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RESEARCH ARTICLES

Effect of activated charcoal and phytohormones to improve in vitro regeneration in *Vanda tessellata* (Roxb.) Hook. ex G. Don

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Abstract

Vanda tessellata (Roxb.) Hook. ex G. Don. (Orchidaceae) is an epiphytic orchid species, commonly known as grey orchid, and explored enormously for its significant horticultural, medicinal, phytochemical and pharmacological properties. The present study describes an in vitro culture approach via asymbiotic seed germination assisted by phytohormones and enhanced proliferation of shoots and roots using activated charcoal for large scale production of *V. tessellata*. The maximum frequency of seed germination (100%) was achieved on Murashige and Skoog's (MS) medium fortified with 1.5 mg L^{-1} indole-3 butyric acid (IBA). The seedlings developed from the protocorms were used for further proliferation and development of multiple shoots (24.8 ± 0.52 shoots per protocorm with 5.2 ± 0.35 cm average length) on MS medium augmented with 0.5 mg L^{-1} each of 6-benzylaminopurine (BAP) and indole-3 acetic acid (IAA) + 100 mg L^{-1} activated charcoal (AC) after 4th subculture. The optimal rooting of individual shoots was achieved on MS medium with 1.0 mg L^{-1} IBA. The well-developed plantlets were hardened using soilrite® + cocopeat + coconut husk complex in a greenhouse for 4 weeks. The plantlets were acclimatized under relatively increased temperature and low humidity for 4–5 weeks in the greenhouse with 98% survival rate. The protocol can be utilized for the development of strategies for large scale stable production of *V. tessellata* plantlets.

Keywords Asymbiotic seed germination · Epiphytic · Grey orchid · Shoot proliferation · *Vanda tessellata*

Introduction

Vanda tessellata (Roxb.) Hook. ex G. Don. (syn. *Vanda roxburghii* R. Br.), of the family Orchidaceae is popularly known as grey orchid. It is a widely cultivated horticultural and epiphytic orchid that forms huge clumps on tree trunks or rocks in thick forests and sacred groves (Basu 2010), and distributed in India, Nepal, Sri Lanka, Myanmar, and the Indo-China regions (Khan et al. 2019). Gray orchid has stout, scandent stem of about 30–60 cm length with branched aerial roots. The leaves are succulent and linear (15–20 cm long). The plants have 6–10 flowered racemes of 15–20 cm long peduncle with yellow sepals, tessellated with brown lines and white margins. Petals are shorter than the sepals (lip 16 mm long), yellow colored with brown lines and white margins (Shamsul Islam et al. 2016).

The intense fragrance of the flowers and roots of *V. tessellata* is due to the presence of industrially significant volatile oils such as linalool, methyl benzoate, cinnamic aldehyde, and methyl cinnamate (Usman et al. 2012). Grey orchid has also been explored for the treatment of various human ailments (Prakash and Bais 2016). The roots are highly medicinal and possess anti-inflammatory and analgesic properties. These are used in the treatment of bronchitis, dyspepsia, hiccup, inflammations, piles, syphilis, and scorpion stings. The root extract is applied in rheumatism and other nervous system problems (Uddin et al. 2015). A new chemical compound, 2,7,7-tri methyl bicyclo [2.2.1] heptane has also been extracted from this plant which possess aphrodisiac properties (Subramoniam et al. 2013).

The demand for orchids in the floriculture/horticulture industries is increasing at an alarming rate, but large-scale production to convene the demand is limited (Khuraijam et al. 2017). Likewise, there is a growing interest to propagate the orchids at a commercial scale for industrial inputs (Hinsley et al. 2018). Unfortunately, nearly all the orchid

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species cannot be grown due to some specific ecological constraints. Moreover, the conventional propagation of orchids through vegetative cuttings, lateral buds, or seeds is very slow (Vij and Pathak 2012). The symbiosis of mycobionts (fungi) with orchid seeds is essential for successful seed germination and establishment of seedlings under natural conditions, due to the non-availability of sufficient stored food in the seeds of terrestrial orchids (Alghamdi 2019). The availability of a specific fungal inoculum to the particular orchid seed also hampered owing to deforestation and changed micro-ecosystem of the natural forests. Therefore, asymbiotic germination of seeds could be a viable option to ensure almost 100% seed germination in orchids under in vitro conditions. Moreover, in vitro propagation offers a substitute solution for the production of a large number of clones with genetic and phenotypic similarity within a short duration (Leyva-Ovalle et al. 2020).

Activated charcoal (AC) stabilizes the nutrients and growth hormones of the culture medium and thereby promotes effective in vitro organogenesis (Sharma et al. 2012; Chutipaijit and Sutjaritvorakul 2018). The basic adsorbing property of AC is due to the presence of fine pores with more surface area, which suck up the toxic secondary products and phenolic exudates, thus improves overall cell growth and metabolism for effective organogenesis. Another favorable effect of AC is that it gradually releases the adsorbed nutrients to support stable plant growth under in vitro conditions (Thomas 2008).

Available literature revealed that in vitro propagation of *V. tessellata* has been reported through the culture of shoot tips (Rahman et al. 2009), shoot segments (Bhattacharjee and Islam 2014), immature seeds (Bhattacharjee et al. 2015), and protocorm like bodies (Bhattacharjee and Islam 2017). However, the rate of shoot multiplication, *ex vitro* management of plantlets, and low transplant survival are the major issues of tissue culture of grey orchid. To the best of our knowledge, the role of activated charcoal in the proliferation of shoots has not been studied for large scale proliferation in grey orchid. Therefore, the present study was undertaken to improve the existing protocols in terms of rate of shoot multiplication in *V. tessellata* utilizing activated charcoal and *ex vitro* acclimatization of plantlets.

Materials and methods

Plant material, culture conditions, and in vitro seed germination

The mature pods of *V. tessellata* were collected from the coastal areas of Puducherry, India. The pods were sterilized by a systematic treatment of 0.5% (v/v) NaOCl (Hi-Media, India) for 5 min, 0.1% (w/v) Bavistin (BASF, India) for the next 10–15 min, thereafter surface sterilization with 0.1% (w/v) $HgCl_2$ (Hi-Media, India) for 4–6 min and followed by washed for 5–8 times with sterilized distilled water under aseptic conditions before inoculation. Murashige and Skoog (1962) medium with 3% sucrose, 0.2% phytagel (Sigma-Aldrich, USA) and six concentrations (0.5–3.0 mg L^{-1}) of auxins [indole-3 acetic acid (IAA), indole-3 butyric acid (IBA) and α-naphthalene acetic acid (NAA)] (Hi-Media, India) was used for asymbiotic in vitro seed germination. The culture medium's pH was rendered to 5.8 ± 0.02 and autoclaved at 121 °C for 20 min. The seeds were inoculated and initially maintained under diffused light with 20–25 µmol m^{-2} s^{-1} spectral photon flux density (SPFD) for a week, and thereafter incubated at culture room with 40–50 µmol m^{-2} s^{-1} SPFD light intensity provided by cool and white fluorescent tubes (Philips, India), at 25 ± 2 °C and 55–60% relative humidity (RH). The frequency of seed germination was monitored for 4–8 weeks and calculated as:

Number of germinated seeds/Total number of seeds × 100.

Shoot proliferation and rooting

The seedlings were sub-cultured once in 4 weeks on MS medium fortified with various concentrations (0.25–1.0 mg L^{-1}) of 6-benzylaminopurine (BAP) and auxins (IAA or NAA) with activated charcoal (50–200 mg L^{-1}) for multiple shoots induction. The robust shoots were separated from proliferated culture clumps and transferred individually to full and half strengths of MS media containing four concentrations (0.5–3.0 mg L^{-1}) of IBA or NAA and incubated under diffused light (20–25 µmol m^{-2} s^{-1} SPFD) for 4 weeks to achieve in vitro root induction.

Hardening and acclimatization of plantlets

Healthy and well-rooted plantlets were taken out from the nutrient medium and washed thoroughly to confiscate the traces of medium from the roots. The plantlets were transplanted to soilrite® (a chemically inert horticultural graded perlite procured from Keltech Energies Ltd., Bangalore, India) in eco-friendly paper cups, moistened with quarter strength MS macro-salts, and covered with perforated transparent cups for 3–4 weeks before transferring to a potting mixture of various combinations (soilrite® + manure + coconut husk, soilrite® + cocopeat + coconut husk, soilrite® + vermicompost + coconut husk) in the ratio of 1:1:1 and maintained in the greenhouse. The plantlets were gradually shifted to a higher temperature and low humidity zone for further acclimatization up to 4–5 weeks.

Experimental design and data analysis

The experiments were carried out with 20 replicates per treatment and repeated thrice. The significance of differences among each treatment was drawn using Duncan's multiple range tests (DMRT) at $P < 0.05$. Statistical analysis of the data was done by ANOVA using statistical software 'SPSS' version 17 (SPSS, Chicago, USA).

Results and discussion

Asymbiotic seed germination

The frequency and morphogenic potential of protocorm and seedling development from the seeds differed with the type and concentration of auxins experimented. The maximum germination frequency (100%) and protocorm development were observed on MS medium fortified with 1.5 mg L^{-1} IBA, followed by 1.0 mg L^{-1} IAA (70.25%) and NAA (62.28%) (Table 1; Fig. 1a). The protocorm development (development of chlorophyllous cells from the seeds) was observed after 2 weeks on IBA containing medium. The conversion of protocorms into seedlings was also recorded higher on MS medium augmented with IBA as compared to IAA and NAA. The organogenesis from the protocorms, such as the formation of shoots (1.7 ± 0.10), the number of leaves (5.4 ± 0.25), and roots (3.0 ± 0.00) were notably higher on 1.5 mg L^{-1} IBA than other concentrations and types of auxins used (Table 2; Fig. 1b). The increased concentrations of auxins induced detrimental effects in morphogenic response, and no sign of growth was observed in control experiments even after 8 weeks of incubation. This could be due to the absence of sufficient endogenous growth regulating substances in the seeds of *V. tessellata*.

Table 1 Effect of auxins on asymbiotic seed germination in *V. tessellata*

Conc. of auxins (mg L^{-1})			Seed germination (%) (Mean ± SE)
IAA	IBA	NAA	
0.0	0.0	0.0	00.00 ± 0.00^{a}
0.5	–	–	51.50 ± 0.26^{d}
1.0	–	–	70.25 ± 0.20^{i}
1.5	–	–	64.10 ± 0.31^{h}
2.0	–	–	58.00 ± 0.17^{fg}
2.5	–	–	53.15 ± 0.29^{de}
3.0	–	–	40.55 ± 0.13^{bc}
–	0.5	–	79.10 ± 0.42^{j}
–	1.0	–	88.00 ± 0.30^{k}
–	1.5	–	100.0 ± 0.69^{l}
–	2.0	–	91.26 ± 0.21^{k}
–	2.5	–	80.10 ± 0.28^{j}
–	3.0	–	73.60 ± 0.32^{i}
–	–	0.5	51.00 ± 0.43^{d}
–	–	1.0	62.28 ± 0.16^{g}
–	–	1.5	57.30 ± 0.33^{ef}
–	–	2.0	50.37 ± 0.20^{d}
–	–	2.5	44.00 ± 0.29^{c}
–	–	3.0	38.60 ± 0.24^{b}

Incubation time—4 weeks, mean values in the column with different letters indicate significant differences according to DMRT at $P < 0.05$

The results revealed that IBA could improve seed germination and development of seedling in grey orchid. Auxins are important plant growth regulators which guide morphogenic responses underlying shoot and root development from the meristems (Vanneste and Friml 2009). The morphogenesis of protocorms and the development of leaves were influenced by the polar auxin transport inhibitors (Novak et al. 2014). The natural auxins (IBA and IAA) readily form conjugates and release free auxins when required by the plant (Woodward and Bartel 2005). Indole-3 butyric acid performs as an independent auxin under in vitro conditions and readily conjugates with the available sugar and amino acids, and release free auxins due to its stability to oxidation (Wiesman et al. 1989). The metabolic profile of IAA reveals its least stable nature, compared with IBA and NAA, but the level of NAA sustains a long period in cultures, which causes toxicity to the germinating seeds (Centeno et al. 1999). The selection of proper culture medium and the growth regulator has a positive effect on seed germination and organogenesis due to the differences in maintaining equilibrium and supply of organic and inorganic nutrients (Teixeira da Silva 2014).

In vitro proliferation of shoots

The seedling derived shoots were repeatedly sub-cultured on fresh MS medium fortified with various combinations of auxins and cytokinins. The mutual effects of cytokinin (BAP) and auxins (IAA, IBA or NAA) was experimented to determine the best combination for in vitro shoot proliferation in *V. tessellata*. Multiple shoots were induced with improved leaf number and length while sub-culturing of seedlings on MS medium containing cytokinins and auxins. Highest number of shoots (24.8 ± 0.52 shoots with 5.2 cm length) were produced after 4th sub-culture on MS medium incorporated with 0.5 mg L^{-1} each of BAP and IAA. Comparatively less number of shoots was regenerated on BAP and NAA (11.4 shoots with 3.6 cm length) (Table 3; Fig. 1c, d). Incorporation of IBA with BAP resulted in the formation of compact callus from the root and hindered the shoot growth (data not shown), hence avoided in shoot amplification experiments. In this study, the reduced concentrations of auxins combined with BAP promoted shoot proliferation. The shoots were continuously sub-cultured at

Fig. 1 Asymbiotic seed germination and regeneration competence of *Vanda tessellata.* **a** Asymbiotic seed germination and development of protocorms on MS + 1.5 mg L^{-1} IBA. **b** In vitro germinated seedlings from protocorms on MS + 1.5 mg L^{-1} IBA. **c** Multiplication of shoots on MS medium containing 0.5 mg L^{-1} each of BAP + IAA, additives, and 100 mg L^{-1} AC after 3rd sub-culture. **d** Shoot proliferation and elongation on the optimized medium and phytohormones after 4th sub-culture. **e** In vitro rooting of shoots on MS medium + 1.0 mg L^{-1} IBA. **f** Hardening of micropropagated plants in the greenhouse. **g** *Ex vitro* acclimatized plantlets of *V. tessellata*

Table 2 Organogenesis from protocorms (seedling development) on optimized concentrations of plant growth regulators

Conc. of PGRs (mg L^{-1})	Organogenesis from protocorms (Mean ± SE)				
	No. of shoot	Shoot length (cm)	No. of leaf per shoot	Number of roots per shoot	Root length (cm)
IAA 1.0	1.1 ± 0.00^{b}	1.8 ± 0.21^{b}	2.0 ± 0.30^{a}	1.0 ± 0.27^{b}	0.2 ± 0.00^{b}
IBA 1.5	1.7 ± 0.10^{c}	3.0 ± 0.13^{c}	5.4 ± 0.25^{b}	3.0 ± 0.00^{c}	0.4 ± 0.11^{c}
NAA 1.0	1.0 ± 0.18^{a}	1.0 ± 0.15^{a}	2.0 ± 0.00^{a}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}

Incubation time—4 weeks, mean values in the column with different letters indicate significant differences according to DMRT at $P < 0.05$

PGRs Plant Growth Regulators,

4 week interval and there was a significant rise in number of shoots and leaves in MS medium up to 4th sub-culture (Fig. 1d).

Among the discrete concentrations of activated charcoal (AC) used, MS medium with 100 mg L^{-1} AC with the optimized PGRs exerted proliferation of shoots (Fig. 2). The incorporation of AC helps in shoot multiplication by adsorption of phenolics and toxic compounds (Thomas 2008). Fortification of nutrient medium with additives enhances the level of nitrogen and assists in the effective proliferation of shoots. A number of reports available on the use of additives and activated charcoal in nutrient medium to improve the number and quality of shoots in orchids (Jiang et al. 2003; Aktar et al. 2007; Zahara et al. 2017). The presence of AC in growth medium significantly improves the culture growth under in vitro and *ex vitro* conditions as reported in European, Brazilian and other orchids (Van Waes 1987; Moraes et al. 2005; Prizao et al. 2012).

The number of shoots regenerated in the present study was significantly high as compared to the previous reports (Rahman et al. 2009; Bhattacharjee et al. 2015; Bhattacharjee and Islam 2014; 2017). The authors reported that MS

Table 3 Effect of plant growth regulators (PGRs) on multiple shoot regeneration from seedlings

Conc. of PGRs (mg L^{-1})			Number of shoots (Mean ± SE)	Shoot length (cm) (Mean ± SE)
BAP	IAA	NAA		
0.25	0.25	–	11.0 ± 0.21^{h}	2.8 ± 0.14^{f}
0.5	0.25	–	14.5 ± 0.36^{j}	4.1 ± 0.20^{j}
0.75	0.25	–	12.8 ± 0.20^{i}	3.5 ± 0.26^{hi}
1.0	0.25	–	09.0 ± 0.17^{g}	3.0 ± 0.20^{fgh}
0.25	0.5	–	16.0 ± 0.10^{j}	4.7 ± 0.28^{kl}
0.5	0.5	–	24.8 ± 0.52^{l}	5.2 ± 0.35^{l}
0.75	0.5	–	19.5 ± 0.33^{k}	5.0 ± 0.30^{kl}
1.0	0.5	–	06.0 ± 0.29^{de}	4.6 ± 0.31^{k}
0.25	1.0	–	08.3 ± 0.21^{fg}	3.0 ± 0.47^{fgh}
0.5	1.0	–	11.6 ± 0.26^{hi}	3.4 ± 0.30^{ghi}
0.75	1.0	–	09.0 ± 0.40^{g}	2.9 ± 0.23^{fg}
1.0	1.0	–	05.9 ± 0.12^{de}	2.0 ± 0.18^{de}
0.25	–	0.25	04.7 ± 0.16^{bcd}	0.8 ± 0.21^{ab}
0.5	–	0.25	08.9 ± 0.22^{g}	1.3 ± 0.13^{bc}
0.75	–	0.25	06.0 ± 0.16^{de}	1.0 ± 0.10^{ab}
1.0	–	0.25	03.0 ± 0.10^{ab}	0.5 ± 0.14^{a}
0.25	–	0.5	08.0 ± 0.24^{fg}	2.8 ± 0.17^{f}
0.5	–	0.5	11.4 ± 0.18^{hi}	3.6 ± 0.22^{i}
0.75	–	0.5	07.6 ± 0.10^{efg}	3.0 ± 0.18^{fgh}
1.0	–	0.5	04.0 ± 0.00^{bc}	2.3 ± 0.20^{e}
0.25	–	1.0	03.5 ± 0.11^{abc}	1.3 ± 0.16^{bc}
0.5	–	1.0	07.0 ± 0.13^{ef}	1.7 ± 0.14^{cd}
0.75	–	1.0	05.0 ± 0.00^{cd}	1.0 ± 0.00^{ab}
1.0	–	1.0	02.0 ± 0.00^{a}	1.0 ± 0.00^{ab}

Mean values (after 4th sub-culture) in the column with different letters indicate significant differences according to DMRT at $P < 0.05$

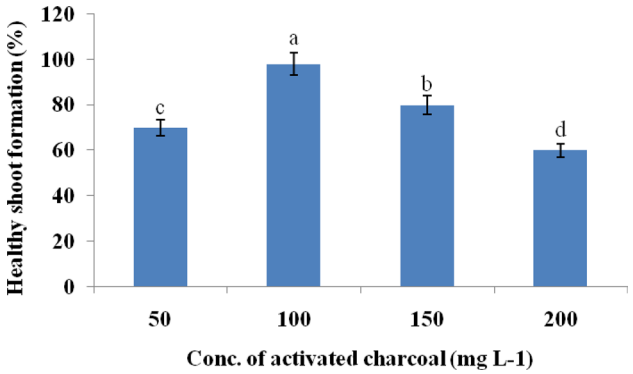

Fig. 2 Effect of activated charcoal (AC) on the proliferation of healthy shoots. (Error bars represent standard error. Different letters indicate significant differences between the multiplication rate by DMRT at $P < 0.05$)

medium supplemented with NAA and BAP induced 7.52 shoots (Bhattacharjee and Islam 2014), and BAP and NAA produced 10.8 shoots through somatic embryogenesis (Bhattacharjee and Islam 2017). The combination of BAP and NAA generated 13.19 shoots (Rahman et al. 2009), and the same concentration of PGRs in the medium induced 16.5 shoots from the immature seeds (Bhattacharjee et al. 2015).

Induction of roots from the shoots

The rooting response of *V. tessellata* in the present study was higher than the existing reports. The individual integration of auxins in the medium showed varying responses in the development of roots. Of the various concentrations of auxins and strengths of MS medium tested, maximum response (100%) and a number of roots (7.2 roots per shoot with 4.0 cm length) was recorded on MS + 1.0 mg L^{-1} IBA (Table 4; Figs. 1e and 3). Root development was marginal in MS medium with 1.5 mg L^{-1} NAA. The stimulatory effect of auxins on in vitro rooting had been reported in several orchid species (Novak et al. 2014; Mirani et al. 2017). Amendment of auxins in half-strength MS medium was found ineffective in rooting experiments (Fig. 3). The present findings are superior and contradictory to the earlier reports on in vitro rooting of *Vanda* species. As per Bhattacharjee and Islam (2014), half-strength MS medium augmented with IAA gave better roots (6.1 roots) in *V. tessellata*. Rahman et al. (2009) reported that MS medium with NAA and IBA induced maximum roots within 28 days. A combination of MS medium with NAA, IAA, and 250 mg L^{-1} cefotaxime was reported fine in rooting of *Vanda* hybrids 'Dr. Anek' (Baby et al. 2019) and 'Sansai Blue' (Baby and Valsala 2019). Half strength MS medium augmented with 0.5 mg L^{-1} each of IAA and NAA induced 5.0 roots from somatic embryo-derived shoots of *V. tessellata* (Bhattacharjee and Islam 2017).

The exogenous application of auxins promotes rooting in orchid species under in vitro conditions (Panwar et al. 2012), but, the presence of higher concentrations of auxins inhibits rhizogenesis as well as shoot growth, as these move from shoot apex to the root primardia (Ozel et al. 2006).

Hardening of plantlets in greenhouse

The hardening set up with the upper covering of transparent polythene cups provides an ambient micro-environment for stabilization of roots and shoots on sudden *ex vitro* transfer. After 4 weeks (Fig. 1f), plantlets were transferred to sterile potting mixtures and exposed to 12-h photoperiod, higher temperature (32 ± 2 °C) and reduced RH (40–50%) and maintained for 12 weeks (Fig. 1g). Out of the three potting mixtures tested, soilrite® + cocopeat + coconut husk complex in the ratio of 1:1:1 supported the acclimatization process and the maximum percentage of survival rate (98%) was recorded under the greenhouse conditions (Fig. 4). Formation of new leaves

Table 4 Effect of auxins on in vitro induction of roots

Conc. of auxins (mg L^{-1})		Root induction frequency (%) (Mean ± SE)	Number of roots/plantlet (Mean ± SE)	Root length (cm)/ shoot (Mean ± SE)
IBA	NAA			
0.0	0.0	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}
0.5	–	88.3 ± 0.43^{f}	5.0 ± 0.22^{g}	3.1 ± 0.16^{f}
1.0	–	100.0 ± 0.37^{h}	7.2 ± 0.19^{i}	4.0 ± 0.12^{j}
1.5	–	94.0 ± 0.20^{g}	6.0 ± 0.00^{h}	3.8 ± 0.23^{i}
2.0	–	89.6 ± 0.34^{fg}	5.2 ± 0.27^{g}	3.4 ± 0.19^{h}
2.5	–	80.0 ± 0.41^{de}	4.0 ± 0.33^{f}	2.9 ± 0.10^{g}
3.0	–	73.8 ± 0.29^{bc}	4.0 ± 0.20^{f}	2.5 ± 0.25^{f}
–	0.5	74.0 ± 0.31^{bc}	3.4 ± 0.26^{e}	2.0 ± 0.10^{c}
–	1.0	88.2 ± 0.23^{f}	4.0 ± 0.11^{f}	2.6 ± 0.19^{e}
–	1.5	83.0 ± 0.17^{e}	3.6 ± 0.10^{e}	2.0 ± 0.22^{d}
–	2.0	76.4 ± 0.21^{cd}	3.0 ± 0.19^{d}	1.8 ± 0.16^{d}
–	2.5	71.7 ± 0.26^{bc}	2.2 ± 0.30^{c}	1.3 ± 0.28^{c}
–	3.0	69.5 ± 0.20^{b}	1.9 ± 0.18^{b}	1.0 ± 0.30^{b}

Incubation time—4 weeks, mean values in the column with different letters indicate significant differences according to DMRT at $P < 0.05$

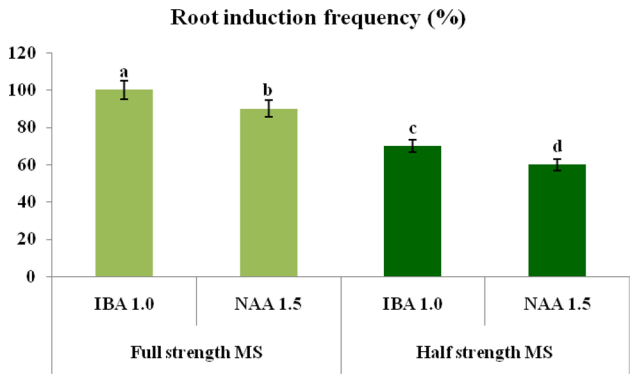

Fig. 3 Effect of strengths of MS medium on in vitro induction of roots. (Error bars represent standard error. Different letters indicate significant differences between the strength of MS medium by DMRT at $P < 0.05$.)

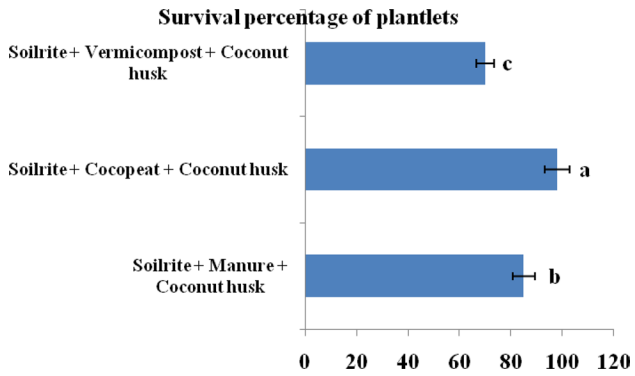

Fig. 4 The survival rate of in vitro raised *Vanda tessellata* plantlets in the greenhouse on three different substrates after 3 months. (Error bars represent standard error. Different letters indicate significant differences between the substrate responses by DMRT at $P < 0.05$.)

and roots was observed on this potting mixture and almost all the shoots survived after 4–5 weeks of *ex vitro* hardening.

Conclusion

The present report illustrates an enhanced in vitro regeneration system for *Vanda tessellata* via asymbiotic seed germination using auxins and proliferation of shoots with the aid of activated charcoal. The shoots were effectively multiplied, rooted, and acclimatized in the greenhouse. This protocol could be used for large scale propagation, conservation, and sustainable utilization of this important orchid species.

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Author contributions MM, MSS, and LR: Conceptualization, investigation, methodology. MSS, MM, and PS: Writing the original draft. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no known competing interests in this paper.

Human and animal rights This research did not involve experiments with human or animal participants.

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