed by air dried aseptically. All the cultural procedures are carried out inside laminar air flow cabinets. Seeds were extracted from the capsule by using a sterile surgical blade and spread (approximately 200 mg of seeds) it over liquid media (five different media: Murashige and Skoog (MS)[25], Half strength MS, Knudson (KC)[18], Vacin and Went (VW)[34], and Gamborg et al (B5)[12], inside the test tubes. Each test tube contains 15 ml of the media. The pH of all the media were maintained at 5.8 with the help of 2 N NaOH followed by autoclaving at 121 °C for 15 minutes. The culture tubes were placed in the dark for 3 weeks at 25 ± 2 ℃ and 65 -75 % relative humidity followed by 15-h light with 2.5 K light intensity and 9-h dark period. Each experiment was repeated three times with 10 replicates per treatment.

Figure1: (A) Flowering plant of *D. jenkinsii* in wild. (B) Seeds of *D. jenkinsii*

2.2. Plant growth regulators

Murashige and Skoog the most effective medium for seed germination and protocorm development was selected and supplemented with different concentrations of 6-Benzylaminopurine (BAP), Kinetin, α-Naphthaleneacetic acid (NAA), Indole-3-acetic acid (IAA) to study their effects in subsequent growth of the protocorms and plantlets.

2.3. Seed germination

Seeds were germinated in five different media MS, Half strength MS, KC, VW and B5. Seeds were inoculated in 50 ml test tubes containing 15 ml of media. Seeds germination data were collected following 60 days of culture.

2.4. Protocorm induction

Protocorm were induced in MS, Half MS, KC, VW, B5 media. Protocorm formation data were recorded at an interval of 15 days up to 90 days.

2.5. Shoot and root regeneration

Protocorm were randomly selected and sub cultured in MS medium with different concentrations and combinations of cytokinins (BAP, Kinetin) and auxins (IAA, NAA). All the data regarding shoot and root regeneration were collected after 75 days of protocorm subculture.

2.6. Hardening and acclimatization

In vitro well rooted plantlets were washed properly with sterile water to remove residual medium completely then transferred to pots containing sterile mixture of brick, charcoal, pebble and coconut husk in a ratio 1:1:1:2(v/v). Plantlets were initially covered with sterilized perforated poly bags to maintain relative humidity and sprayed with half strength MS media on alternate days. Plantlets were kept in culture room for 25 days and later transferred to greenhouse condition. Survival rates of the plantlets were calculated after 90 days of transfer.

2.7. Statistical analysis

10 replicates were maintained for each treatment and the whole experiment was repeated three times. The data were represented as mean ± standard error and one-way analysis of variance (ANOVA) was performed using IBM SPSS V23.0 statistical package (Armonk, New York, USA). The means were compared by Tukey's HSD test (P= 0.05).

3. Results

3.1. Seed germination in different media

Seeds were germinated on MS, Half MS, Knudson C, VW and B5 media. Appearance of the embryo from the testa was recognised as germination [3, 25]. Highest percentage (93.7%) of seeds was germinated on MS media. The percentages of seed germination in other media were 81.1% (Half MS), 72.5 % (KC media), 59.2% (VW) and 64.2 % (B5). On MS medium germination started after 3 weeks of inoculation. On the other hand on Half MS medium and KC medium germination started after 4 weeks of culture and in VW and B5 medium germination began after 6 weeks of inoculation.

3.2. Protocorm formation

On MS medium protocorm appears after 3 to 4 weeks of germination. Appearance of protocorm was 1 week slower in Half MS and KC media and 2 to 3 weeks slower in VW and B5 media as compared to MS medium.

3.3. Shoot and root regeneration from protocorms

Protocorms which were derived from MS, Half MS, Knudson C, VW and B5 media were sub cultured in MS media supplemented with BAP, Kinetin, NAA and IAA in different combination and concentrations for shoot and root regeneration. MS medium supplemented with BAP (2 µM) and NAA (0.5 µM) gives maximum number of shoot and leaves. On the other hand maximum no. of roots were obtained with combination (i) BAP (2 µM) + NAA (0.5 µM) and (ii) BAP (0.5 µM) + IAA (0.4 µM). Lowest number of root and shoot were observed in MS media without addition of any PGR.

Table I: Effect of MS, Half MS, KC, VW and B5 media on seed germination and protocorm development

Media	Percentage of seed germination*	Percentage of protocorm formation	No. of days taken for prtotocorm formation
MS	93.7±0.54[a]	74.6±0.70[a]	37.5±0.98[a]
Half MS	81.1±0.60[b]	63.2±0.83[b]	45.5±0.88[b]
KC	72.5±0.52[c]	55.3±0.89[c]	63.8±0.68[c]
VW	59.2±0.80[d]	56.2±0.92[d]	71.6±0.82[d]
B5	64.2±0.55[e]	44±0.98[e]	85±0.73[e]

*Germination percentage were recorded after 60 days of inoculation
Values are Mean ± SE of 10 replicates from the 3 repeated experiments. Means followed by same superscript in the column are not significantly different at $p<0.05$ as per Tukey's HSD

3.4. Acclimatization and hardening.

The well rooted plantlets were transferred to pot containing mixture of sterile crushed bricks, charcoal, pebbles and coconut husk with ratio 1:1:1:2. The roots of the plantlets were washed carefully with sterile distilled water to remove the remnant of the media. Initially the plantlets were kept in culture room for 20 to 25 days and then transferred in to the green house. The temperature of the green house was maintained at 25± 2 ºC with relative humidity 60 to 70% and 16 hours light and 8 hours dark period. About 70% plants were survived in green house condition.

4. Discussions

In our investigation we found that asymbiotic seed germination technique can be used for mass propagation of *D. jenkinsii* seedlings and thus this can be applied successfully for conservation purposes. Asymbiotic media influenced the seed germination and subsequent growth of the seedlings [7]

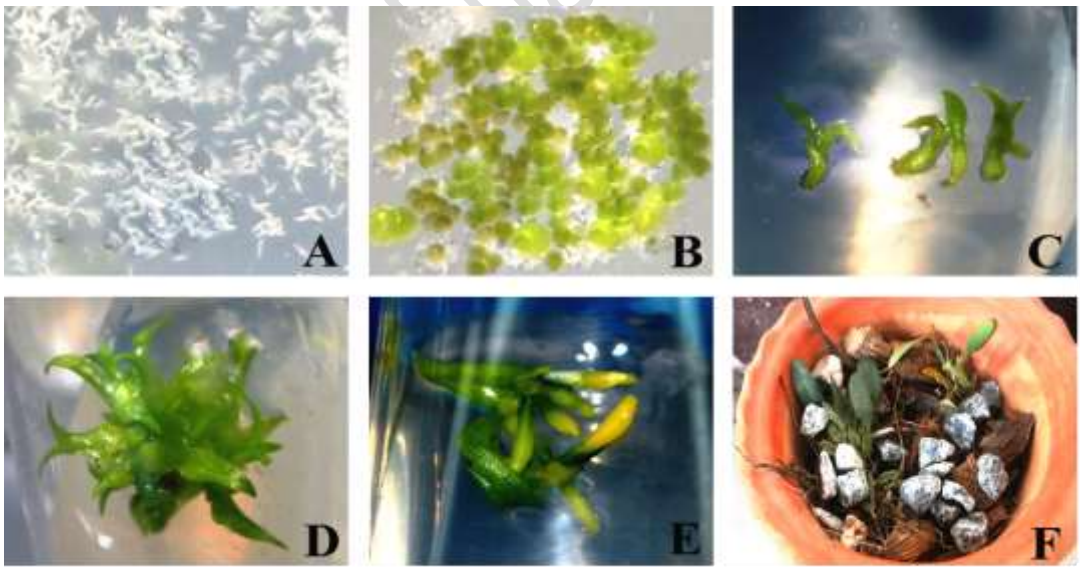

Figure 2: Different stages of development of plantlets from seeds of *D. jenkinsii* (A) Seeds after 7 days of inoculation (B) Germination and protocorm formation after 60 days of culture (C) Leaf initial stage (D) Multiple shoot formation (E) Seedlings with root and shoot (F) Hardened plantlets after 90 days

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Table II: Effect of plant growth regulators on development of plantlet from protocorm on MS media after 75 days of subculture.

PGR (µM)		Shoot No.	Shoot Length	Leaf No.	Root No.	Root Length
Control		0.8±0.2^{a}	0.69±0.09^{a}	1.4±0.16^{abc}	1.2±0.13^{a}	1.19±0.17^{ab}
BAP	NAA					
0.5	0.5	1.4±0.16^{abcd}	1.6±0.22^{abc}	3.6±0.27^{ghij}	2.2±0.25^{abcd}	1.7±0.26^{abc}
1	0.5	2±0.25^{bcdef}	2.9±0.31^{defg}	3.1±0.23^{efgh}	2.6±0.31^{bcd}	3.5±0.17^{fg}
2	0.5	4.6±0.22^{g}	3±0.25^{efg}	4.7±0.30^{j}	3.2±0.33^{d}	2.2±0.16^{bcde}
2.5	0.5	2.5±0.26^{ef}	3.4±0.30^{efg}	1.4±0.16^{abc}	2.1±0.31^{abcd}	2.12±0.23^{bcd}
3	0.5	1.4±0.22^{abcd}	1.6±0.16^{abc}	1.2±0.13^{a}	1.4±0.16^{ab}	1.77±0.10^{abcd}
Kinetin	NAA					
0.5	0.5	1.3±0.15^{abc}	2.4±0.30^{bcdef}	2.9±0.28^{defg}	2.4±0.27^{abcd}	0.95±0.16^{a}
1	0.5	1.6±0.22^{abcde}	2.8±0.29^{cdefg}	1.5±0.22^{abc}	1.8±0.20^{abc}	1.11±0.15^{a}
2	0.5	1.9±0.23^{bcdef}	3.5±0.27^{fg}	3.3±0.37^{ghi}	2.6±0.30^{bcd}	1.86±0.11^{abcd}
2.5	0.5	1.4±0.16^{abcd}	1.6±0.22^{abc}	1.8±0.25^{abcde}	2.1±0.23^{abcd}	2.53±0.19^{cde}
3	0.5	1.2±0.13^{ab}	1.7±0.26^{abcd}	2.7±0.21^{cdefg}	1.9±0.28^{abcd}	2.3±0.3^{bcde}
BAP	IAA					
0.5	0.2	1.5±0.16^{abcde}	1.4±0.16^{ab}	1.9±0.23^{abcdef}	1.7±0.26^{abc}	2.67±0.21^{def}
0.5	0.4	2.3±0.21^{cdef}	3.7±0.21^{g}	4.3±0.30^{hij}	3.2±0.29^{d}	3.1±0.28^{efg}
0.5	0.6	2.4±0.22^{def}	2.7±0.3^{cdefg}	4.5±0.40^{ij}	2.1±0.23^{abcd}	3.91±0.17^{g}
0.5	0.8	2.7±0.21^{f}	2.5±0.22^{bcdefg}	3.6±0.30^{ghij}	1.7±0.21^{abc}	1.84±0.15^{abcd}
0.5	1	1.8±0.2^{abcdef}	2.3±0.21^{bcdef}	1.6±0.22^{abcd}	1.3±0.15^{a}	0.95±0.096^{a}
Kinetin	IAA					
0.5	0.2	1.6±0.22^{abcde}	2.2±0.25^{bcde}	3.2±0.33^{ghi}	2.4±0.22^{abcd}	1.15±0.19^{ab}
0.5	0.4	2.2±0.32^{bcdef}	2.4±0.22^{bcdef}	1.3±0.15^{ab}	1.8±0.25^{abc}	1.54±0.14^{ab}
0.5	0.6	1.7±0.26^{abcdef}	3.2±0.25^{efg}	3.6±0.30^{ghij}	2.6±0.27^{bcd}	1.86±0.12^{abcd}
0.5	0.8	1±0.16^{abcd}	2.4±0.22^{bcdef}	2.6±0.37^{bcdefg}	2.8±0.39^{cd}	1.6±0.14^{abcd}
0.5	1	1.2±0.13^{ab}	2.2±0.25^{bcde}	1.7±0.26^{abcd}	1.4±0.16^{ab}	1.19±0.12^{a}

Values are Mean ± SE of 10 replicates from the 3 repeated experiments. Means followed by same superscript in the column are not significantly different at $p<0.05$ as per Tukey's HSD.

Different nutrients are required for seed germination of different orchid species [2, 15, and 21]. Nitrogen is an important element which helps in plants growth and development [7]. The sources of nitrogen affect the seed germination process of various orchid species [1, 26, and 30]. The nitrogen source is different in all the media used in this experiment. MS medium contains NH_4 NO_3 and KNO_3 as source of nitrogen while KC medium contains $Ca(NO_3)_2 4H_2O$, KNO_3 and $(NH_4)_2$ SO_4.. B5 and VW media consist of KNO_3 & $(NH_4)_2$ SO_4 as source of nitrogen. Ammonium nitrate in MS medium may be a convenient source of nitrogen for seed germination and seedling development of *Dendrobium jenkinsii*. Presence of different vitamins like pyridoxine, thiamine and nicotinic acid in MS media may also promote the germination rate of *D. jenkinsii.* Our result is similar to those obtained in *Cymbidium mastersii* by Mohanty et al (2012) and Cattelya sp. by Mead and Bullard (1979). The requirement of nutrient for germination of orchid varies species to species due to their physiological difference [33]. In our study additional plant growth regulators are not required for seed germination which indicates that already sufficient endogenous hormones are present inside the seeds [20, 24]. Development of plantlets from orchid seeds may be formed directly or through formation of secondary protocorms [28].It is observed that when protocorms of *Dendrobium jenkinsii* were sub cultured in MS basal medium it did not give rise to any secondary protocorms which may be due to absent of sufficient endogenous hormone. Addition of exogenous hormones auxin and cytokinins also did not result any secondary protocorm formation; rather resulted in formation of shoot buds and roots. Our result is not similar with

Cymbidium mastersii [21], *Cymbidium nativity* [10] and *C. lapine dancer* [16]. Protocorms supplemented with BAP 2 µM and NAA 0.5 µM yield highest number of shoots suggesting synergistic effect of both the hormones which was also reported in *Dendrobium candidum* by Yih-Juh Shiau et al 2005. Induction of shoot and root was observed in all the concentrations of BAP + NAA. Cytokinin and auxin plays an important role in shoot formation and elongation in many plant species [11, 27]. Nasiruddin et al (2003) has been used BAP and NAA successfully for shoot induction in *Dendrobium formusum*. High concentrations of BAP results decreased no. of shoots and roots and our finding is similar with *Cymbidium devonianum* Paxt. [3]. The synergistic effect of cytokinin and auxin vary on shoot and root formation of *Dendrobium jenkinsii*. Hardening is an important process for survival of the in vitro grown plantlets [6]. In this study we successfully acclimatized 70% of the plantlets.

Conclusion

Our present research study is the first to report in vitro asymbiotic seed germination and plant regeneration of *Dendrobium jenkinsii.* An efficient and simple protocol for mass propagation of *Dendrobium jenkinsii* has been established through asymbiotic seed germination. This protocol will facilitate for mass propagation and conservation of the orchid. This will also help commercial production of the *Dendrobium jenkinsii* and thus will help to stop illegal collection of the orchid from natural population.

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