J Agric Rural Dev Volume14(1&2):29-33, December 2022 *ISSN 1810-1860*

SCHOOL OF AGRICULTURE AND RURAL DEVELOPMENT
কৃষি ও পল্লী উন্নয়ন স্কুল

JARD
Journal of Agriculture & Rural Development

Micropropagation of *Vanda* orchid as influenced by different culture media

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ABSTRACT

The mature pods of Vanda sp. were used as ex-plant. Murashige and Skoog (MS) medium with different supplements and additives viz. MS + 0.2 g/l charcoal, MS + 2.0 mg/l BAP + 1.0 mg/l IAA + 0.2 g/l charcoal and MS + without charcoal were tested for large- scale multiplication of native orchid, Vanda sp. Among the treatments, MS + 2.0 mg/l BAP + 1.0 mg/l IAA + 0.2 g/l charcoal was found to be the best for seed germination. In this medium, minimum days were required for seed germination (45–50 days) and plantlet formation (64-68 days). The highest shoot length (4.3 cm), leaf number (4.2), leaf length (3.1 cm), leaf width (0.4 cm), root length (1.8 cm) were also obtained in MS + 2.0 mg/l BAP + 1.0 mg/l IAA + 0.2 g/l charcoal. Root number was highest when media were supplemented with charcoal. MS medium supplemented with high concentration of BAP and low concentration of IAA was found to be efficient for maximum multiplication of shoot and root. The in vitro developed healthy rooted plantlets of Vanda orchid were successfully acclimatized in plastic pots containing coconut fibre. This protocol might be useful for rapid multiplication of Vanda orchid through seed culture.

Key words: orchid, *vanda*, micropropagation, charcoal, BAP, IAA

INTRODUCTION

Orchid seeds are very small, only 1.0-2.0 mm long and 0.5-1.0 mm wide, having no food reserve. A single orchid capsule contains 2-3 million seeds. The seeds consist of a thickened testa, enclosing an embryo of about 100 cells with no endosperm (Pyati, 2019). They do not have the capacity for directly utilizing the substrate available in nature. Thus in spite of very large number of seeds produced, only 2-3% seed germinate in nature with the help of specialized mycorrhizal fungus *Rhizoctonia* spp. (Bhowmik and Rahman, 2017). For appropriate germination of orchid seeds, *in vitro* germination is imperative for multiplication rather than *in vivo* (Kaur and Bhutani, 2014). Knudson (1922) for the first time succeeded to germinate orchid seeds *in vitro* without fungal association. Now, the asymbiotic seed germination technique is utilized for the production of many orchids and it is shown to be an efficient tool for the production of orchids for conservation and reintroduction into their natural habitats (Johnson and Kane, 2007; Sebastinraj and Muhirkuzhali, 2014; Islam *et al*., 2015; Pyati, 2019). In Bangladesh, a good number of orchids with beautiful flowers are available. So, like other countries, i.e. Thailand and the Philippines, appropriate propagation technique for large scale production of native orchids may be a profitable source of earning foreign exchange in Bangladesh. In literature, many nutrient media formulations are reported for successful *in vitro* propagation of different orchid genera and species (BoLong *et al*., 2010; Hossain *et al*., 2010; Pant *et al*., 2011; Shibu *et al*., 2012; Panwar *et al*., 2012; Paudel and Pant, 2013; Salam *et al*., 2013; Hossain, 2014; Bhattacharjee and Islam, 2014; Parmar and Pant, 2016; Regmi *et al*., 2017). The present investigation was made to find out the suitable media for large scale multiplication and faster development of *Vanda* orchid.

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MATERIALS AND METHODS

The mature pods of *Vanda* sp. were collected and surface sterilized by submerging them in 0.1% $HgCl_2$ solution with 2-3 drops of Tween-20 for 15 min. The pods were washed 3-4 times with sterile distilled water and were transferred to a sterile plastic pot in laminar air flow cabinet. The pods were then dipped in absolute ethanol and flamed rapidly for a few seconds holding with forceps. They were then splitted longitudinally with a sterile scalpel and the seeds were taken by forceps and transferred to the agar solidified nutrient medium for seed germination. Three different culture media namely, MS (Murashige and Skoog, 1962) + 0.2 g/l charcoal, MS + 2.0 mg/l BAP + 1.0 mg/l IAA + 0.2 g/l charcoal and MS + without charcoal were used. In per litre of the medium 30 g sucrose and 8.0 g of agar were used and pH was adjusted to 5.8 prior to autoclaving. Seeds were sown in culture flask (50 ml) containing 20 ml of the medium. Culture flasks (100 ml) containing 35 ml of fresh medium were used for sub-culturing. The media were autoclaved at 121^0C for 20 minutes at 1.2 kg/cm^2 pressure. The inoculated culture vessels were incubated at 25^0C ± 1^0C under 16h photoperiod with a light intensity of approximately about 2000 lux under white fluorescent tubes.

The experiment was carried out as completely randomized design (CRD) with three replications. Data were collected and subjected to analysis of variance. Means were separated by least significant difference (LSD) test using R statistical software program version 4.3.1.

RESULTS AND DISCUSSION

Days to seed germination, protocorm formation, shoot initiation and plantlet formation are shown in Table 1 and effect of charcoal, charcoal with growth regulators on plantlet development are shown in Table 2. Seeds germinated on MS medium became greenish within 60 days and produced light green globular structures. The overall shoot initiation up to plantlet establishment procedures are shown in Figure 1 (a - c).

Table 1. Response of various supplements in MS medium on days to seed germination, protocorm formation, shoot initiation and plantlet formation in *Vanda* orchid

Treatments	Days to seed germination	Days to protocorm formation	Days to shoot initiation	Days to plantlet formation
MS + Without charcoal	53-55	70-75	60-62	72-75
MS + 0.2 g/l charcoal	50-52	60-68	50-55	68-70
MS + 2.0 mg/l BAP+1.0 mg/l IAA+0.2g/l charcoal	45-50	60-68	54-58	64-68

MS = Murashige and Skoog, BAP = Benzylamino purine, IAA = Indole-3-acetic acid

Seed germination was recorded in all the treatments within 55 days. It was observed that maximum days (53 – 55) were needed for seed germination in MS medium without charcoal supplementation and minimum days (45 - 50) were required in MS media containing 2.0 mg/l BAP + 1.0 mg/l IAA + 0.2 g/l charcoal. The result is in agreement with the findings of Islam *et al*. 2015 who noted 40 days required for germination of orchid seeds. Seeds developed into protocorms with commencement of the germination process. Protocorm formation was started within 75 days. Results showed that protocorm formation started 60 – 68 days both in charcoal supplemented media (MS + 0.2 g/l charcoal and MS + 2.0 mg/l BAP + 1.0 mg/l IAA + 0.2 g/l charcoal). On the other hand, 70 -75 days required for protocorm formation in MS media without charcoal (Table 1). Similar observations were reported by Kaur (2021) where they reported that in basal medium, orchid protocorm formation was achieved in 70 days. When sub-cultured in the new fresh medium, the germinated protocorm increased 2-3 folds within 3-4 weeks. For the development of plantlets, protocorm were sub-cultured for 3-4 times. Shoot initiation was observed within 50-55 days in MS + 0.2 g/l charcoal and 54-58 days in MS + 2.0 mg/l BAP + 1.0 mg/l IAA + 0.2 g/l charcoal while in 60 – 62 days needed in without charcoal supplemented media. The minimum days (64 – 68) were required for plantlet formation in MS + 2.0 g/l BAP + 1.0 mg/l IAA + 0.2 g/l charcoal whereas 68 - 70 days in MS + 0.2 g/l charcoal. Kaur (2021) reported that *Vanda testacea* protocorm differentiated into plantlets within 95 days in NAA fortified medium. The difference might be due to the different species and hormonal composition they used in the medium. From the study it was noted that plant

growth regulators supplemented media took lesser time for germination, protocorm proliferation and further differentiation of leaf primordia to shoot formation than plant growth regulator free medium. Similar observation was reported by Bhowmik and Rahman (2017).

Table 2. Effect of charcoal and charcoal with growth regulators on plantlet development of *Vanda* orchid after 4th subculture

Treatments	Length of shoot (cm)	Number of leaf /plantlet	Leaf length (cm)	Leaf width (cm)	Average number of root /plantlet	Length of root (cm)
MS + Without charcoal	2.5 b	2.0 c	1.0 b	0.2 a	0 c	0 c
MS + 0.2 g/l charcoal	2.0 b	3.1 b	1.5 ab	0.3 a	2.2 b	1.0b
MS + 2.0 mg/l BAP+1.0 mg/l IAA+0.2g/l charcoal	4.3 a	4.2 a	3.1 a	0.4 a	3.6 a	1.8a
CV (%)	22.35	7.08	22.66	33.33	8.03	33.11
LSD (0.05)	1.29	0.44	0.85	0.199	0.31	0.62

Means bearing same letters do not differ significantly at 5% level of probability

MS = Murashige and Skoog, BAP = Benzylamino purine, IAA = Indole-3-acetic acid

a b c

Figure 1: (*a) Shoot initiation (b) Multiple shoots with roots (c) Established plantlets of Vanda orchids.*

In order to induce rapid growth and development, protocorm were transferred to different culture media with or without charcoal. Data were recorded for shoot length, leaf number, leaf length, leaf width, root number, root length and results have been presented in Table 2. Significant variation in shoot length was observed among the treatments. The highest shoot length (4.3 cm) was observed in MS + 2.0 mg/l BAP + 1.0 mg/l IAA + 0.2 g/l charcoal followed by MS + without charcoal (2.5 cm). On the other hand, the lowest (2.0 cm) was found from MS + 0.2 g/l charcoal. Results indicated that the addition of charcoal with growth regulators increased the shoot length. Number of leaves was significantly influenced with the addition of plant growth regulators. The highest leaf number (4.2) was obtained from the treatment containing MS + 2.0 mg/l BAP + 1.0 mg/l IAA + 0.2 g/l charcoal followed by MS + 0.2 g/l charcoal (3.1). Whereas, the lowest leaf number was obtained in MS + without charcoal (2.0). Leaf length was significantly varied among the treatments. The highest leaf length (3.1 cm) was observed from MS + 2.0 mg/l BAP + 1.0 mg/l IAA + 0.2 g/l charcoal followed by MS + 0.2 g/l charcoal (1.5 cm) while the lowest from MS + without charcoal (1.0 cm). Although there was no significant differences were observed among the

treatments. The highest width of leaf (0.4 cm) was observed from MS + 2.0 mg/l BAP + 1.0 mg/l IAA + 0.2 g/l charcoal followed by MS + 0.2 g/l charcoal (0.3 cm) while the lowest from MS + without charcoal (0.2 cm).

Significantly different results in root number were observed among the treatments. The highest number of roots (3.6) was observed in MS + 2.0 mg/l BAP + 1.0 mg/l IAA + 0.2 g/l charcoal and lowest from MS + 0.2 g/l charcoal (2.2). On the other hand, MS + without charcoal did not produce any root (Table 2). Results indicated that medium supplemented with activated charcoal found best for number of root formation. Activated charcoal is suitable for root induction because it creates dark conditions of the media in accordance to the original environment of the underground root (Setiaji *et al*., 2021). The addition of activated charcoal in medium improves the growth of *Vanda*. The positive effect of activated charcoal are improved aeration, established polarity of micronutrients, stabilizes substrate temperature and adsorbs toxic substances (Phenolic compounds), because of the nature of activated charcoal which has small pores and a large surface area (Thomas, 2008; Zeng *et al*., 2015). Nagaraju *et al*. (2001) also reported such stimulation by activated charcoal in the medium. Similar findings were reported by Paul *et al.* (2019). A significant difference was found on root length of plantlet among the treatments. The highest root length (1.8 cm) was found in MS + 2.0 mg/l BAP + 1.0 mg/l IAA + 0.2 g/l charcoal and the lowest from MS + 0.2 g/l charcoal (1.0cm). The plantlets with a good root system were transferred from the culture room and kept in laboratory at room temperature for 5-7 days for hardening. Then the plantlets were removed from the culture vessels and washed carefully to remove the adhering agar. They were then planted to plastic pots with coconut fibre and the plantlets were established there (Figure 1c). The results of this study allow the establishment of a protocol for mass multiplication of *Vanda* orchid. Full-strength MS medium fortified with 2.0 mg/l BAP + 1.0 mg/l IAA + 0.2 g/l charcoal may be recommended for the effective *in vitro* germination, protocorm development and plantlet formation. This protocol is an efficient means for the large-scale propagation of *Vanda* orchid.

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