NOVEL INSIGHTS INTO THE EFFECTS OF *IN VITRO* CONDITIONS ON MICROPROPAGATION AND SUBSEQUENT *EX VITRO* ACCLIMATISATION OF *DENDROBIUM* HYBRID

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ABSTRACT

Micropropagation of Dendrobiums has been achieved using various explants on media supplemented with growth regulators. However, the plantlets have different growth stages and growth rates. Due to sudden changes in environmental conditions, these plantlets may not survive after ex-vitro transfer. To provide enough materials for acclimatisation in the greenhouse and field conditions, it is crucial to devise a method to form plantlets in vitro that are morphologically uniform with well-developed leaves and established root systems. With its significant findings, the present study compared the effects of various supplements and light sources on plantlets during in vitro cultivation and ex-vitro acclimatisation. Young seedlings of Dendrobium cv Sonia, approximately 0.5 to 1.0 cm, were cultured onto ½ MS medium supplemented with BAP, NAA, 10% (v/v) coconut water, 10% mashed banana, and 10% (v/v) beetroot juice. The cultures were kept in the culture room and the greenhouse for 16 weeks. Among the treatments, the medium supplemented with 1.0 mg/L NAA helped increase root length significantly ($P<0.05$) in plantlets. Cultures with controlled temperature and lighting had better PLBs and shoot development in the culture room. In contrast, cultures in the greenhouse exposed to natural day and night conditions had better root development. After two weeks of acclimatisation, plantlets kept in the greenhouse had a higher survival rate and grew well compared to plantlets kept in the culture room.

Keywords: Dendrobium, in vitro culture, greenhouse, acclimatisation

INTRODUCTION

Mass propagation methods have been developed and established to meet the growing demand for orchids, ornamental plants, and many other high-value plants. However, the micropropagation technique is limited in developing countries due to the high cost of the media components and operations. Our research, which has practical implications, showed that using white sugar as a carbon source can reduce 94% of the cost compared to sucrose. Another experiment showed that food-grade agar can be used as a gelling agent in a media to minimise the total cost by 32 to 76%. However, the plantlets have different growth stages and growth rates and do not have well-developed root systems. Due to sudden changes in environmental conditions, these plantlets may not survive after *ex-vitro* transfer.

Our research, with its unique focus on devising a method to form plantlets *in vitro* that are morphologically uniform, with well-developed leaves and established root systems, offers a fresh perspective. The success of the micropropagation method hinges on producing plants through tissue cultures at low costs and with a high survival rate. This urgency and relevance underline the novelty and importance of our research and its potential impact on plant biology.

The present study compared the effects of various supplements and light sources on plantlets during *in vitro* cultivation and *ex-vitro* acclimatisation.

MATERIALS AND METHODS

Plant Materials

Young seedlings of *Dendrobium* cv. Sonia were initiated and maintained. These young seedlings were used to generate the stock cultures as a source of explants. The seedlings were subcultured and maintained on MS (Murashige and Skoog, 1962) media supplemented with 8 g/L agar, 1 g/L activated charcoal, 20 g/L sucrose, 10% (v/v) coconut water and 10% (v/v) mashed banana. The subculturing was repeated every 4-5 weeks to get sufficient stock of explants to initiate the experiments.

Preparation of Extracts

Fresh coconut water was collected one day before media preparation from young coconuts purchased from local stores in the Nilai area to prepare extracts. The water was carefully filtered to remove any impurities from the coconut husk. Fresh bananas were also bought from local stores one day before media preparation. These bananas were sliced into small pieces and mashed before being

added to the media. Similarly, fresh beetroots were purchased from local stores one day before media preparation. The beetroots were processed using a food processor to extract the juice, which was then added to the media.

Preparation of Culture Media and Culture Conditions

We used 50ml of 1/2 MS medium for our experiments throughout the study. The medium was supplemented with 30 g/L sucrose, 8 g/L agar, 2 g/L activated charcoal, and different supplements (0.5 mg/L BAP, 0.1 mg/L NAA, 1.0 mg/L NAA, 10% (v/v) coconut water, 10% (v/v) mashed banana, or 10% (v/v) beetroot juice). The medium was adjusted to pH 5.7 ± 0.1 with 1M NaOH or 1M HCl before autoclaving at 121ºC for 15 minutes under 1 kg cm^{-2} pressure. A total of 50 ml autoclaved medium was poured into each glass jar.

All cultures in the culture room were incubated at 25 ± 2ºC under 12/12 (light/dark cycle), and irradiance was provided by white, fluorescent tubes (Philips, Thailand) of 24 $\mu molm^{-2}s^{-2}$. All cultures in the greenhouse were incubated under shaded sunlight of 35 $\mu molm^{-2}s^{-2}$ with 35 ± 2ºC and 30 ± 2ºC day/night temperature.

Effect of Supplements and Culture Conditions

The effect of supplements on the growth and development of *in vitro* cultures was studied by culturing 0.5 grams of 0.5 cm to 1.0 cm young seedlings on 1/2 MS medium supplemented with 30 g/L sucrose, 8 g/L agar, 2 g/L activated charcoal, and different supplements (0.5 mg/L BAP, 0.1 mg/L NAA, 1.0 mg/L NAA, 10% (v/v) coconut water, 10% (v/v) mashed banana, or 10% (v/v) beetroot juice). The cultures were kept at 2 culture conditions to evaluate the effect of different culture conditions on the growth and development of cultures:

[1] controlled conditions in the culture room with 25 ± 2ºC temperature and 12/12 (light/dark cycle) and irradiance provided by white, fluorescent tubes of 24 $\mu molm^{-2}s^{-2}$.
[2] natural condition in the greenhouse with 35 ± 2ºC and shaded sunlight of 35 $\mu molm^{-2}s^{-2}$.

Acclimatisation of Cultures

Twelve cultures from each treatment were subcultured for 1 month before acclimatisation. A total of 5 rooted cultures from each treatment were washed, treated with fungicides (Mancozeb, 25 g/L) and rooting hormone (Spectra rooting powder, 10 g/L), and then transferred in an orchid potting mixture (wood charcoal and coconut husk) for acclimatisation in the greenhouse for 2 weeks. The plantlets were misted with water twice per day. The fertiliser (100 ml/L) was sprayed once per week. All *ex-vitro* experiments were set up in a completely random design.

Assessment of Growth and Statistical Analysis

Fresh weight, number of shoots per culture, number of shoots with roots, number of shoots without roots, height of shoots, number of roots per culture, number of roots per shoots, and length of roots of cultures were recorded after 16 weeks in culture. All *in vitro* experiments were set up in a completely random design. Each treatment consisted of 5 cultures and was replicated four times. The data was presented as mean ± standard error (SE) values of 8 cultures. All data was subjected to Analysis of Variance (ANOVA), and means were compared using Tukey's test at $p < 0.05$.

RESULTS AND DISCUSSION

Through visual observation, there was a mixture of elongated shoots and PLBs in all the cultures that were kept in the culture room, but there were more elongated shoots than PLBs. The cultures in the greenhouse also contained a mixture of elongated shoots and PLBs, but there were more elongated shoots than PLBs through visual observation (Figure 1 and Figure 2). When the cultures in the culture room were compared with those in the greenhouse, the greenhouse cultures had shoots with more extended and broader leaves. However, there were generally fewer shoots and PLBs produced in all the treatments kept in the greenhouse.

Effect of Supplements on Fresh Weight

The growth of *Dendrobium* cv. Sonia plantlets were established on 1/2 semi-solid MS medium that was supplemented with 30 g/L sucrose, 8 g/L agar, 2 g/L activated charcoal, and different supplements (0.5 mg/L BAP, 0.1 mg/L NAA, 1.0 mg/L NAA, 10% (v/v) coconut water, 10% (v/v) mashed banana, or 10% beetroot juice). The results showed that the plantlets multiplied further from an original clump of 0.5 grams of shoots after 16 weeks of cultivation.

The addition of cytokinin (0.5 mg/L BAP), auxin (0.1 – 1.0 mg/L NAA), and complex organic additives (10% coconut water and 10% mashed banana), however, increased the number of PLBs and shoots produced per culture. The mean fresh weight of cultures ranged from 5.31 g to 9.01 g (Table 1). The PLBs accounted for more than 40% of the fresh weight, the shoots without roots for 15 – 27%, and the rooted shoots for 22 – 35% of the fresh weight, suggesting that the treatments produced more PLBs than shoots. The lowest responding cultures were observed on medium containing 10% beetroot juice, while the highest were on medium containing 0.1 mg/L NAA. However, the differences over the control were not significant.

Table 1: Effect of supplements on mean fresh weight of culture, PLBs, shoots without roots and shoots with roots of *Dendrobium* cv. Sonia, after 16 weeks of culture on 1/2 MS medium

Treatments	Supplements	Mean fresh weight of culture (g ± SE)	Mean fresh weight of PLBs (g ± SE) (%)	Mean fresh weight of shoots without roots (g ± SE) (%)	Mean fresh weight of shoots with roots (g ± SE) (%)
T0	Control	7.01 ± 0.77[a]	3.27 ± 0.61[a] (46.65)	1.32 ± 0.34[a] (18.83)	2.42 ± 0.40[a] (34.52)
T1	0.5 mg/L BAP	8.08 ± 1.40[a]	4.36 ± 1.13[a] (53.96)	1.76 ± 0.35[a] (21.78)	1.97 ± 0.32[a] (24.38)
T2	0.1 mg/L NAA	9.01 ± 0.89[a]	5.29 ± 1.02[a] (58.71)	1.71 ± 0.25[a] (18.98)	2.01 ± 0.24[a] (22.31)
T3	1.0 mg/L NAA	7.60 ± 0.98[a]	4.21 ± 0.96[a] (55.39)	1.17 ± 0.20[a] (15.39)	2.22 ± 0.32[a] (29.21)
T4	10% Coconut Water	7.32 ± 1.06[a]	3.69 ± 0.88[a] (50.40)	1.43 ± 0.26[a] (19.54)	2.21 ± 0.31[a] (30.19)
T5	10% Mashed Banana	7.62 ± 1.67[a]	3.67 ± 1.30[a] (50.13)	1.68 ± 0.42[a] (22.05)	2.28 ± 0.33[a] (29.92)
T6	10% Beetroot Juice	5.31 ± 1.08[a]	2.29 ± 0.74[a] (43.12)	1.45 ± 0.32[a] (27.31)	1.57 ± 0.23[a] (29.57)

**Means ± SE followed by the different letters within a column significantly differ at the P<0.05 by Tukey's multiple range test. Sixteen cultures were used in each treatment.

Effect of Supplements on Number and Height of Shoots

Shoots with and without roots were carefully separated from the cultures in all treatments. The mean number of shoots per culture is presented in Table 2. Shoot growth and development were observed in the control group after 16 weeks in an *in vitro* culture. Adding cytokinin, auxin, and complex organic additives has slightly increased the mean number of shoots produced in cultures. Likewise, the mean number of rooted shoots in these treatments was higher than in the control. However, NAA at 1.0 mg/L and coconut water at 10% appeared to reduce the mean number of shoots with and without roots. The regenerated shoots have a height of approximately 0.3 to 4.3 cm. Adding 10% mashed banana in the culture medium produced taller shoots.

Table 2: Effect of supplements on the mean number of shoots per culture, number of shoots with roots and number without roots of *Dendrobium* cv. Sonia, after 16 weeks of culture on 1/2 MS medium.

Treatments	Supplements	The mean number of shoots with roots	Mean number of shoots without roots	Height of shoots (cm)
T0	Control	20.44 ± 2.79[a]	23.31 ± 4.12[a]	0.30 – 2.50
T1	0.5 mg/L BAP	20.25 ± 2.30[a]	34.38 ± 6.97[a]	0.30 – 2.40
T2	0.1 mg/L NAA	20.00 ± 2.73[a]	32.44 ± 4.19[a]	0.30 – 2.60
T3	1.0 mg/L NAA	18.25 ± 3.07[a]	18.06 ± 2.51[a]	0.30 – 3.50
T4	10% Coconut Water	18.25 ± 2.50[a]	21.81 ± 2.97[a]	0.30 – 2.60
T5	10% Mashed Banana	22.31 ± 4.53[a]	29.25 ± 6.68[a]	0.30 – 4.30
T6	10% Beetroot Juice	19.06 ± 2.97[a]	26.31 ±5.36[a]	0.30 – 2.00

**Means ± SE followed by the different letters within a column significantly differ at the P<0.05 by Tukey's multiple range test. Sixteen cultures were used for each treatment.

Effect of Supplements on Number and Length of Roots

After 16 weeks of culture in 1/2 MS basal medium supplemented with 3% sucrose and 2 g/L activated charcoal, rooted shoots produced approximately 2.73 roots with a mean length of 0.85 cm (Table 3). Adding a low concentration of BAP and NAA and 10% organic additives did not significantly affect the mean number of roots produced in cultures. The mean number of roots per shoot ranged between 2.44 to 2.90. However, adding a higher concentration of NAA (1.0 mg/L) significantly increased the mean length of roots compared to the control. On the other hand, 10% of beetroot juice in the culture medium significantly inhibited the growth of roots. The mean length of roots in beetroot-containing medium was 0.72 cm. On the other hand, NAA significantly increased the length of roots of *Dendrobium* species (Goswami *et al.*, 2015). Moreover, medium supplemented with 1.0 mg/L NAA significantly increased the length of roots and root number of *Alstroemeria* cv. Furgo compared to medium supplemented with 0.1 mg/L NAA (Seyyedyousefi *et al.*, 2013), which agreed with the results of the present experiment.

Table 3: Effect of supplements on mean number of roots per shoot and length of roots of *Dendrobium* cv. Sonia, after 16 weeks of culture on 1/2 MS medium

Treatments	Supplements	Mean number of roots per shoot (number ± SE)	Mean length of roots (cm ± SE)
T0	Control	2.73 ± 0.25^{a}	0.85 ± 0.02^{bc}
T1	0.5 mg/L BAP	2.44 ± 0.25^{a}	0.92 ± 0.02^{ab}
T2	0.1 mg/L NAA	2.53 ± 0.30^{a}	0.93 ± 0.02^{ab}
T3	1.0 mg/L NAA	2.90 ± 0.30^{a}	0.96 ± 0.02^{a}
T4	10% Coconut Water	2.87 ± 0.34^{a}	0.88 ± 0.02^{abc}
T5	10% Mashed Banana	2.79 ± 0.39^{a}	0.83 ± 0.02^{c}
T6	10% Beetroot Juice	2.72 ± 0.22^{a}	0.72 ± 0.02^{d}

**Means ± SE followed by the different letters within a column significantly differ at the P<0.05 by Tukey's multiple range test. Sixteen cultures were used for each treatment.

Pyati (2022) revealed that micropropagation of some important medicinal and ornamental *Dendrobiums* has been achieved using various explants on media supplemented with growth regulators. BAP is the most used cytokinin to induce regeneration and proliferation. In shoot proliferation, there is a synergistic effect between cytokinins, auxins and organic supplements (e.g. coconut water and banana extract). The beneficial effect of coconut water in enhancing the growth of orchids *in vitro* may be correlated to the fact that coconut water contains sugars, amino acids, minerals, vitamins, and phytohormones (Yong *et al*., 2009).

In the present study, medium supplemented with 10% mashed banana increased the fresh weight of PLBs and shoots. This observation is consistent with the findings of other researchers. Fresh weight of PLBS and shoots of *Dendrobium* cv. Sonia was increased in the medium supplemented with banana extract (Obsuwan and Thepsithar, 2014; Islam *et al.*, 2016). Similarly, 10% Sabri banana pulp gave a better performance on fresh weight of PLBs, several shoots and leaves per explant in *Dendrobium* orchid (Akter *et al*., 2007), while 10% Mas (AA) banana pulp increased PLB proliferation in *Phalaenopsis violacea* orchid (Gnasekaran *et al*., 2010). Bananas contain a high content of sucrose concentration (Kaur and Bhutani, 2012), iron, potassium, vitamins B6 and B2 and tryptophan (Gnasekaran *et al.*, 2010), Vitamin C or ascorbic acid, provitamin A (β-carotene, α-carotene, β-cryptoxanthin), and mineral composition (Wall, 2006), which may contribute to the increase in the number of shoots in *Dendrobium* cv. Sonia cultures.

Ascorbic acid is an antioxidant that can prevent oxidation and is involved in cell division and elongation (Smirnoff, 1996). Ascorbic acid was found to increase the number of shoots of banana cv. Cavendish (Ko *et al.*, 2009). Beetroot juice contains high levels of inorganic nitrate, sugars, betalains and oxalic acid (Wruss *et al*., 2015). These complex compounds may have contributed to the shoot formation in *in vitro* cultures. In sweet potatoes, a 1/2 MS medium that was supplemented with 6-benzyl adenine (BA) (2.0 mg L-1) and oxalic acid (100 mg L-1) yielded the greatest shoot proliferation (Yaser Hassan Dewir et al., 2020).

Effect of Different Culture Conditions on Growth and Development of *In Vitro* Cultures

As shown in Table 4, there were no significant differences in mean fresh weight and number of shoots produced in cultures maintained for 16 weeks in the culture room and the greenhouse. However, the conditions in the culture room significantly promoted the growth of PLBs and shoots without roots in the cultures. The mean fresh weight of PLBs and shoots without roots in the culture room was 4.87 g and 2.12 g, respectively. The mean fresh weight of PLBs accounted for 57.09%, while the shoots without roots were 24.85% of the mean fresh weight of the culture. Consequently, the mean number and dry weight of shoots without roots were significantly higher in the cultures kept in the culture room than in the greenhouse. On the other hand, the mean fresh weight of PLBs and shoots without roots in the greenhouse accounted for around 44.06% and 13.95% of the mean fresh weight of the culture. As such, the mean number and dry weight of shoots without roots in the greenhouse cultures were significantly lower than those in the culture room.

The greenhouse's higher temperature and light intensity conditions appeared to have induced rooting in *in vitro* cultures. The average temperature in the greenhouse was 35 ± 2 ºC during the day and 30 ± 2 ºC at night, while light intensity was 35 $\mu molm^{-2}s^{-2}$. In most studies on *Dendrobium* micropropagation, the temperature ranged from 22 ºC to 29 ºC, with an average of 25 ± 2 ºC. The photoperiod was 10-16 hours with 30 $\mu molm^{-2}s^{-2}$ to 60 $\mu molm^{-2}s^{-2}$ illumination intensities (Teixeira da Silva et al., 2015). Interestingly, the mean fresh weight of rooted shoots was 2.65 g, accounting for 42.00% of the mean fresh weight of the cultures, while the mean fresh weight of PLBs was 2.78 g (44.06% of the mean fresh weight of the culture). These results indicated that the greenhouse conditions had balanced the formation between PLBs and rooted shoots in the cultures. Moreover, the mean number of roots per shoot and length of roots were significantly higher in cultures kept in the greenhouse. The mean number of roots per shoot was 3.35, with a mean length of 0.95 cm.

Table 4: Effect of different culture conditions on the growth and development of *Dendrobium* cv. Sonia, after 16 weeks in culture.

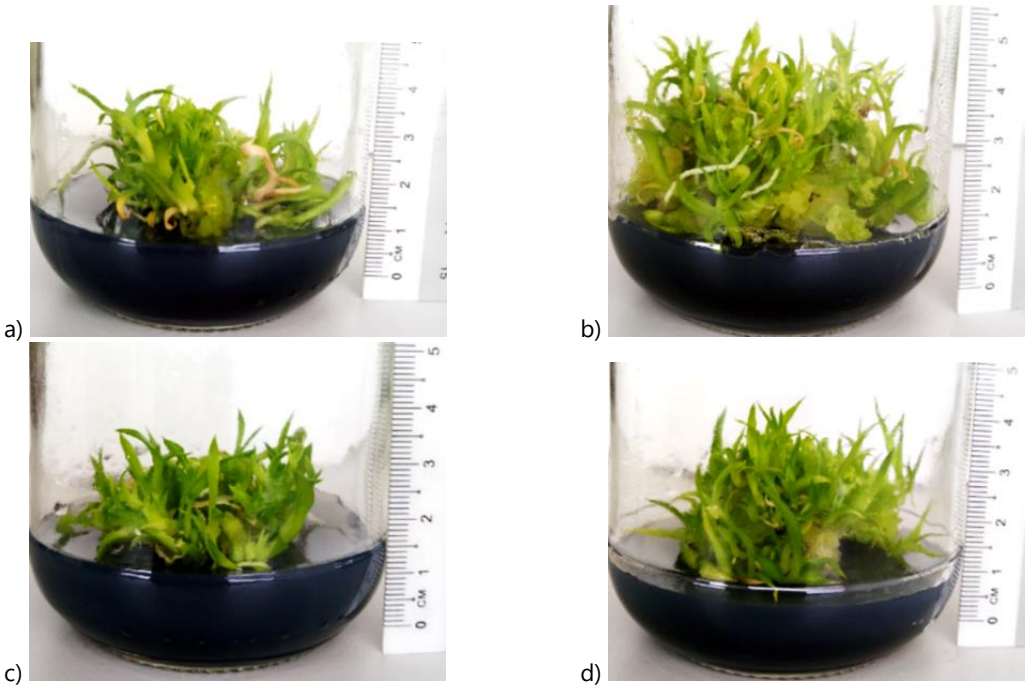
Growth parameters	Culture Room	Greenhouse
Mean Fresh Weight of Culture (g ± SE)	8.53 ± 0.86[a]	6.31 ± 0.88[a]
Mean Fresh Weight of PLBs (g ± SE) (%)	4.87 ± 0.63[a] (57.09%)	2.78 ± 0.63[b] (44.06%)
Mean Fresh Weight of Shoots without Roots (g ± SE) (%)	2.12 ± 0.15[a] (24.85)	0.88 ± 0.10[b] (13.95)
Mean Fresh Weight of Shoots with Roots (g ± SE) (%)	1.41 ± 0.06[b] (16.53%)	2.65 ± 0.27[a] (42.00%)
Mean Number of Shoots per Culture	48.82 ± 5.03[a]	43.79 ± 2.71[a]
Height of Shoots (cm)	0.30 – 4.30	0.30 – 3.50
Mean Number of Shoots with Roots	16.73 ± 1.26[b]	22.86 ± 1.21[a]
Mean Number of Shoots without Roots	32.09 ± 3.97[a]	20.93 ± 2.00[b]
Mean Number of Roots per Shoots	2.07 ± 0.1[b]	3.35 ± 0.07[a]
Mean Length of Roots (cm ± SE)	0.68 ± 0.03[b]	0.95 ± 0.04[a]

**Means ± SE followed by the different letters within a column significantly differ at the P<0.05 by Tukey's multiple range test. Eight cultures were used for each treatment.

Acclimatisation of Plantlets

The full-grown shoots with roots obtained from shoot cultures were kept in the culture room, and the greenhouse was transferred to pots containing wood charcoal and coco peat, which gradually hardened off in the greenhouse. After 2 weeks of acclimatisation in the greenhouse, it was found that the survival rate was lower in plantlets from the culture room compared to plantlets from the greenhouse. Approximately half of the plantlets from the culture room died, while most of the plantlets from the greenhouse survived. New roots started developing, while old roots became longer and turned green in plantlets from both cultures. The shoots and leaves were not growing much in plantlets from the culture room, but the shoots and leaves in plantlets from the greenhouse were growing longer and bigger after 2 weeks. Visual morphological abnormalities were observed in plantlets from the culture room but not in the greenhouse. Overall, plantlets from the greenhouse grew better than those from the culture room (Figure 3).

Figure 1: Dendrobium cv. Sonia plantlets after 16 weeks of cultivation on 1/2 MS medium supplemented with different supplements in the culture room: a) control; b) 0.5 mg/L BAP; c) 0.1mg/L NAA; d) 1.0 mg/L NAA; e) 10% coconut water; f) 10% mashed banana; g) 10% beetroot juice



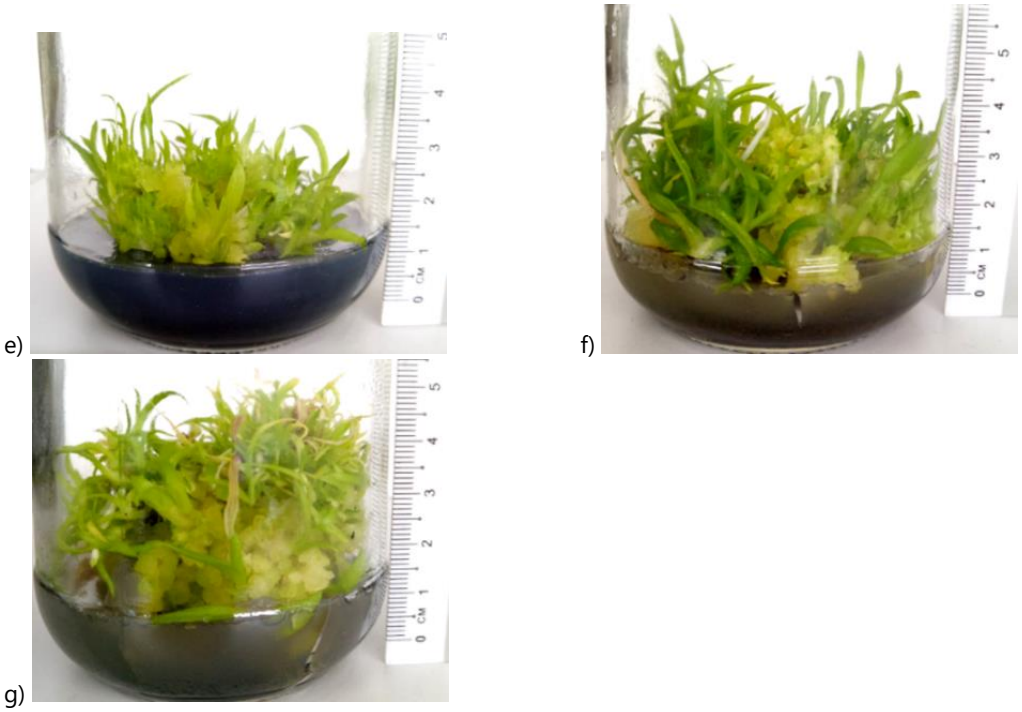


Figure 2: *Dendrobium* cv. Sonia plantlets after 16 weeks of cultivation on 1/2 MS medium supplemented with different supplements in the greenhouse: a) control; b) 0.5 mg/L BAP; c) 0.1mg/L NAA; d) 1.0 mg/L NAA; e) 10% coconut water; f) 10% mashed banana; g) 10% beetroot juice

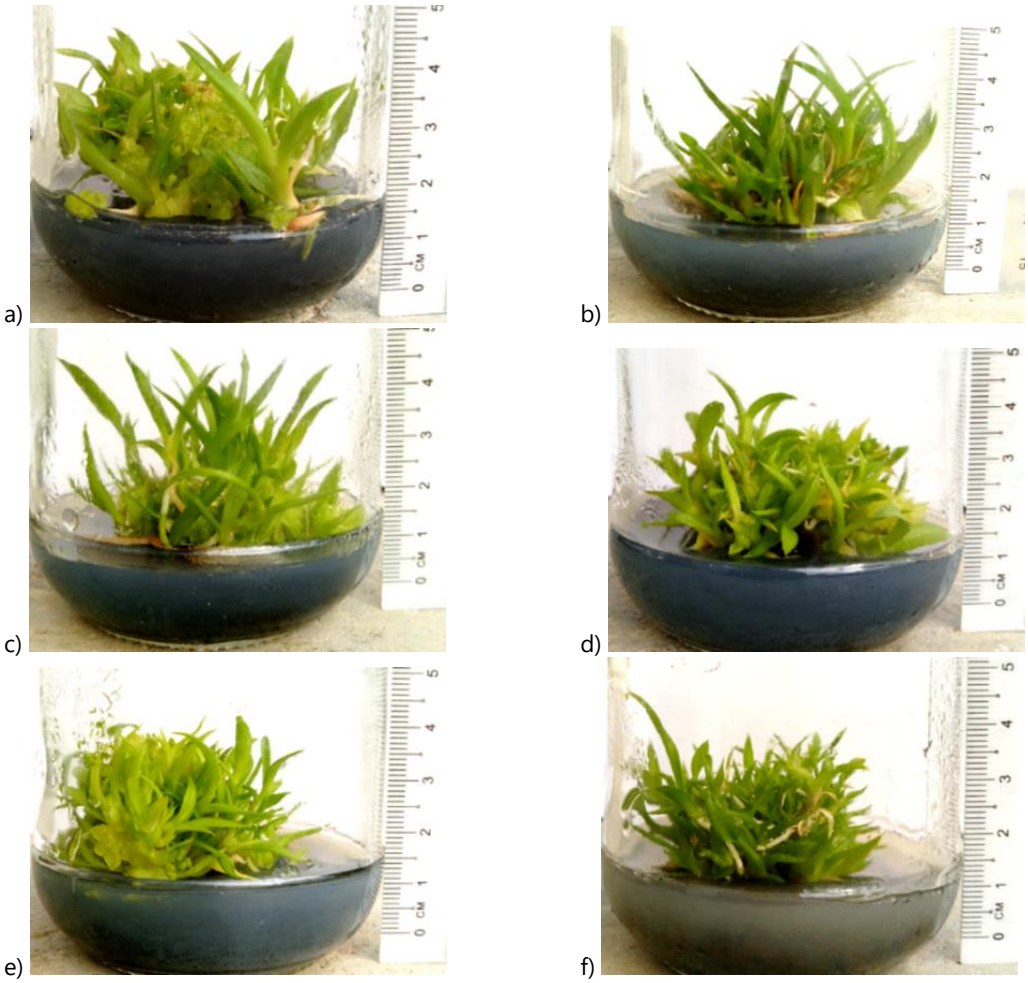


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Figure 3: *Dendrobium* cv. Sonia plantlets after 2 weeks of acclimatisation in the pots containing wood charcoal and coco peat in the greenhouse:
a – c. Plantlets derived from the culture room
d – f. Plantlets derived from the greenhouse

a)  b)  c) 

d)  e)  f) 

CONCLUSION

The medium supplemented with 1.0 mg/L NAA helped increase root length significantly in plantlets. Cultures with controlled temperature and lighting had better PLBs and shoot development in the culture room. In contrast, cultures in the greenhouse exposed to natural day and night conditions had better root development. After two weeks of acclimatisation, plantlets kept in the greenhouse had a higher survival rate and grew well compared to plantlets kept in the culture room.

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