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In vitro seed germination of *Dendrobium macrostachyum.*

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ABSTRACT

Dendrobium macrostachyum belongs to the family Orchidaceae is one of the widespread species in South India. It is an epiphytic herb, stem tufted, leaves membranous and deciduous during flowering season with small green flowers and narrow petals. The seeds of orchids have neither functional storage organs, nor a true seed coat so lack of metabolic machinery do not let them to germinate in adequate percentage in nature, only 0.2-0.3% gets germinate, these may be overcome by adopting *in vitro* tissue culture technique. In the present study full and half strength Prasad and Mitra (PM) media was evaluated for seed germination of *Dendrobium macrostachyum*. Besides, effect of organic additives *viz.* coconut water, peptone, casein hydrolysate and plant growth regulators such as BAP+NAA, Kn+NAA, TDZ+NAA+2,4-D and Kn+NAA+2,4-D was studied. Both full and half strength PM media supplemented with organic additive and the hormonal combination of 3 mg/L BAP and 1 mg/L NAA showed excellent growth (100%) followed by 4 mg/L BAP and 1 mg/L NAA (75%). Germination of seeds and protocorm development was recorded after 10 days and 25 days respectively. The first leaf primordial was recorded on 40th day from the day of inoculation. The present investigation revealed that PM medium with 3 mg/L BAP and 1 mg/L NAA showed excellent growth at both full and half strength and were most effective for high frequency germination of seeds in *Dendrobium macrostachyum.*

Keywords: *Dendrobium macrostachyum,* Prasad and Mitra (PM) media, *in vitro* seed germination, plant growth regulators

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INTRODUCTION

The Orchidaceae is one of the largest plant family having 35,000 species [1,2]. Orchids are represented by 125 genera and 1500 species in India. In South India 70 genera and 250 species are recorded, among these 51 genera and 173 species are present in Karnataka in which 67 species are endemic [3,4]. Orchids grow in nature through seeds but in absence of appropriate hosts the seeds do not germinate in adequate percentage [5]. A single orchid capsule/pod contains millions of seeds. The seeds of orchids have neither functional storage organs, nor a true seed coat so lack of metabolic machinery do not let them germinate, only 0.2- 0.3% gets germinated in nature, these problems may be overcome by adopting *in vitro* tissue culture technique [6,7]. The frequency of callus like bodies and protocorm like body (PLBs) production in orchids are influenced by many factors, such as genotypes, type of explants and composition of media [8]. Nutrient composition is considered to be major sources of variation in plant tissue culture [9].

Dendrobium which contains more than 1,340 species and possibly thousands of hybrids which are distributed throughout the world is the second largest genera of Orchidaceae [10]. Among them *Dendrobium macrostachyum* is one of the widespread species in South India (Fig 1). It is an epiphytic herb, stem tufted, leaves membranous and deciduous during flowering season with small green flowers and narrow petals. It is commonly known as 'Marathilotti' found abundantly in plains [3]. This plant is used as a painkiller by tying plant materials overnight on the parts of body to relieve from pain. The tender shoot tips are used as an ear drop for earache and also to treat boils, pimples and other skin eruptions [11].

Different culture media have been used for efficient seed germination in orchid tissue culture [12]. Among them, Prasad and Mitra medium (PM media) was found to be efficient for PLBs formation and leaflet generation [12, 13]. For *in vitro* growth of PLBs and seedlings, some complex organic additives were reported satisfactory while some were unsatisfactory and even inhibitory [14]. A large number of complex additives like coconut water, peptone and casein hydrolysate are very effective in providing good environment for germination of seeds. Earlier report on *in vitro* germination utilizing *Dendrobium macrostachyum* is very scanty with only the report of Dutta *et al.* (2011) which has shown that on MS medium supplemented with IAA (50µg/100ml) and Kn (50µg/100ml), protocorms was developed after 3 weeks of inoculation and further development into plantlets from the protocorms was observed after 8 weeks [15]. Therefore, the present study was undertaken to observe the effects of both full and half strength PM media and organic additives on seed germination, formation of PLBs and leaflets generation of *Dendrobium macrostachyum* with different combination plant growth regulators such as BAP+NAA, Kn+NAA, TDZ+NAA+2,4-D and Kn+NAA+2,4-D.

MATERIALS AND METHODS

The mature pods of *Dendrobium macrostachyum* were collected during November and December, 2014 near the forest area from Hosanagara Taluk, Shivamogga District which was authenticated by Dr. Prashantha K M, Lecturer, Department of Botany, Sahyadri Science College, Shivamogga to investigate the *in vitro* seed germination potentialities using organic additives and different hormone concentration in PM media.

Culture media, inoculation and incubation conditions for seed germination

Both full and half strength PM media was used for *in vitro* seed germination of *Dendrobium macrostachyum*. Double distilled water was used to prepare the media. Media is supplemented with organic additives; casein hydrolysate, peptone and coconut water. The pH of the medium was adjusted at 5.8±0.2 by using 0.1N NaOH or HCl prior to gelling with agar. Media was heated till the agar is dissolved. Hormones are freshly prepared. Media is supplemented with different type of hormones *viz*. TDZ (Thidiazuron a Cytokinin), Kinetin (Kn), 6-benzylaminopurine (BAP), 2,4-dichlorophenoxyacetic acid (2,4- D) and Napthelene acetic acid (NAA) and these hormones are used in different combination. 100mL of the media were dispensed into 250 ml culture bottles and autoclaved at 121^{o}C for 20 min at 15 lbs pressure.

Incubation

Culture bottles with inoculated seeds were maintained in a culture room at 12/12h continuous light (60 µmol m^2/s) and dark conditions at 24±2°C. The cultures were monitored regularly and the data was recorded at one week intervals. The seeds were allowed to germinate and differentiate into protocorms and seedling development.

Inoculation

Collected mature pods were initially washed under running tap water to remove the external particles attached to it. Then they were washed with detergent containing 2-4 drops of Tween-20 for 15 min, and then rinsed with tap water until the detergent is washed away clearly. In inoculation chamber the pods were surface sterilized using 0.1% mercuric chloride solution for 10-15min followed by 70% ethanol for 30sec and sterile distilled water for 3-4 times. Surface sterilized pods were dried on Whatman filter paper and dissected longitudinally with the help of sterilized surgical blade and forceps to expose the powdery seeds. The powdery seeds were scooped, inoculated and spread on the surface of agar gelled PM media under aseptic condition.

RESULTS

In present study, full and half strength PM media was used for the *in vitro* seed germination of *Dendrobium macrostachyum.* PM medium fortified with coconut water (15%), casein hydrolysate (200mg/L), peptone (100mg/L) with different combination of hormones was found to be most efficient media for seed germination of *Dendrobium macrostachyum.* Germination of seeds and protocorm development was observed after 10 and 25 days of inoculation respectively. Both full and half strength media was effective for the germination (Table 1).

Hormone Combinations				
PM media	**Kn+NAA+2,4-D**	**TDZ+NAA+2,4-D**	**Kn+NAA**	**BAP+NAA**
Full strength	No growth	No growth	Average growth	Good growth
Half strength	No growth	No growth	Average growth	Good growth

Table 1: Effects of different Plant Growth Regulators on full and half strength PM media.

Besides the effect of organic additives *viz.* coconut water, peptone, casein hydrolysate the other important addition to the media was plant growth regulators such as BAP+NAA, Kn+NAA, TDZ+NAA+2,4-D and Kn+NAA+2,4-D with different concentration was used in the study. Both full and half strength PM media supplemented with organic additive and the hormonal combination of 3mg/L BAP and 1mg/L NAA showed excellent germination of seeds in *Dendrobium macrostachyum* (100%) followed by 4mg/L BAP and 1mg/L NAA (75%) (Table 2).

In the Kn and NAA supplemented PM media, the combination of 5mg/L Kn and 2mg/L NAA and 6mg/L Kn and 0.5mg/L NAA showed average growth of germination (above 75%) (Table 3). The PM media supplemented with TDZ+NAA+2, 4-D and Kn+NAA+2,4-D however not showed any germination results.

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BAP mg L^{-1}	NAA mg L^{-1}	*Dendrobium macrostachyum*
1	0.25	---
1	0.5	---
1	1	---
1	2	---
2	0.25	---
2	0.5	---
2	1.0	---
2	2.0	---
3	0.25	---
3	0.5	++
3	**1.0**	**++++**
3	2.0	++
4	0.25	+
4	0.5	++
4	**1.0**	**+++**
4	2.0	+
5	0.25	---
5	0.5	---
5	1.0	---
5	2.0	---

Data were recorded on the basis of observations of 20 cultures per combination.
--- = Shows no growth, + = showing (upto 25%) growth, ++ = good growth (with in 50%),
+++ = very good growth (Above 75%), ++++ = (100%) Excellent growth

Table 2: Effect of BAP and NAA on orchid seed germination.

Kn mg L^{-1}	NAA mg L^{-1}	*Dendrobium macrostachyum*
4	0.5	---
4	1.0	---
4	2.0	---
5	0.5	---
5	1.0	+
5	**2.0**	**++**
6	**0.5**	**+++**
6	1.0	++
6	2.0	---
7	0.5	---
7	1.0	---
7	2.0	---

Data were recorded on the basis of observations of 20 cultures per combination.
--- = Shows no growth, + = showing (upto 25%) growth, ++ = good growth (with in 50%),
+++ = very good growth (Above 75%), ++++ = (100%) Excellent growth

Table 3. Effect of Kn and NAA on orchid seed germination.

Stages of seed germination

Within 7-10 days of culture, the undifferentiated tissues of embryos of the seeds swelled up by imbibing water and nutrients, increasing cell number through repeated cell divisions. Upon germination, the embryo swelled and turned to a green callus like structures which in turn swelled and turned into more or less round shaped Protocorm like bodies (after 20-30 days). Further growth of protocorm development formed the

vegetative apex of the stem with a leaf primordial (after 30 days) and continues to enlarge and start to produce leaflets (Fig 2). First leaf primordial was recorded on 40th day (Table 4).

The results clearly demonstrated that both full and half strength PM medium among organic additives; coconut water (15%), peptone (100mg/L) and casein hydrolysate (200mg/L) and hormonal combination of 3 mg/L BAP and 1mg/L NAA showed excellent growth (100%) at both full strength and half strength followed by 4 mg/L BAP and 1mg/LNAA (75%) were most effective for high frequency germination of seeds in *Dendrobium macrostachyum*.

Name	Days for germination from the day of inoculation	Days for protocorm development from the day of inoculation	Days for first leaf production from the day of inoculation
Dendrobium macrostachyum	10	25	40

Table 4. Time taken by orchid seeds on PM medium

(a)
(b) (b)

Figure 1: *Dendrobium macrostachyum* - (a) Habit (b) Flower

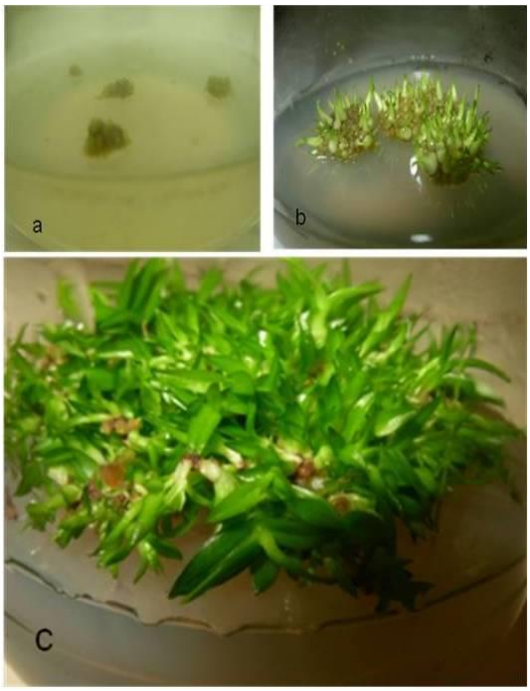

Figure 2: Germination and growth *of Dendrobium macrostachyum* seeds on PM media (a) 10th day after inoculation, (b) 25th day, (c) after germination

DISCUSSION

Influence of culture media and organic additives

PM media is enriched with vitamins and organic additives. Addition of vitamins and additives into the medium was reported to be promotive for seed germination and seedling growth of many orchids [16], Mariat (1949) reported that vitamin B favors germination and differentiation in orchids [17].

In general addition of organic additives coconut water, peptone, and casein hydrolysate is ideal for improved germination, early protocorm formation and seedling development. Coconut water (CW) encourages seed germination and protocorm and callus regeneration which has been reported in several orchid species [18-20]. Coconut water contains many undefined organic supplements and hormones [21]. Similar work was carried out for *in vitro* seed germination by using coconut water in many of the orchid species [22].

Essential amount of organic nitrogen, amino acids and proteins of casein hydrolysate helps in growth of seeds of many orchids *in vitro e.g. Malaxis*, *Phalaenopsis, Doritaenopsis* and *Vanda* [23-25]. Peptone in media enhances the germination rate and also favours the healthy protocorm development. It has also been proved that peptone is useful in inducing differentiation in the protocorms on PDA medium. It has growth promotory nature which has been correlated with its peptides and other unknown contents whereas amino acids, amides and vitamin contents of this additive are considered responsible for its benign nature [26,27]. MasliniJapar Ali *et al.* (2011) in his study on orchids *in vitro* seed germination evaluated that addition of 15% CW or 2 g/L peptone into half MS enhanced the germination of *D. tetrachromum* seeds [28].

Effects of Plant Growth Regulators

The seeds germinated on most of the hormone concentration used in study, but percentage, time of seeds germination and production of protocorm like bodies was found to vary. Some time it depends on species of orchids, because some orchids are media specific [29]. Both auxin and cytokinins in most of the orchids show absolute requirement for induction of callus and their maintenance [30-33]. BAP is used in media because it is known to enhance germination frequency in many orchid species [34]. MS media supplemented with BA alone or, with or without the presence of NAA showed faster growth of seedlings [21]. Swar and Pant (2004), who found that MS medium supplemented with BAP (1mg/L) and NAA (1mg/L) was most effective for seed germination of *Cymbidium iridioides* [35]. It is also found that media during subculture supplemented with BAP and NAA is essential for embryogenic potential of callus and also for induction of shoots in orchids [36].

Morphogenesis of seed and protocorm development

Orchid seeds are unique and its germination is different from other seeds. The pod contains large number of seeds which are very minute; dust like; undifferentiated embryo and lacks endosperm. Self-pollination in certain orchids is not possible and even if possible as in the case of *Vanda*, requires 4-6 month for pod development [37]. Since orchid seeds are unique, exogenous water and nutrients is obligatory for germination [38].

A PLB is an organ that has a similar morphology, structure and function, as an enlarged seed-derived zygotic embryo, the protocorm that develops in *in vitro* culture of orchid [39, 40]. Formation of protocorm is considered to be a peculiarity of post seminal development in orchids and shape of the protocorm is taxon specific round, oval, elongated, disk-shaped, branched, thorn-shaped, spherical or spindle-shaped [41]. During protocorms formation, the basal part functioning as "storage organ", consists of larger paranchymatous cells and are covered with hairs homologous to the hypocotyls of the embryo of flowering plants [42]. Early in germination, chlorophyll appears in the protocorms cells of epiphytic orchids [43]. The apical part of the protocorms, consisting of small number of cells formed a ‘tubercle’ which turns into shoot apex. As the shoot organs were formed, it became asymmetrical. In the early development of protocorm, a leaf primordium appeared at upper part that looked like a closed ridge. As the primordium keeps growing, an opening formed

by the edges of the ridge gradually moves to a lateral position. Interpretations of the first foliar organ of orchids differ. It is referred to as either a cotyledon or as a leaf proper [40, 41].

The results revealed both full and half strength PM media supplemented with organic additives and different concentration and combination of plant growth regulators is efficient media for germination of *Dendrobium macrostachyum*. Standardization of protocol for *in vitro* seed germination of *Dendrobium macrostachyum* was evaluated.

CONCLUSION

The protocol offers an ideal method for *ex-situ* conservation of this orchid and as a means for the rapid propagation and cultivation which is a prerequisite for commercialization.

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