



In vitro rapid propagation technology system of *Dendrobium moniliforme* (L.) Sw., a threatened orchid species in China

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Abstract

Dendrobium moniliforme is a threatened and medicinal orchid species in China. The establishment of in vitro rapid propagation technology system will facilitate preservation and utilization of germplasm resources in *D. moniliforme*. In this study, the effects of different plant growth regulators (PGRs) on seed germination, proliferation of cluster shoots, rooting of shoots were tested under asymbiotic culture condition, and the best acclimatization condition of plantlets was screened. Our research results were shown as follows: germination rate (GR) was significantly increased by adding N6-benzyladenine (BA, 0.1 mg/L) during asymbiotic culture. In Murashige and Skoog (MS) medium supplemented with α -naphthaleneacetic acid (NAA, 1.0 mg/L) and BA (1.0 mg/L), the proliferation coefficient of cluster shoots was 6.1 times comparing with the control group. The MS medium with NAA (0.5 mg/L) and indole-3-butyric acid (IBA, 0.5 mg/L) promoted the highest rooting rate (RR). For plantlet transplanting, the equal mixing volume ratio of pine bark, turfy soil, and peanut shells was the best substrate. In a word, this rapid propagation system provides strong technical support for *D. moniliforme* to expand proliferation and germplasm resource protection.

Keywords *Dendrobium moniliforme* · Seed germination · Rapid propagation technology system · Plant growth regulators · Asymbiotic culture

Introduction

Orchidaceae is one of the largest families of angiosperms, as well as one of the most threatened taxa in the world (Wraith and Pickering 2018). With low natural reproduction rates and over exploitation to satisfy various purposes, the endangered status of many orchids remains unimproved (Pujasatria et al. 2020). Since in situ conservation and management of rare populations alone are not sufficient to ensure species survival, relocation and proliferation are the key to conserving their germplasm resources (Seaton et al. 2010); (Arcidiacono et al. 2021). The natural propagation of orchids often hindered by low seed germination and slow growth, therefore, in vitro tissue culture is a reliable conservation strategy that not only provides commercial material but also

reduces habitat pressure (Kang et al. 2020); (Arcidiacono et al. 2021).

Dendrobium moniliforme is a perennial epiphytic herb and one of the most widely distributed species in the genus *Dendrobium* of the Orchidaceae family, mainly in East Asia, including China, Japan, Korea, and Nepal (Ye et al. 2017); (Meng et al. 2019); (Shah et al. 2019). It has important economic values such as wide applications in ornamental horticulture, modern medicine, and cosmetic industries (Lee et al. 2012a; Lee et al. 2012b); (Baek et al. 2016). Due to over-exploitation, habitat destruction and shrinkage, the distribution of *D. moniliforme* in China is threatened with extinction. Therefore, it is listed in List of National Key Protected Wild Plants (new edition) in China and Appendix II of the CITES list. The conservation of *D. moniliforme* has attracted a lot of attention from scholars. (Lo et al. 2008) screened the suitable basic medium for *D. moniliforme* seed germination and tested the effect of sucrose concentration and organic additions on plant growth. There are also research data to confirm activated charcoal (AC) and sucrose can enhance shoot proliferation and rooting (Bae et al. 2014). Using the mixture of Hyponex, peptone, sucrose and charcoal, (Kim et al. 2016) cultivated

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plantlets under asymbiotic culture condition, and eventually succeeded in restoring the natural planting status of *D. moniliforme*. For the effect of plant growth regulators (PGRs) on propagation, only protocorm-like body (PLB) formation was involved (Bae et al. 2014). PGRs have been widely used in rapid propagation techniques for orchids and other ornamental species (Rademacher 2015); (Kumar et al. 2022). However, the effect of PGRs on seed germination of *D. moniliforme* and their effects on plant growth and development has not been reported. Using asymbiotic culture condition, our group have already observed the post-embryonic development and seedling morphogenesis (Liu et al. 2022). In this study, we focus on the effects of different PGRs on seed germination, cluster shoots induction, rooting, and screen the best acclimatization condition of plantlets. This study successfully established a technical system for the rapid propagation of *D. moniliforme*, which is not only beneficial to the conservation of germplasm resources, but also has great application value in the production practice of *D. moniliforme*.

Materials and methods

Collection of plant material and seed viability determination

D. moniliforme capsules were harvested in six months after pollination from Huoshan County, Anhui Province, China. The indehiscent mature fruits were yellowish green. Then, they were kept in the refrigerator at 4 °C for the next experiment.

The viability of *D. moniliforme* seeds was evaluated using improved Vujanovic's method of 2,3,5-triphenyltetrazolium chloride (TTC) test (Vujanovic et al. 2000) before asymbiotic culture. Seeds were taken from three capsules randomly, then stained with 1% (w/v) TTC in phosphate-buffered saline (PBS, pH 6.5) at 30 ± 1 °C for 30 h in dark condition. After rinsed three times with sterile water, the seeds were transferred to concave slides and observed under a stereomicroscope (Olympus SX7, Tokyo, Japan). The experiment was repeated three times. The number of red seed embryos and the total number of seed embryos in each field of view were counted, and then the seed viability (SV) was calculated. The formula for calculating SV is as follows:

$$\text{Seed viability(\%)} = \frac{\text{Number of red seed embryos}}{\text{Total number of seed embryos}} \times 100\%$$

Surface disinfection of capsules and culture conditions

Surface sterilization of capsules and inoculation of the seeds have been done using improved Tan's method (Tan et al. 2014). The capsules were washed with detergent and then rinsed three

times with running water. In a super-clean bench, the capsules were sterilized with 75% alcohol for 3 min followed by three times rinse in sterile water. Then, the capsules were transferred into 2% NaOCl for 10 min followed by five times rinse in sterile water. Under aseptic conditions, one end of a capsule was cut by aseptic scalpel. And then the seeds were poured into a 2 mL centrifuge tube and made a suspension with 2 mL sterile water followed by tenfold dilutions. Using a sterilized pipette, 10 µL of the seed suspension as a sample drop was transferred onto the medium surface. There were 20 drops in per petri dish. The cultures were carried on a growth chamber with a light intensity of 20 µE, 16 h light/8 h dark under long daylight conditions and a controlled temperature of 26 ± 1 °C.

Screening of PGRs components on promoting seed germination

Four concentration gradients (0.1 mg/L, 0.5 mg/L, 1.0 mg/L, 2.0 mg/L) were set with gibberellic acid (GA₃), indole-3-acetic acid (IAA) and N6-benzyladenine (BA) respectively, while no PGR as control. The culture medium was Murashige and Skoog (MS), which contained 3% sucrose and 0.7% agar (pH 5.8). According to the method mentioned above, seeds were sown with 10 drops per treatment, each drop contained at least 10 seeds, and three times replicated. The seeds were observed with a stereomicroscope on 20 days after culture (DAC), while germination rate (GR) of seeds was calculated. Also, the number of days with > 50% of germination seeds was recorded. The formula for calculating GR is as follows:

$$\text{Germination rate(\%)} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds} \times \text{SV}} \times 100\%$$

Selection of the optimum combination of different PGRs for proliferation of cluster shoots

Plantlets of about 1 cm in height and 2–3 leaves were selected for this test. MS was used as the basic medium and no PGR was added as control. Then the treat meant was carried out with four gradients of 0.1 mg/L, 0.5 mg/L, 1.0 mg/L and 2.0 mg/L for α-naphthaleneacetic acid (NAA) and BA, respectively, with 20 samples per group and three replications. On 40 DAC, plant height and number of shoots were measured. The proliferation coefficient of cluster shoots on 40 DAC were calculated based on the new shoots number divided by the plantlet number at the start of culture. Three times repeated.

$$\text{Proliferation coefficient of cluster shoots} = \frac{\text{Number of new shoots}}{\text{Total number of plantlets}}$$

Screening of the optimum combination of PGRs for rooting

The cluster shoots of approximately 3–4 cm in height were selected for rooting medium screening. Two PGRs, NAA and indole-3-butyric acid (IBA), were mixed in MS medium containing 2% carbon powder, and also were set with four concentration gradients (0.5 mg/L, 1.0 mg/L, 1.5 mg/L, 2.0 mg/L). After 60 d of incubation, the length, number, and rooting rate (RR) were measured. Three times repeated. The formula for calculating RR is as follows:

$$\text{Rooting rate (\%)} = \frac{\text{Number of rooting plantlets}}{\text{Total number of plantlets}} \times 100\%$$

Acclimatization and transplant

After 7 d of acclimatization under natural conditions, *in vitro* plantlets about 5 cm in height with healthy roots were washed with tap water to remove the rooting medium, then they were transplanted into plastic boxes with 6 cells in each one at a controlled temperature of 26 ± 1 °C. The boxes were covered with clear plastic lids to prevent drying in the early stages of acclimatization, and the lids were gradually opened each week until they were completely removed after 4 weeks. From the fifth week onward, the boxes were watered every two days. The transplanting substrates were selected from pine bark, turfy soil and peanut shells and mixed in varied ratios and divided into 7 groups as follows: (a) 1:0:0, (b) 0:1:0, (c) 0:0:1, (d) 1:1:0, (e) 0:1:1, (f) 1:0:1, (g) 1:1:1. Two plantlets were planted in each cell and 24 plantlets were planted in each group with three replications. The survival rate, number of new shoots and height of plantlets were counted after 6 weeks.

$$\text{Survival rate (\%)} = \frac{\text{Number of surviving plantlets}}{\text{Number of transplanted plantlets}} \times 100\%$$

Statistics

The data were analyzed for significant differences. One-way analysis of variance (ANOVA), Duncan's test, and homogeneity of variance tests were analyzed with IBM SPSS Statistics 21 software. Means were separated using Duncan's multiple range test (DMRT) at $p < 0.05$.

Results

Seed viability and germination

Seed viability test has been done because it will be avoided effect the statistic results of the following seed germination.

Seeds were taken from the mature capsules (Fig. 1a). Before TTC staining, the color of seeds was almost pale yellow (Fig. 1a1), and the immature embryo was ellipsoidal surrounded by membranous seed coat (Fig. 1c). After 30 h, some seeds stained red, it meant the embryo has viability. Normally, 57.06% of the seeds in capsules were viable (Fig. 1b). Under *in vitro* culture condition, the viable seed size enhanced gradually, the embryo grew into sphere, and then the seed coat broke (Fig. 1d, e).

The effect of PGRs on seed germination

Using GA₃, IAA and BA, we investigated the effect of PGRs on seed germination. The results are shown in Table 1. GA₃ and IAA had poor promoting effects, and GR of seeds was lower than that of the control group. On the contrary, the promoting effect of BA was the best one. When BA concentration was 0.1 mg/L, GR of *D. moniliforme* was 99.39% on 7 DAC. However, GR showed a decreasing trend with the increase of BA concentration. Figure 2 shows the status of seed germination under different concentration of BA treatment. Under the condition of BA 0.1 mg/L, the GR was the highest one, also the spherical embryos in the seed were yellow-green and were almost equal in size, indicating relatively synchronous development (Fig. 2a). The 0.5 mg/L BA treatment was similar with the 0.1 mg/L BA, but the growth status of embryo was less synchronous (Fig. 2b). Also 1.0 mg/L BA treatment was similar with 2.0 mg/L BA, the spherical embryos were vitrified and did not develop synchronously (Fig. 2c, d). In addition, with GR > 50% as node, the culture time was recorded one by one except the treatment which was never reached 50%. It is obviously that the shortest germination time is 7 days, and GR is also the highest one accordingly (Table 1).

Effect of PGRs on the proliferation of cluster shoots

The results of the proliferation of cluster shoots under different combination of NAA and BA are shown in Fig. 3. As a general rule, it is not conducive to the growth of cluster shoots that the BA and NAA are too much lower or higher in *D. moniliforme*. Figure 3a is showing the development status of cluster shoots. The number of shoots in 0.5 mg/L BA + 2.0 mg/L NAA culture condition was the worst one comparing with the control group and the other treatments (Fig. 3a-c). When the concentration of NAA was 1.0 mg/L, the number of cluster shoots increased with the increase of BA concentration and then decreased, the maximum proliferation coefficient was 6.1 when BA was 1.0 mg/L (Fig. 3b). Simultaneously, the growth of cluster shoots was the best one (Fig. 3a), and the length of shoots could reach 2.205 cm

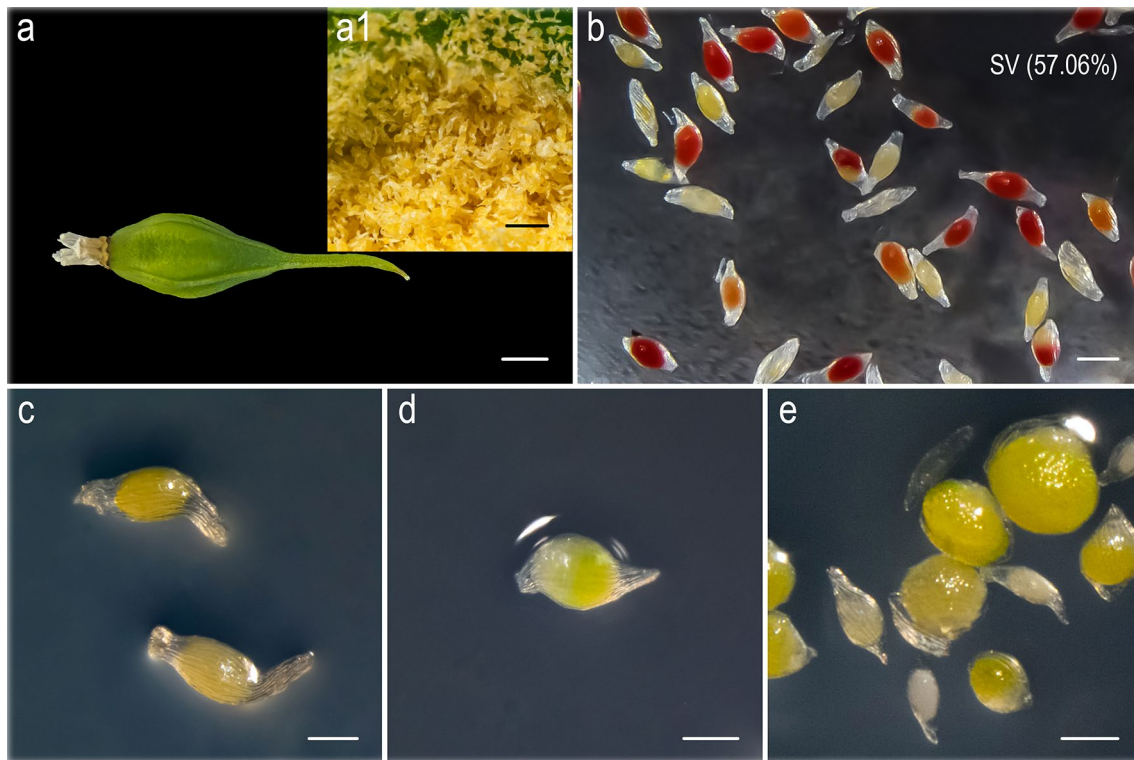


Fig. 1 Seed morphology, viability and germination. **a** The mature capsule of *D. moniliforme*. **a1** The seed in mature capsule. **b** Viable seeds, showing in red color. **c** Seed morphology on 0 DAC. **d**

Expanded seed embryo not separated from seed coat. **e** Spherical embryo breaking through seed coat after germination. Scale bar: **a** 1 cm, **a1** 500 μ m, **b** 200 μ m, **c-e** 100 μ m

Table 1 Effect of PGRs on seed germination

PGRs (mg/L)			GR (%)	Time of germination (d, GR > 50%)
GA ₃	IAA	BA		
0	0	0	73.37 \pm 0.86 ^b	9
0.1			57.26 \pm 0.95 ^b	14
0.5			67.80 \pm 1.21 ^b	10
1.0			52.22 \pm 0.71 ^d	18
2.0			45.68 \pm 0.43 ^d	/
	0.1		38.33 \pm 0.96 ^d	/
	0.5		61.42 \pm 1.36 ^b	13
	1.0		53.40 \pm 1.48 ^c	17
	2.0		45.45 \pm 0.69 ^d	/
		0.1	99.39 \pm 0.19 ^a	7
		0.5	95.25 \pm 0.64 ^b	8
		1.0	66.98 \pm 1.40 ^b	12
		2.0	59.12 \pm 0.50 ^b	13

Data indicate the mean \pm SD of three replicates and means with the same letter are not significantly different ($p < 0.05$) according to DMRT

(Fig. 3c). As a whole, the optimal concentration of PGRs is BA 1.0 mg/L + NAA 1.0 mg/L on the proliferation of cluster shoots.

Effect of PGRs on rooting

The effects of the PGRs on rooting ratios (RR) and the plantlet growth status are shown in Fig. 4. Overall, appropriate auxin and cytokinin ratio is beneficial to cluster shoots rooting in *D. moniliforme*. The highest RR could be achieved 93.33% when NAA was 0.5 mg/L and IBA was 0.5 mg/L, likewise, the number of roots was higher with an average of 5.9 (Fig. 4b–d). In particular, the longest roots were induced on the medium with NAA 1.5 mg/L and IBA 0.5 mg/L, with a length of 1.692 cm (Fig. 4c), but the RR was only 46.67% (Fig. 4d), and the number of roots was also lower with an average of 3.7 (Fig. 4b). Therefore, NAA at 0.5 mg/L plus IBA at 0.5 mg/L was the best combination for the plantlet rooting of *D. moniliforme*. Accordingly, the plantlet growth was the best one comparing with other culture conditions (Fig. 4a).

The effect on acclimatization and transplanting

The transplanting conditions and the plantlets growth status of *D. moniliforme* are shown in Fig. 5. There was no doubt that the single pine bark and peanut shells were not a suitable substrate for the growth of plantlets comparing with turfy soil (Fig. 5a–c, h). Although the survival rate was

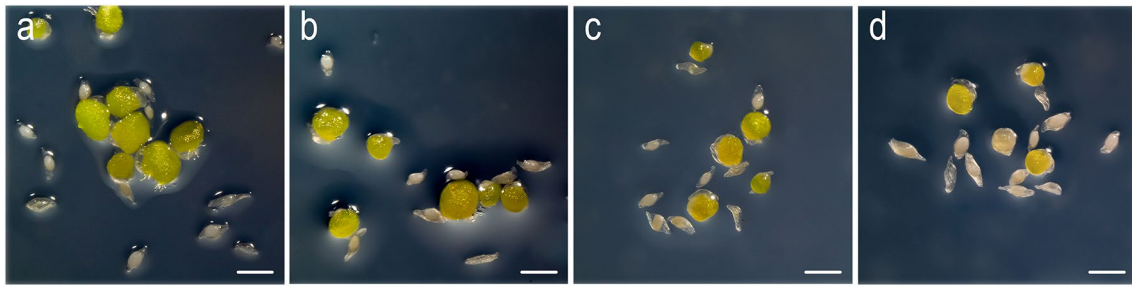


Fig. 2 Effects of BA on seed germination. **a** BA 0.1 mg/L, **b** 0.5 mg/L, **c** 1.0 mg/L, **d** 2.0 mg/L. Scale bar: 200 μm

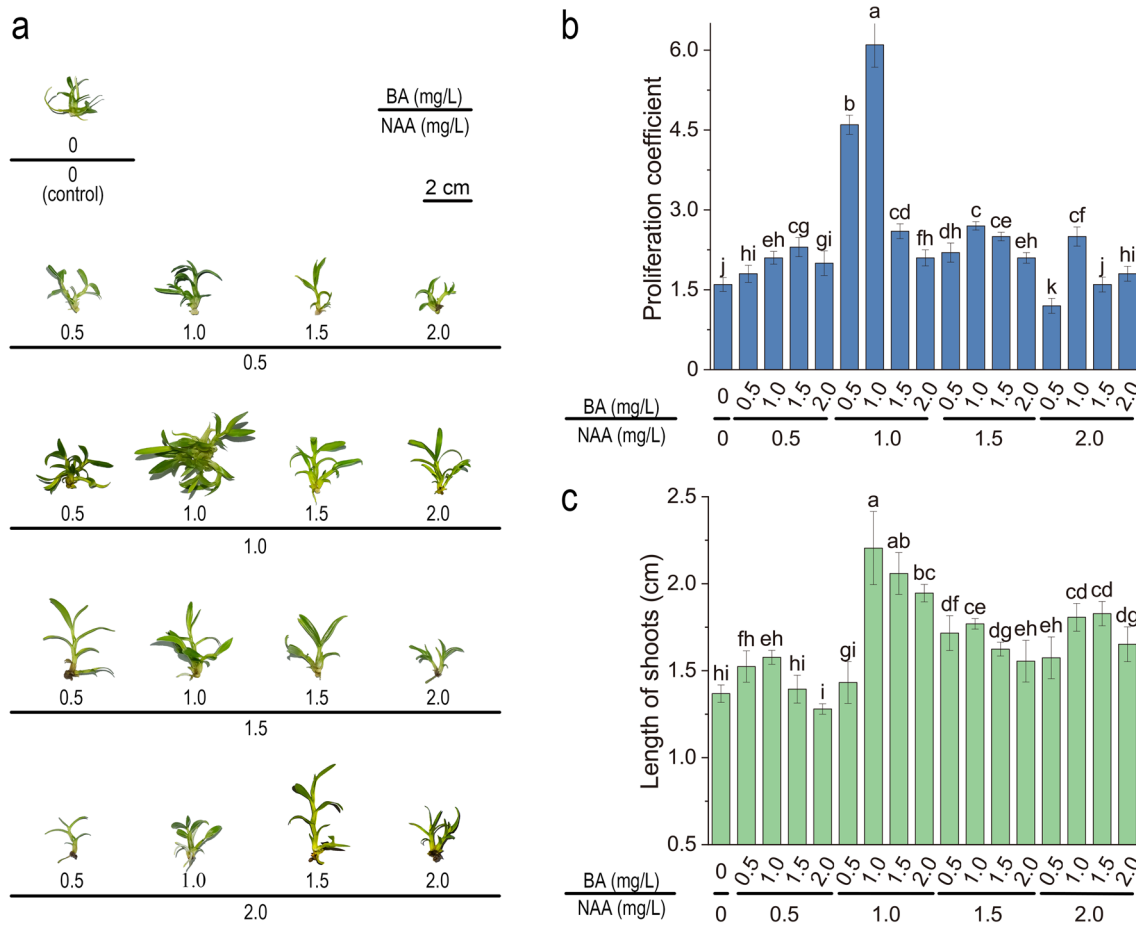


Fig. 3 Effects of PGRs on cluster shoots proliferation. **a** The cluster shoots morphology under different PGRs combination, Scale bar: 2 cm. **b** The difference of proliferation coefficient. **c** The difference

of shoots length. Note: The bars represent the standard error, and the different letters on the top of the bars represent significant differences in individual traits analyzed by DMRT at $p < 0.05$

higher in the single substrate of turfy soil, the number of new shoots was lower, and the plants grew poorly (Fig. 5b). It was worth noting that the survival rate of the plantlets could reach more than 90% in all combinations containing turfy soil, and the new shoot numbers and plantlet heights were much better (Fig. 5h). The new shoot number of plantlet which was planted in the mix substrate of pine bark and turf

soil was less than that of peanut shells and turf soil substrate (Fig. 5d, e, h). Although the survival rate was slightly lower, the plantlet was higher when it was planted in a mixture of pine bark and peanut shells, but the new shoot number was a little bit lower (Fig. 5f, h). In particular, plantlets transplanted in a substrate of peanut shells: turfy soil: pine bark (1:1:1) had the highest survival rate of 93.06%, and their

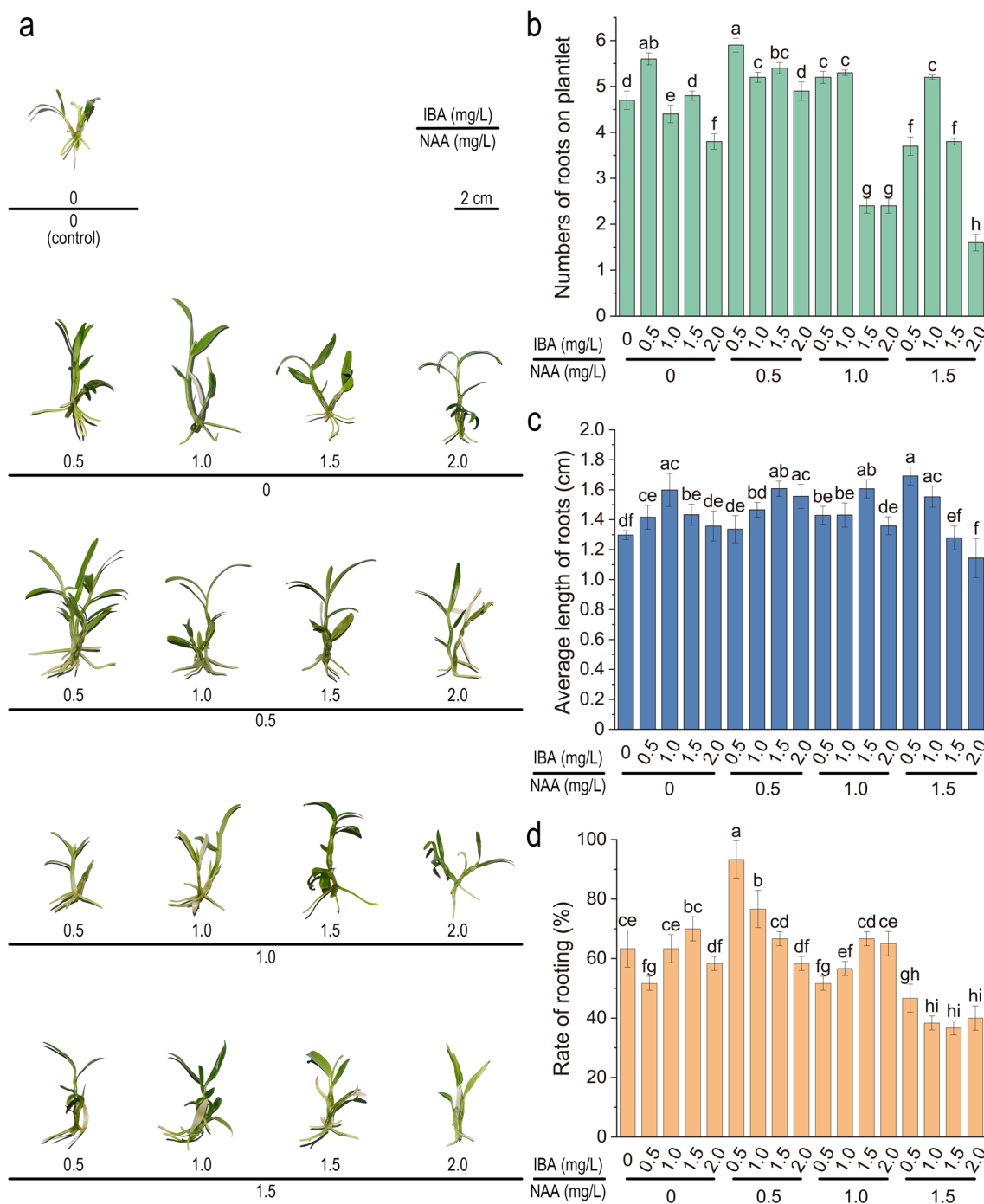


Fig. 4 Effects of PGRs on rooting. **a** The rooting plantlet morphology under different PGRs combination, Scale bar: 2 cm. **b** The difference of root number. **c** The difference of average root length. **d** The differ-

ence of RR. Note: The bars represent the standard error, and the different letters on the top of the bars represent significant differences in individual traits analyzed by DMRT at $p < 0.05$

plant heights was also the highest at 5.629 cm, with a higher number of new shoots (Fig. 5g, h). Therefore, the transplanting substrate with a mixture of pine bark, turfy soil and peanut shells were more favorable for the transplanting culture of *D. moniliforme*.

Discussion

Under asymbiotic culture, appropriate BA concentration has been shown to promote seed germination in orchids. It was suggested that the addition of BA in the medium could accelerate GR of *Calanthe tricarinata* (Godo et al. 2010). (Pierce

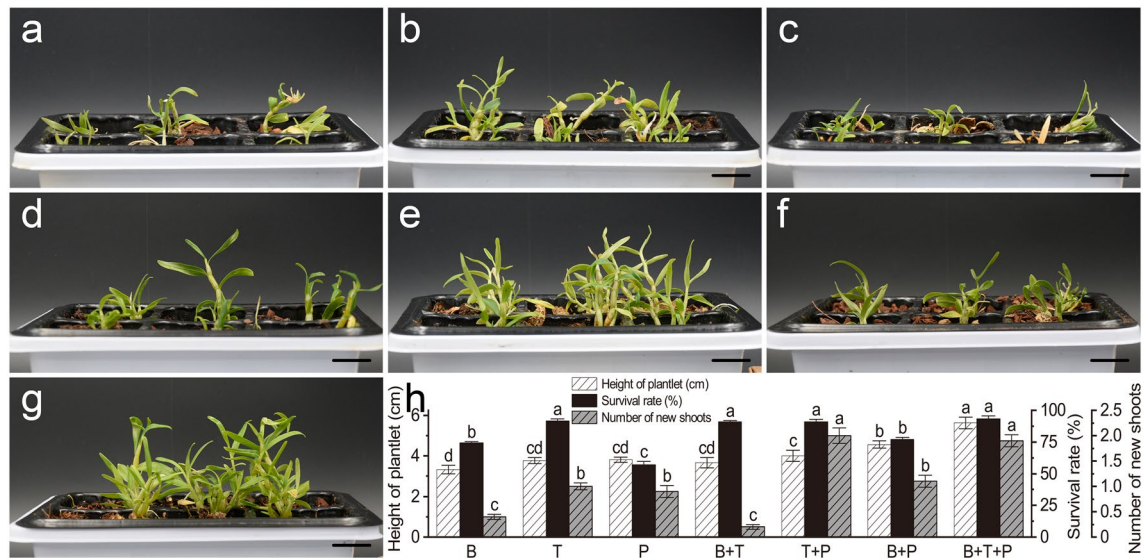


Fig. 5 Effects of different substrate composition on acclimatization and transplanting. **a–g** The different plantlet growth status, growing in the composition of mix substrates which is pine bark: turfy soil: peanut shells, i.e. **a** 1:0:0, **b** 0:1:0, **c** 0:0:1, **d** 1:1:0, **e** 0:1:1, **f** 1:0:1, **g**

1:1:1. **h** The relative data of plantlet growth. Note: The different letters on the top of the error bars represent significant differences in individual traits analyzed by DMRT at $p < 0.05$. Scale bar: 2 cm

and Cerabolini 2011) found that BA, GA₃, kinetin and thidiazuron (TDZ) all significantly increased GR of immature seeds in *Pseudorchis albida*, among which BA was the most effective (50.5%). The same result was confirmed in the seed germination in *Cypripedium candidum* seeds (Pauw et al. 1995) and *Calanthe tricarinata* (Godo et al. 2010). As we all known, cytokinin is involved in maintaining meristematic tissue function (Kurakawa et al. 2007). Meanwhile, it is also known from morphological and anatomical analyses that the embryo is undifferentiated in orchid seeds. The embryo has not yet established axial tissues when a seed has germinated (Liu et al. 2022). Therefore, the immature embryo needs cell division and differentiation by increasing its size rather than radicle elongation to break out of the seed coat (Zhang et al. 2015); (Kunakhonnuruk et al. 2018). These findings may be able to explain the reason why BA promotes the seed germination of orchids. Our research work confirmed this view by showing that the highest GR (99.39%) and the shortest germination time (7 DAC) of *D. moniliforme* seeds in the medium supplemented with 0.1 mg/L BA.

PGRs significantly influence the induction of cluster shoots, and the selection of suitable PGRs is particularly crucial in the culture process. The main goal of plant tissue culture is to produce the maximum number of cluster shoots during clonal propagation (Bhattacharyya et al. 2016). Previous studies proved that the regeneration and proliferation of cluster shoots are closely related to the type and concentration of cytokinin (Amoo et al. 2014). Actually, adding BA into MS medium not only induced more shoots, but also, they were healthier than those one induced by other PGRs in

Phaleonopsis hybrid ‘Little gem’ (Chung et al. 2016). Presently study has confirmed that BA played an important role during proliferation of protocorms or in vitro multiplication, thus resulted in maximum number of shoots, root length on MS medium with 1.0 mg/L BA in *Cymbidium aloifolium* (Kumar et al. 2022). Similarly, the highest number of cluster shoots was produced when the MS medium contains 15 μM BA and 15 μM NAA in *D. longicornu* (Dohling et al. 2012). In this study, the highest number of cluster shoots and the most increased shoot length of *D. moniliforme* was recorded when MS medium contained 1.0 mg/L BA and 1.0 mg/L NAA. It’s worth noting that the induction rate by meta-topolin (mT) in combination with NAA was superior to that of by BA in combination with NAA in the induction of cluster shoots in *D. nobile* (Bhattacharyya et al. 2016). In recent years, a suggestion has been made to utilize mT instead of BA for greater efficiency (Saeiahagh et al. 2019). Although mT improves the adaptability and high genetic fidelity of plantlets (Lata et al. 2016), and BA has the potential to induce variation in vitro clones (Biswas et al. 2009), undeniably, BA is still widely used in the tissue culture of orchids (Chung et al. 2016); (Kumar et al. 2022). Furthermore, considering of the cost it should be spent, BA is more readily available and affordable in the rapid propagation technology systems which are utilized widely (Ivanova and Staden 2008).

Two effect factors of auxin are noticed mostly during cluster shoot rooting, i.e., IBA and NAA. IBA has been widely utilized in the rooting culture of orchids (Bhattacharyya et al. 2016). In *Bulbophyllum odoratissimum*, different

types of auxins reflected a wide range of variations in its rooting process. The highest number of roots per explant was found in 0.5 mg/L IBA with better root length after 8 weeks of inoculation comparing with NAA enriched medium (Prasad et al. 2021). Similarly, (Bhattacharyya et al. 2018) found that using IBA alone in *D. aphyllum* could induce higher RR and longer roots than that of NAA. In particular, the effects of IBA better than other auxin in promoting orchid rooting have been discussed adequately (Mahendran and Bai 2009); (Mohanty et al. 2012). In contrast to the above findings, we found that the highest rooting percentage (93.33%) and the number of roots (5.9) were achieved when the IBA concentration was 0.5 mg/L, and the NAA was 0.5 mg/L. It meant that the proper combination of NAA and IBA were necessary for getting the maximum RR and the highest number of roots during the rooting culture of *D. moniliforme*.

In orchid species cultivation, the substrate is essential since it influences the quality of the final plantlet, mainly performing the supportive function of the root system (Faria et al. 2018). Research data show that turfy soil provides sufficient nutrients and retains moisture well (He et al. 2016), while peanut shells and pine bark are good drainage (Faria et al. 2018). In spite of *Dendrobium* orchids are mainly epiphytes, when it is grown in artificial conditions, aeration, capillary action, water and nutrient-holding capacities of the substrate should be taken into consideration (Teixeira da Silva et al. 2017). In this study, the highest survival rate of the plantlet was achieved when the turfy soil, peanut shells and pine bark were mixed at a volume ratio of 1:1:1. Obviously, any kind of substrate alone is not good enough for the growth of *D. moniliforme*, and the proper proportion of substrates is crucial to improve the survival rate when the plantlets are transplanted into the natural environment.

Conclusion

This study established a protocol for asymbiotic germination, in vitro culture, and greenhouse acclimatization of *D. moniliforme*. The results show that seed germination, cluster shoot proliferation, and plantlet rooting require suitable types and concentrations of PGRs in *D. moniliforme*. Appropriate concentration of BA was conducive to rapid germination of seeds, likewise, the proper concentration proportion of NAA and BA were also promoted plantlet proliferation. Furthermore, transplant acclimatization needs a loose and airy mixed substrate. Obviously, peanut shells: turfy soil: pine bark (1:1:1) is the best mixed substrate for the growth of plantlets. Overall, this rapid, low-cost, and reproducible micropropagation protocol can be used commercially or for conservation purposes. It will provide an important solution to the sustainable use of *D. moniliforme*.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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