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Coconut water to the rescue of *Dendrobium ovatum* (L.) Kraenzl., a RET species of orchid through enhanced proliferation of shoots from PLBs under *in vitro* conditions

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Abstract
Dendrobium is a slow-growing epiphytic orchid and the third largest genus in the Orchidaceae family. Though an orchid plant produces thousands of micro seeds owing to low level of mycorrhizal interactions, most of the seeds fail to grow out into plantlets. The present study was aimed at standardizing a protocol for culturing micro seeds of *Dendrobium ovatum* to obtain plantlets asymbiotically. Emergence of Protocorm-like bodies (PLBs) and their conversion into young plantlets was found the best in half-strength MS semi-solid media. PLBs formation was found earliest (20 days after culture) on MS half-strength semi-solid medium as compared to Lindeman media. Addition of 15 per cent coconut water (CW) to the media further accelerated the production of plantlets within 15 days of culture initiation. Among the different BAP treatments, half MS + 2 mg L^{-1} BAP + NAA (0.5 mg L^{-1}) + 15% CW produced 9.20 shoots per explant, shoot length of 4.20 cm and the number of leaves was 6.40 at 90 days after culture. It can be inferred that addition of 15 per cent coconut water in the media is well suited for the mass multiplication of *Dendrobium ovatum* orchid and it further accelerate the morphological traits such as shoot length and number of leaves due to stimulative effect of growth regulator content leading to high-quality plantlets production.

Keywords: *In vitro*, *Dendrobium*, coconut water, protector like bodies and Asymbiotic

Introduction
Among the plant kingdom in the world, Orchidaceae is surely one of the most diverse and largest family comprising of 25,000 to 35,000 species and 700 to 800 genera. Dendrobium is the third largest genus in the Orchidaceae family and is known to contain 1,189 species globally (Leitch, 2009) [16]. *Dendrobium* sp. command tremendous appreciation from people all over the world. These are not only valued for their diverse and distinctly beautiful flowers but also their ethnomedicinal properties. In traditional healing systems, dendrobium has been used since ancient times in many parts of the world as in the treatment of several diseases.
Habitat depletion in the Western Ghats and its buffer zones has been a source of concern due to environmental and man-made disasters such as indiscriminate selection, deforestation of host trees, and illegal specimen trade. There is also a chance that biotic stress is putting a lot of pressure on wild endemic orchids, putting them at risk of extinction (Kishor *et al*., 2006) [15]. As a result, preserving and conserving the germplasm of these medicinally important wild endemic orchid resources is critical. Thousands of powdery fusiform micro seeds are produced by *D. ovatum* capsules, but only a small percentage (1%) germinate in nature. Orchid seeds have low germination rate due to their small, powdery non-endosperm seeds and their requirement for mycorrhizal association (Rao, 1977 and Richardson *et al*., 1992) [25, 26]. The mycorrhizal alliance assists seeds in obtaining nutrients for development, especially simple sugars, which must be metabolized for seed germination (Arditti, 1967) [2].
Dendrobium orchids reproduce by seeds, but in the absence of suitable hosts, the seeds do not germinate in sufficient numbers. Tissue culture is a method that can be used to solve these barriers. The nutrient composition is regarded to be one of the most important sources of variation in plant tissue culture (Khanna and Raina, 1998) [14]. A large number of complex additives like coconut water, banana pulp, peptone, tomato juice, slap honey and beef extract

can be very effective in providing undefined mixture of organic nutrients and growth factors. For *in vitro* growth of PLBs and seedlings, some complex organic additives were reported satisfactory while some were unsatisfactory and even inhibitory (Arditti, 1967) [2]. For this, suitable media and organic additives are needed to be identified for large-scale utilization in orchid tissue culture. Therefore, addition of coconut water in the culture media has shown to be effective for enhancing the development of cultured cells and tissues has it possesses a wide spectrum of growth factors, and has been successfully used in orchid production (Pyati *et al*., 2002) [22]. Although, micropropagation is a common practice in plant tissue culture and many efforts have been paid in various plant species (Alam *et al*., 2010 [1]; Chugh *et al*., 2009 [5] and Pyati *et al*., 2002 [22]).

Hence, this study was undertaken to establish an *in vitro* culture condition under the effect of different BAP concentration and natural product (coconut water) on the *in vitro* growth and growth attributes of *Dendrobium ovatum* suitable for large-scale production.

Materials and Methods

Planting material

Dendrobium ovatum mother plants with the capsules were collected from the Ponnampet taluk, Kodagu district. The mother plants were cultivated in the small mud pots containing a mixture of coconut husk, charcoal and brick pieces. The plants were maintained in an orchidarium (a shade net house) at the Plant Tissue Culture Laboratory, Department of Horticulture and used for obtaining explants (capsules) for the experiment. The shade net house conditions were maintained at 26-28 ºC and 60-90% relative humidity (RH) by misting the plants with water twice a day.

Surface sterilization of capsules

The capsules were harvested from the mother plants and were washed with tap water before transferring them to a sterile glass bottle containing 100-200 ml of sterile distilled water with 1-2 drops of Tween-20. They were thoroughly washed with intermittent shaking for 15 minutes and rinsed with sterile distilled water for three times (five minutes per rinse) to remove all the traces of Tween-20 from the surface of the capsules. The rinsed capsules were dipped in 100 per cent ethanol and flamed for few seconds using a spirit lamp with the aid of sterile forceps. The sterilized capsules were cut longitudinally (Fig. 4a) using a sterile scalpel and the seeds were transferred to the bottles containing media.

Media preparation and inoculation

Semi-solid and liquid Murashige and Skoog (MS) and Lindemann media of different strengths (full and a half) were employed for the regeneration study. The organic additive, CW at 15 per cent was added to study the effect on the growth and development of protocorms and plantlets formation. The basal MS and Lindemann media were amended with 3% (w/v) sucrose. The pH of the media was adjusted to 5.7 by the addition of 1N HCl/NaOH as required and the media were heated and brought to boil for melting of agar (6 g/L). Completely boiled media measuring 30 ml was then dispensed into each of the sterilized culture bottles. They were labelled and then autoclaved at 15 psi pressure at 121 °C temperature for 22 minutes. The autoclaved culture bottles containing media were cooled at room temperature and then stored for 2-4 days to make sure that there was no contamination in the media. After inoculation, the bottles were maintained in the growth room under a photoperiodic regime of 16:8 hours of light: dark cycle at 25±2°C.

Multiple shoot induction and plantlet elongation

To evaluate the regeneration efficiency, the protocorms which developed from the germinated orchid seeds were isolated aseptically and transferred into fresh culture bottles containing half-strength semi-solid MS medium supplemented with different concentration of BAP with constant NAA (0.5 mg/L) and addition of 15 per cent coconut water.

Rooting and acclimatization

Newly formed adventitious shoots of 4-5 cm height (90 days) were placed in a rooting medium for root development. Half-strength semi-solid MS medium supplemented with IBA was used for effective rooting. The well-rooted *in vitro* grown plantlets were successfully hardened in a potting mixture containing coconut husk, charcoal, and brick pieces in 1:1:1 ratio and eventually established under natural conditions using the methods of Rahman *et al*. (2009) [23].

Results and Discussion

Effect of different media on growth and proliferation of PLBs

Among the two different media used, MS media had a significant influence on early formation of the PLBs from the seeds as compared to Lindeman media (Table 1). The seeds grown on MS half-strength semi-solid and MS half-strength liquid medium took as early as 20.00 and 23.20 days for PLBs formation while MS full-strength semi-solid, and MS full-strength liquid medium took 27.40 and 32.80 days respectively. The formation of PLBs lagged in Lindeman media. The seeds grown on half-strength semi-solid and half-strength liquid Lindeman medium took 55.80 and 62.20 days while Lindeman full-strength semi-solid and full-strength liquid medium took as many as 66.60 and 74.20 days respectively. Out of different combinations of MS media, the formation of PLBs was earlier in half-strength semi-solid medium (Fig. 4b). By passing through several stages of embryo development, the culture medium was useful for conditioning the explants and encouraging their early growth (Fig. 4c). The choice of basal media depends on plant species and the intended use of culture medium.

The above study revealed that Murashige and Skoog (1962) [19] media is the widely recognized media for plant tissue culture. This could be due to a comprehensive composition of salts, which are crucial for the ideal growth, and development of plant cultures under *in vitro* conditions. Generally speaking, MS media apart from the composition of its salts, the concentrations of salts also proved to be the most suitable. Similarly, reduced MS media concentrations significantly increased PLBs formation in *Grammatophyllum* orchid and *Brassocattleya* orchid (Sopalun *et al*., 2010 and Cardoso and Ono, 2011) [28, 4]. Cardoso and Ono (2001) reported that MS media concentrations must be adjusted for each plant species to achieve optimal *in vitro* growth and morphogenesis development and the most beneficial MS salt delivers enough nutrients to boost metabolism and cell growth.

Table 1: Effect of different media on days taken for the formation of Protocorm Like Bodies (PLBs) through seed culture in *Dendrobium ovatum* (L) Kraenzl

Media	Days taken for the formation of PLBs
T_1-MS full-strength semi-solid medium	27.40
T_2- MS full-strength liquid medium	32.80
T_3-MS half-strength semi-solid medium	20.00
T_4-MS half-strength Liquid medium	23.20
T_5-Lindeman full-strength semi-solid medium	66.60
T_6-Lindeman full-strength liquid medium	74.20
T_7-Lindeman half-strength semi-solid medium	53.80
T_8-Lindeman half-strength liquid medium	62.20
S. Em. ±	0.94
CD at 1%	3.63

Effect of coconut water on multiple shoot induction and plantlets elongation

Number of shoots

A maximum of 10.20 shoots were produced in the MS medium supplemented with 15 per cent coconut water at 90 days after culture (Table 2). On par results of 9.20 shoots were recorded in the media supplemented with 2 mg L^{-1} BAP + NAA (0.5 mg L^{-1}) + CW 15 per cent at 90 days after culture. The minimum (5.00) shoots per explant were recorded in media containing 1 mg L^{-1} BAP + NAA (0.5 mg L^{-1}) + CW 15 per cent at 90 days after culture. The study revealed that addition of CW (coconut water) promotes the possibility of replacing plant growth regulators with organic additive.

The earlier research on the similar aspects has revealed the promotive benefits of coconut water included in the media. According to Sudeep *et al*. (1997) coconut water (15%) increased the number of shoots in *Dendrobium nobile* with half MS medium without growth regulators as it possesses a wide spectrum of growth factors and has been successfully used in orchid production (Pyati *et al*., 2002) [22]. Similarly, shoot regeneration from the protocorm of *Vanda teres* was reported by Sinha and Roy (2003) [27] in half MS medium with coconut water. Bhadra and Hossain (2003) [3] observed that the elongated seedlings of *Goedrum* grew on half MS medium. CW at 15% in half-strength MS medium was optimal for the proliferation of *Dendrobium* 'Alya Pink' PLBs and resulted in a fourfold increase in fresh weight in 4 weeks (Nambiar *et al*., 2012) [20].

Table 2: Effect of different concentrations of BAP with constant NAA (0.5 mg L^{-1}) + coconut water at 15 per cent on the number of shoots formed at 30, 60 and 90 days after culture in *Dendrobium ovatum* (L) Kraenzl

Treatments	Number of shoots/explants		
	30 (DAC)	60 (DAC)	90 (DAC)
Control (Basal half MS + 15% CW)	5.00	7.80	10.20
Half MS + 0.5 mg L^{-1} BAP with NAA (0.5 mg L^{-1}) + 15% CW	3.60	4.40	6.40
Half MS + 1mg L^{-1} BAP + NAA (0.5 mg L^{-1}) + 15% CW	2.80	3.80	5.00
Half MS + 1.5 mg L^{-1} BAP + NAA (0.5 mg L^{-1}) + 15% CW	4.40	5.40	8.40
Half MS + 2 mg L^{-1} BAP + NAA (0.5 mg L^{-1}) + 15% CW	4.80	7.00	9.20
S. Em. ±	0.42	0.49	0.53
CD at 1%	1.71	1.97	2.13

DAC: Days After Culture

Shoot length (cm)

A maximum shoot length of 4.52 cm was recorded in the media containing 2 mg L^{-1} BAP + NAA (0.5 mg L^{-1}) + CW 15 per cent at 90 days after culture. A minimum shoot length of 2.66 cm was recorded in the media containing 1.5 mg L^{-1} BAP + NAA (0.5 mg L^{-1}) + CW 15 per cent at 90 days after culture (Table 3 and Fig. 4d).

Table 3: Effect of different concentrations of BAP with constant NAA (0.5 mg L^{-1}) + CW 15 per cent on shoot length (cm) at 30, 60 and 90 days after culture in *Dendrobium ovatum* (L) Kraenzl

Treatments	Shoot length (cm)/explant		
	30 (DAC)	60 (DAC)	90 (DAC)
Control (Basal half MS + CW 15%)	1.74	2.70	3.12
½ MS + 0.5 mg L^{-1} BAP with NAA (0.5 mg L^{-1}) + CW 15%	2.24	3.38	3.80
½ MS + 1mg L^{-1} BAP + NAA (0.5 mg L^{-1}) + CW 15%	2.08	3.14	3.46
½ MS + 1.5 mg L^{-1} BAP + NAA (0.5 mg L^{-1}) + CW 15%	1.44	2.20	2.66
½ MS + 2 mg L^{-1} BAP + NAA (0.5 mg L^{-1}) + CW 15%	2.58	3.60	4.52
S. Em. ±	0.14	0.15	0.26
CD at 1%	0.57	0.61	1.06

DAC: Days After Culture

Number of leaves: The number of leaves was significantly higher (6.40) in the media containing 2 mg L^{-1} BAP + NAA (0.5 mg L^{-1}) + CW 15 per cent at 90 days after culture (Table 4). Similar, on par results of 6 leaves were observed in the media containing 0.5 mg L^{-1} BAP + NAA (0.5 mg L^{-1}) + CW 15 per cent at 90 days after culture. The study revealed that addition of CW has positively influenced morphogenic activity after PLBs formation.

Table 4: Effect of different concentrations of BAP with constant NAA (0.5 mg L^{-1}) + CW 15 per cent on the number of leaves at 30, 60 and 90 days after culture in *Dendrobium ovatum* (L) Kraenzl

Treatments	Number of leaves /explants		
	30 (DAC)	60 (DAC)	90 (DAC)
Control (Basal half MS + CW 15%)	2.20	4.00	5.20
½ MS + 0.5 mg L^{-1} BAP with NAA (0.5 mg L^{-1}) + CW 15%	2.60	4.40	6.00
½ MS + 1mg L^{-1} BAP + NAA (0.5 mg L^{-1}) + CW 15%	2.40	4.20	5.60
½ MS + 1.5 mg L^{-1} BAP + NAA (0.5 mg L^{-1}) + CW 15%	2.00	3.40	4.60
½ MS + 2 mg L^{-1} BAP + NAA (0.5 mg L^{-1}) + CW 15%	3.00	4.60	6.40
S. Em. ±	0.18	0.21	0.32
CD at 1%	0.72	0.84	1.30

DAC: Days After Culture

The above study revealed that maximum number of shoots formation was recorded in half MS medium supplemented with 15 per cent coconut water. Further, maximum shoot length and number of leaves were observed in the media supplemented with 2 mg L^{-1} BAP + NAA (0.5 mg L^{-1}) + CW 15 per cent. The presence of coconut water in the media accelerated the morphogenetic development such as shoot length and number of leaves as it possesses a wide spectrum of growth factors, and has been successfully used in orchid production (Fig.1).

The usage of coconut water in tissue culture has been of common interventions off late. The concentration range of coconut water commonly used in orchid tissue culture is 15 per cent (v/v). Cytokinins are assumed the most important growth regulator present in coconut water. It contains amino acid, nucleic acid, purine, sugar, alcohol, vitamins and growth substances. According to Ichihashi and Islam (1999) [12], the liquid endosperm of coconut induces cell division in otherwise non-dividing cells and promotes morphogenesis in orchids. These findings confirm with the studies of Lopez *et al*. (2002) [17].

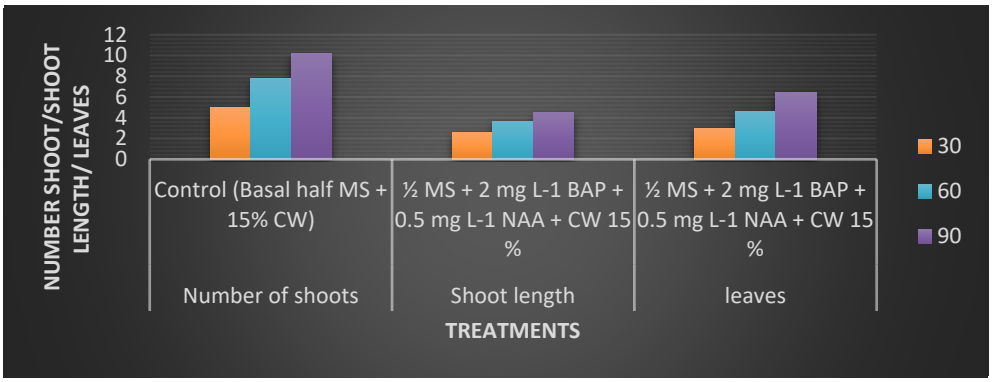

Fig 1: Effect of coconut water on number of shoots, shoot length and number of leaves in control and half MS media with 2 mg L^{-1} BAP + 0.5 mg L^{-1} NAA

Effect of IBA (Indole-3-butyric acid) on root initiation in *Dendrobium ovatum*

For the life of orchid plantlets, the number of roots that are produced during the rooting stage is very important since this will allow the absorption of nutrients from the culture medium. The greater the number of roots, the greater the optimal absorption of nutrients. Growth regulator (Auxin) play an important role in the growth and differentiation of cells and tissues in culture. Indole-3-butyric acid (IBA) has been reported to promote *in vitro* plant rooting in orchids (Hartati, 2019) [10].

Days taken for root formation

It was observed that *Dendrobium ovatum* took as early as 8 days for root formation in the media containing 2 mg L^{-1} IBA (Fig.2). Further, media without hormone took as late as 23.20 days for rooting after root induction.

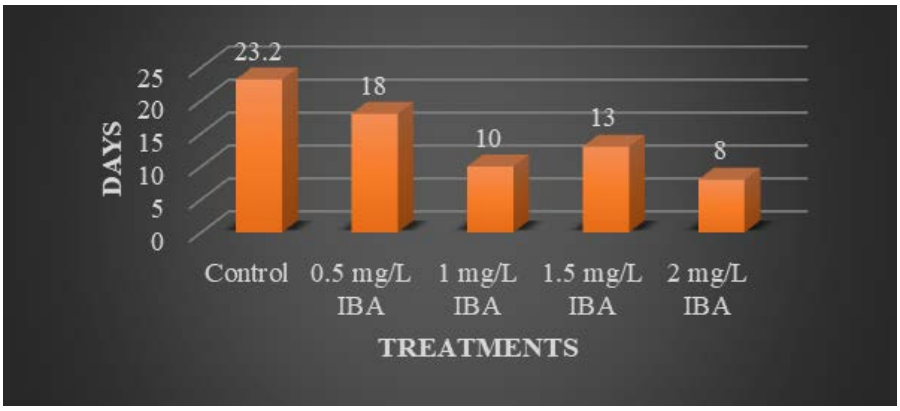

Fig 2: Days taken for root formation at different concentrations of IBA in *Dendrobium ovatum* (L) Kraenzl.

Number of roots per plantlet

The number of roots was found significantly higher (19.20) in media containing 1 mg L^{-1} of IBA at 90 days after root induction. A minimum number of 11.20 roots were observed in control at 90 days after root induction (Fig. 3).

Root length (cm)

Among the different IBA treatments, plantlets in media containing 1 mg L^{-1} IBA produced maximum (6.20 cm) root length at 90 days after root induction (Fig. 4e). A minimum root length of 1.06 cm was recorded in media supplemented with 0.5 mg L^{-1} IBA at 90 days after root induction (Fig. 3).

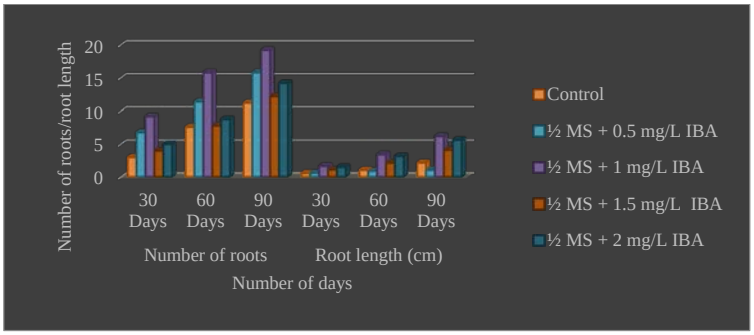

Fig 3: Effect of IBA on number of roots and root length (cm) in *Dendrobium ovatum* at 30, 60 and 90 days after root induction

Acclimatization

After rooting, the plants were transferred for acclimatization in a potting mixture containing coconut husk, charcoal, brick pieces and coco peat in 1:1:1:1 ratio. (Fig. 4f).

The occurrence of roots in shoot culture is the most important morphogenic activity in the complete formation of a plantlet. The role of auxin (IBA) in promoting the occurrence of rooting primordia is a well-established factor and it is very well known that the presence of auxin is the one that triggers events leading to root initiation activity. IBA is by far the most potent auxin as far as induction of roots is concerned. The present study conforms with the earlier reports of the importance of auxins in inducing rooting (George and Sherrington 1984) [8].

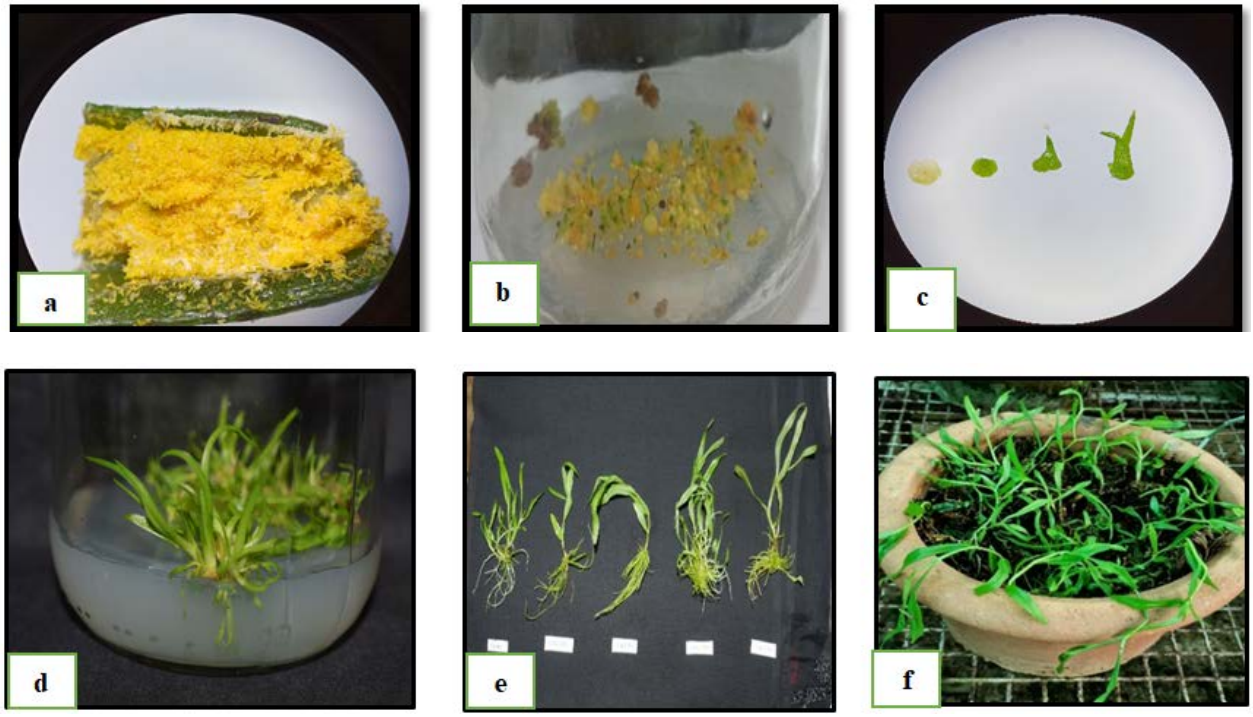

Fig 4 a: Longitudinal cut of *D. ovatum* capsule. **b.** Development of embryos in half MS semi-solid media. **c.** Developmental stages of embryos (PLBs) globular, heart, torpedo and cotyledon. **d.** Multiple shoot induction in the media containing 2 mg L^{-1} BAP + NAA (0.5 mg L^{-1}) + CW 15 per cent. **e.** Rooted plantlets ready for hardening. **f.** Plantlets acclimatized in a potting mixture containing coconut husk, charcoal, brick pieces and coco peat in 1:1:1:1 ratio.

Conclusion

In this study, an efficient *in vitro* micropropagation technique was established supplemented with 15 per cent coconut water to the media. The seeds of *Dendrobium ovatum* germinated well and plantlets were regenerated successfully on MS media in the presence of organic additive (CW 15%). It also promotes the possibility of replacing plant growth regulators with organic additive. In the Western Ghats, orchid seeds take a long time for plants to develop if the environmental conditions are favorable. The development of *in vitro* propagation method would also contribute to the conservation and commercialization of orchids.

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