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Micropropagation of *Dendrobium signatum* Rchb.f.

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ABSTRACT

A study was conducted to elucidate the effects of using different media (MS, ½MS, VW) supplemented with different combinations of 15% coconut water, 10% potato extract and 5% mashed banana with an extra 0.2% activated charcoal on seed germination of *Dendrobium signatum* Rchb.f. in *in vitro* cultures. The results showed that the most suitable media, providing 100% seed germination, were MS added to 10% potato extract, ½MS supplemented with 10% potato extract and MS added to 5% mashed banana. Young plantlets were then transferred to MS and ½MS medium supplemented with various kinds of cytokinin including BA, Kinetin and TDZ at the concentrations of 0, 1 and 2 mg/l, and combined with 0 and 0.5 mg/l of NAA for plantlet development. The results showed that young shoots could be differentiated and regenerated into plantlets in all the treatments. The results revealed that the most suitable medium for shoot proliferation and root induction was ½MS medium with 2 mg/l BA added and combined with 0.5 mg/l NAA.

Keywords: *Dendrobium*, *in vitro* culture, cytokinin, seed germination, organic supplements

INTRODUCTION

Dendrobium signatum Rchb.f. belongs to the genus *Dendrobium*, which is the largest genus of orchid species in Thailand (Seidenfaden, 1985). Genus *Dendrobium* is also one of the most well attended orchids for trade production because of the enormous increase in demand for cut flowers and pot plants over the years (Peyachoknagul et al., 2014). It was reported that many *Dendrobium* species have antidiabetic, anti-cancer and anti-pyretic properties (Pant, 2013). Some phytochemical compounds found in *Dendrobium* are alkaloids, flavonoids, sesquiterpenoids as well as pigments (Singh et al., 2012; Attri, 2016). *D.*

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signatum Rchb.f. is a sympodial epiphytic wild orchid. Its local names are '*Ueang kham kew*' or '*Ueang tin ped*', and it has a geographical distribution in tropical forests ranging from elevations of 200 to 1200 metres and are mostly found in North and Northeast Thailand. The characteristics of the stem are a yellow color, succulence, a diameter of 1.5-2 cm and a height of 30-50 cm. The leaves are also succulent and lanceolate. The leaves grow alternately over the whole length of the stem. The *D. signatum* Rchb.f. blooms in the winter through early summer (approximately March to May) with a short inflorescence positioned on a mature leafless cane towards the apex with two flowers. They are fragrant and long-living (Thaitong, 2005). Since *Dendrobium* is popular in the ornamental market, this species has been collected from the forest with little regard for sustainability. This has initiated the extinction of the *Dendrobium* species (Zhang et al., 2013).

Other causes of the extinction of *Dendrobium* are seed germination and seedling development. In nature, orchid seeds do not have endosperm to support seed germination. Thus, orchid seed germination requires the presence of mycorrhizal fungi, classified as Ascomycota or Basidiomycota, which infect and supply nutrition essential for orchid seed germination (Behie & Bidochka, 2014). In the process of seedling development, the number of seedlings is limited because many protocorms and seedlings are destroyed by pests and aggressive fungi. The reduction of wild orchids has been subsequently increased and this can lead to the extinction of the species in the near future. Therefore, asymbiotic seed germination by tissue culture is essential.

In previous studies, the careful selection of media, cytokinins, auxins and natural supplements and their optimisation have been reported to be the most important factors in orchid propagation (Malabadi et al., 2005; Luo et al., 2009; Parthibhan et al., 2015). The aims of this study were to obtain *in vitro* seed germination and regeneration of *D. signatum* Rchb.f. through optimisation of their aseptic culture conditions. The objectives were: (1) to determine the suitable media for seed germination and regeneration of *D. signatum* Rchb.f., and (2) to determine the optimum concentrations of the organic form of cytokinin and NAA.

MATERIALS AND METHOD

Effect of Different Media and Organic Supplements on Seed Germination

The mature capsules of *D. signatum* Rchb.f. were collected six months after pollination. Capsules were then surface sterilised by dipping in 70% ethyl alcohol and flamed immediately four to five times in laminar air flow. The capsules were then cut longitudinally in a sterilised petri dish. Seeds were scraped from the capsule, mixed with sterile water and pipetted into 200 µl tubes and then cultured on the surface of the medium. Three different basal media were used in the whole experiment consisting of MS (Murashige & Skoog,

1962), ½MS (half strength of MS) and VW (Vacin & Went, 1949) supplemented with several organic ingredients including 15% coconut water, 10% potato extract and 5% mashed banana. *In vitro* cultured seeds were then kept at 25± 2°C under a photoperiod of 16 h light/8 h dark. The percentage of orchid seed germination was obtained by estimating the surface area of seed germination in the tissue culture bottle with a diameter of 4.5 cm. The total surface area of the tissue culture bottle was defined as 100%. After cultivation for eight weeks, the percentage of seed germination was recorded. Observations on the percentage germination of seeds, the number of leaves, length of leaves and number of roots were recorded 16 weeks after culture.

Effects of Different Cytokinin and NAA on Shoot Proliferation and Root Induction

The experiment was performed using young *in vitro* seedlings of *D. signatum* Rchb.f. of approximately 1 cm. height at age 16 weeks. Single plantlets were cultured on MS and ½MS media containing 0, 1 and 2 mg/l of benzyladenine (BA), 6-furfuryl aminopurine (Kinetin) and thidiazuron (TDZ) in combination with 0 and 0.5 mg/l of naphthaleneacetic acid (NAA). Culture conditions were the same as previously. After three months of culture, morphogenetic response to the treatments was evaluated in terms of percentage proliferation of shoot, height of shoot, number of leaves, number of roots and length of root.

All the experiments were set up in completely randomised design (CRD). Each treatment consisted of 10 replicates. The difference among the treatment means was compared based on Duncan's multiple range test (DMRT) analysis.

RESULTS AND DISCUSSION

Effect of Different Media and Organic Supplements on Seed Germination

The effect of different media and organic supplements on seed germination of *D. signatum* Rchb.f. was observed at 60 days after culture on a seed germination medium. It was observed that seeds taken from immature capsules had germinated and green protocorms had formed after culture for eight weeks. (Table 1). Figure 1 shows seed germination of *D. signatum* Rchb.f. cultured in various media supplemented with several organic ingredients after culture for 16 weeks. The most suitable media, providing 100% seed germination, were MS added to 10% potato, ½MS supplemented with 10% potato, and MS added to 5% banana. Naturally, orchid seeds have a poor germination rate because of the small size of the seeds and the lack of cotyledons and endosperm (Maneerattanarungroj, 2007) that contain food reserves in the form of starch grains, oil droplets and small amounts of proteins (Thomas & Michael, 2007). This study found that MS and ½MS were more suitable for seed germination than VW basal media. Miransari and Smith (2014) reported that seeds consume larger amounts of nitrogen

during seed germination. Therefore, the amount of nitrogen in MS and ½MS can influence seed germination of orchids.

Combinations of organic supplements in the medium enhanced seed germination. The best medium for seed germination in treatment was 10% potato extract. Potato extract consists of carbohydrates, amino acids, important vitamins (C, B1, B6) and mineral elements (potassium, iron, magnesium) (Molnár, Virág, & Ördög, 2011). In the *in vitro* culture, potato had useful effects on some orchid species such as *Phalaenopsis* and *Doritaenopsi*s (Thorpe, Stasolla, Yeung, de Klerk, Roberts, & George, 2008). A study of the effect of seedling media or nutrients added to coconut water for orchid growth found that the nutrients could activate low-level seed germination compared to other organic supplements. The composition of coconut water (CW) includes inorganic ions (e.g. phosphorus, potassium), nitrogenous compounds, amino acids, related substances (e.g. alanine, glutamic acid, lysine), enzymes, vitamins and sugar (Sandoval Prando et al., 2014). All these compounds may be the reason for seed germination in orchids (Arditti, 2008). Vijayakumar et al. (2012) reported that MS medium added with 3% sucrose 1.5 mg/l BA and 15% CW showed a higher rate of seed germination of *D. aggregatum*. Banana is frequently used to influence orchid *in vitro*. This study found that MS added to 5% mashed banana could increase the highest percentage for seed germination of *D. sigmnatum* Rchb.f. but 100% germination as a result of media added to mashed banana was observed only in full MS media. Many reports indicated that mashed banana combined with a cultured medium could improve the growth of orchid seedlings. The effect of mashed banana on seed germination in *D. sigmnatum* Rchb.f is similar to other plants such as in *D. wangliangii* and *D. strongylanthum* Rchb.f. (Zhao et al., 2013; Kong et al., 2007)

Table 1
Effect of different media and organic supplements on percentage of seed germination, average values of the number of leaves, leaf length and number of the roots of D. signatum *Rchb.f.*

Medium	**Seed Germination* (%)**	**Average Number of Leaves****	**Average Leaf Length ** (cm)**	**Average Number of the Roots****	**Average Root Length** (cm)**
MS	50 ± 0^{b}	3 ± 2^{bc}	0.3 ± 0.07^{ab}	1 ± 0^{ab}	0.5 ± 0.28^{de}
MS + 15% coconut water	25 ± 0^{a}	3 ± 1.73^{bc}	0.3 ± 0.23^{ab}	1 ± 0.71^{ab}	0.1 ± 0^{ab}
MS + 10% potato extract	100 ± 0^{d}	5 ± 1.22^{c}	0.9 ± 0.24^{d}	4 ± 2.00^{d}	0.3 ± 1^{bc}
MS + 5% mashed banana	100 ± 0^{d}	4 ± 1.22^{c}	0.5 ± 0.20^{bc}	4 ± 0^{d}	0.6 ± 0.12^{ef}

Table 1 (*continue*)

Medium	Seed Germination* (%)	Average Number of Leaves**	Average Leaf Length ** (cm)	Average Number of the Roots**	Average Root Length** (cm)
½MS	50 ±30.62^{b}	3 ± 1.87^{bc}	0.4 ± 0.20^{bc}	1 ± 0^{ab}	0.2 ± 0.14^{b}
½MS + 15% coconut water	25 ± 17.68^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
½MS + 10% potato extract	100 ± 0^{d}	4 ± 1.58^{c}	0.4 ± 0.20^{bc}	3 ± 1.41^{cd}	0.7 ± 0.14^{f}
½MS + 5% mashed banana	75 ± 30.62^{c}	5 ± 1^{c}	0.7 ± 0.37^{cd}	4 ± 0^{d}	0.5 ± 0.17^{de}
VW	50 ± 17.68^{b}	4 ± 1.58^{c}	0.3 ± 0.12^{ab}	1 ± 0^{ab}	0.3 ± 1^{bc}
VW + 15% coconut water	25 ± 0^{a}	2 ± 0^{b}	0.3 ± 0.21^{ab}	1 ± 0^{ab}	0.1 ± 0.07^{ab}
VW + 10% potato extract	75 ± 25.00^{c}	5 ± 1.00^{bc}	0.5 ± 0.23^{bc}	3 ± 0^{cd}	0.2 ± 0.14^{b}
VW + 5% mashed banana	75 ± 0^{c}	3 ± 1.00^{bc}	0.5 ± 0.16^{bc}	2 ± 0^{bc}	0.4 ± 0.16^{cb}

Means followed by the same letter within each column are not significantly different using Duncan's multiple range test at $p<0.05$

* Percentage of seed germination after culture for eight weeks.

** Data were recorded after culture for 16 weeks.

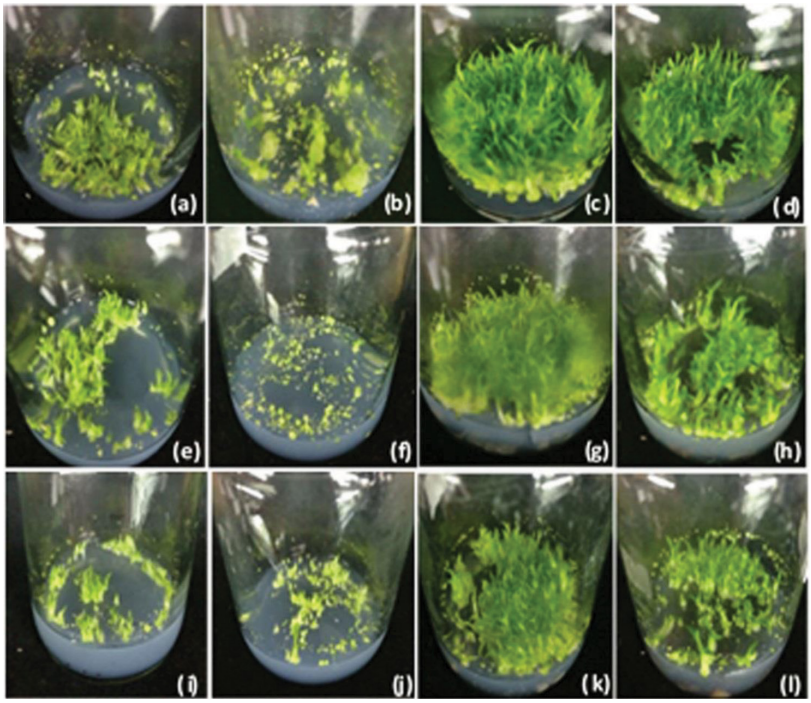

Figure 1. Seed germination of *D. signatum* Rchb.f. cultured in various media supplemented with several organic supplements, (a) MS, (b) MS + 15% coconut water, (c) MS + 10% potato extract, (d) MS + 5% mashed banana, (e) ½MS, (f) ½MS + 15% coconut water, (g) ½MS + 10% potato extract, (h) ½MS + 5% mashed banana, (i) VW, (j) VW + 15% coconut water, (k) VW + 10% potato extract, (l) VW + 5% mashed banana, after culture for 16 weeks

The Effect of Different Cytokinin and NAA on Shoot Proliferation and Root Induction

The results of the study on the effect of different cytokinin and NAA on shoot and root proliferation of the orchid are shown in Figure 2 and Table 2. The combination of cytokinin and auxin promoted the growth of shoots and roots and the vigorous growth of *D. signatum* Rchb.f. The results also showed that young shoots could be differentiated and regenerated into plantlets in all the treatments. The results revealed that the most suitable medium for plantlet development was ½MS medium supplemented with 2 mg/l BA combined with 0.5 mg/l NAA (Table 2). The synthetic kinetin (6-furfuryl aminopurine), benzyladenine (*N*6-benzylaminopurine, *N*6-benzyladenine, BA, BAP), dimethyl aminopurine (DMAP), thidiazuron (TDZ), and the naturally occurring zeatin are used most commonly in orchid culture media (Arditti, 2008). This study showed that the addition of 2 mg/l BA to 0.5 mg/l NAA could induce shoot proliferation and root induction of *D. signatum* Rchb.f. The beneficial effects of cytokinin in promoting the highest shoot proliferation in the *Dendrobium* hybrids, Sonia 17 and 28, which were cultured in half-strength Murashige and Skoog (MS) medium supplemented with 44.4 µM BA (Martin & Madassery, 2006) have been noted in previous studies. In *Dendrobium candidum,* the most suitable for callus induction was MS medium with half-strength macronutrients and full-strength micronutrients combined with 2 mg/l BA and 0.5 mg/L NAA (Zhao et al., 2007). In mass propagation of *Dendrobium* 'Zahra FR 62' half-strength MS medium containing 1 mg/l thidiazuron (TDZ) and 0.5 mg/l N6-benzyladenine (BA) were used, and this resulted in a high protocorm-like body (PLB) (Winarto et al., 2013). As a result of this study, the most commonly used auxins in orchid tissue culture media are the naturally occurring auxin, indoleacetic acid (IAA), synthetic naphthaleneacetic acid (NAA), indolebutyric acid (IBA) and 2,4-dichlorophenoxyacetic acid (2,4-D) (Arditti, 2008). In the present study, 0.5 mg/l NAA combined with 2 mg/l BA applied to the medium induced the highest average of shoot height, number of leaves, number of roots and root length. Some reports explained that the application of a single regulator also influenced shoot proliferation. Sujjaritthurakarn and Kanchanpoom (2011) reported that the highest percentage for PLB induction and the highest number of PLBs per protocorm of dwarf *Dendrobium* were derived from using modified Murashige and Skoog (MS) liquid medium supplemented with 18 µM TDZ.

Table 2
Effect of cytokinin supplemented with naa for percentage of shoot proliferation, shoot regeneration, average of shoot height, number of leaves, number of roots and root length of D. signatum *Rchb.f. cultured for three months*

Number of Medium	Medium	Plant Growth Regulators (mg/l)				Shoot Proliferation (%)	Average of Shoot Height (cm.)	Average Number of Leaves	Average Number of Roots	Average Root Length (cm.)
		BA	**Kinetin**	**TDZ**	**NAA**					
M1	MS	-	-	-	-	33 ± 8.33^{abcd}	2.0 ± 0.15^{bcde}	2.3 ± 0.33^{abc}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}
M2	MS	1.0	-	-	-	25 ± 14.43^{abcd}	1.3 ± 0.23^{ab}	2.0 ± 0.58^{abc}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}
M3	MS	2.0	-	-	-	8 ± 8.33^{ab}	1.4 ± 0.12^{abc}	3.0 ± 1.00^{abcde}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}
M4	MS	-	1.0	-	-	33 ± 22.04^{abcd}	1.7 ± 0.28^{abcde}	4.0 ± 0.58^{abcde}	2.3 ± 2.33^{a}	0.1 ± 0.13^{a}
M5	MS	-	2.0	-	-	0 ± 0.00^{a}	2.0 ± 0.15^{bcde}	3.0 ± 0.58^{abcde}	0.7 ± 0.67^{a}	0.7 ± 0.67^{a}
M6	MS	-	-	1.0	-	17 ± 16.67^{abc}	1.8 ± 0.19^{abcde}	4.3 ± 0.33^{abcde}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}
M7	MS	-	-	2.0	-	42 ± 16.67^{abcde}	1.8 ± 0.32^{abcde}	4.3 ± 1.20^{bcde}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}
M8	MS	-	-	-	0.5	17 ± 16.67^{abc}	1.9 ± 0.09^{bcde}	4.3 ± 0.88^{bcde}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}
M9	MS	1.0	-	-	0.5	42 ± 16.67^{abcde}	1.8 ± 0.39^{abcde}	4.0 ± 2.00^{bcde}	0.3 ± 0.33^{a}	0.0 ± 0.03^{a}
M10	MS	2.0	-	-	0.5	58 ± 22.04^{cde}	1.6 ± 0.20^{abcde}	5.3 ± 1.20^{de}	0.3 ± 0.33^{a}	0.1 ± 0.07^{a}
M11	MS	-	1.0	-	0.5	25 ± 0.00^{abcd}	1.5 ± 0.09^{abcd}	3.7 ± 0.33^{abcde}	1.7 ± 0.88^{a}	0.1 ± 0.06^{a}
M12	MS	-	2.0	-	0.5	17 ± 8.33^{abc}	1.4 ± 0.15^{ab}	2.0 ± 0.00^{abc}	0.0 ± 0.00^{a}	0.0 ± 0.0^{a}
M13	MS	-	-	1.0	0.5	42 ± 8.33^{abcde}	2.1 ± 0.26^{cde}	3.3 ± 0.33^{abcde}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}
M14	MS	-	-	2.0	0.5	58 ± 8.33^{cde}	2.1 ± 0.27^{bcde}	4.7 ± 0.33^{cde}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}
M15	½MS	-	-	-	-	67 ± 8.33^{de}	1.5 ± 0.15^{abcd}	2.0 ± 0.58^{abc}	1.7 ± 1.67^{a}	0.0 ± 0.01^{a}
M16	½MS	1.0	-	-	-	33 ± 22.04^{abcd}	1.9 ± 0.19^{bcde}	2.7 ± 0.33^{abcd}	1.0 ± 1.00^{a}	0.2 ± 0.20^{a}
M17	½MS	2.0	-	-	-	58 ± 16.67^{cde}	1.9 ± 0.25^{abcde}	2.7 ± 0.67^{abcd}	0.3 ± 0.33^{a}	0.2 ± 0.02^{a}
M18	½MS	-	1.0	-	-	25 ± 0.00^{abcd}	1.7 ± 0.12^{abcde}	3.7 ± 0.66^{abcde}	2.3 ± 1.20^{ab}	0.2 ± 0.12^{a}
M19	½MS	-	2.0	-	-	58 ± 22.04^{cde}	2.4 ± 0.31^{e}	4.0 ± 0.58^{abcde}	1.7 ± 0.33^{a}	0.3 ± 0.03^{ab}
M20	½MS	-	-	1.0	-	42 ± 16.67^{abcde}	1.9 ± 0.12^{abcde}	3.7 ± 0.33^{abcde}	1.0 ± 1.00^{a}	0.2 ± 0.20^{a}
M21	½MS	-	-	2.0	-	0 ± 0.00^{a}	1.7 ± 0.38^{abcde}	2.7 ± 1.20^{abcd}	1.3 ± 1.33^{a}	0.7 ± 0.07^{a}
M22	½MS	-	-	-	0.5	25 ± 14.43^{abcd}	1.2 ± 0.07^{a}	1.3 ± 0.33^{a}	0.3 ± 0.33^{a}	0.1 ± 0.10^{a}
M23	½MS	1.0	-	-	0.5	33 ± 8.33^{abcd}	1.4 ± 0.17^{abc}	1.7 ± 0.33^{ab}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}
M24	½MS	2.0	-	-	0.5	58 ± 22.05^{cde}	3.2 ± 0.17^{f}	5.7 ± 1.77^{e}	5.3 ± 2.02^{b}	0.7 ± 0.19^{b}
M25	½MS	-	1.0	-	0.5	42 ± 8.33^{abcde}	2.3 ± 0.32^{e}	4.3 ± 0.88^{bcde}	3.0 ± 2.08^{bc}	0.2 ± 0.10^{a}
M26	½MS	-	2.0	-	0.5	50 ± 0.00^{bcde}	1.4 ± 0.09^{ab}	3.0 ± 0.00^{abcde}	2.0 ± 0.00^{a}	0.0 ± 0.00^{a}
M27	½MS	-	-	1.0	0.5	83 ± 8.33^{e}	1.9 ± 0.12^{bcde}	5.3 ± 0.00^{de}	2.0 ± 1.15^{a}	0.2 ± 0.12^{a}
M28	½MS	-	-	2.0	0.5	67 ± 8.33^{de}	2.2 ± 0.12^{de}	5.3 ± 0.33^{abcd}	0.7 ± 0.67^{a}	0.2 ± 0.23^{a}

Means followed by the same letter within each column are not significantly different using Duncan's multiple range test at $p<0.05$

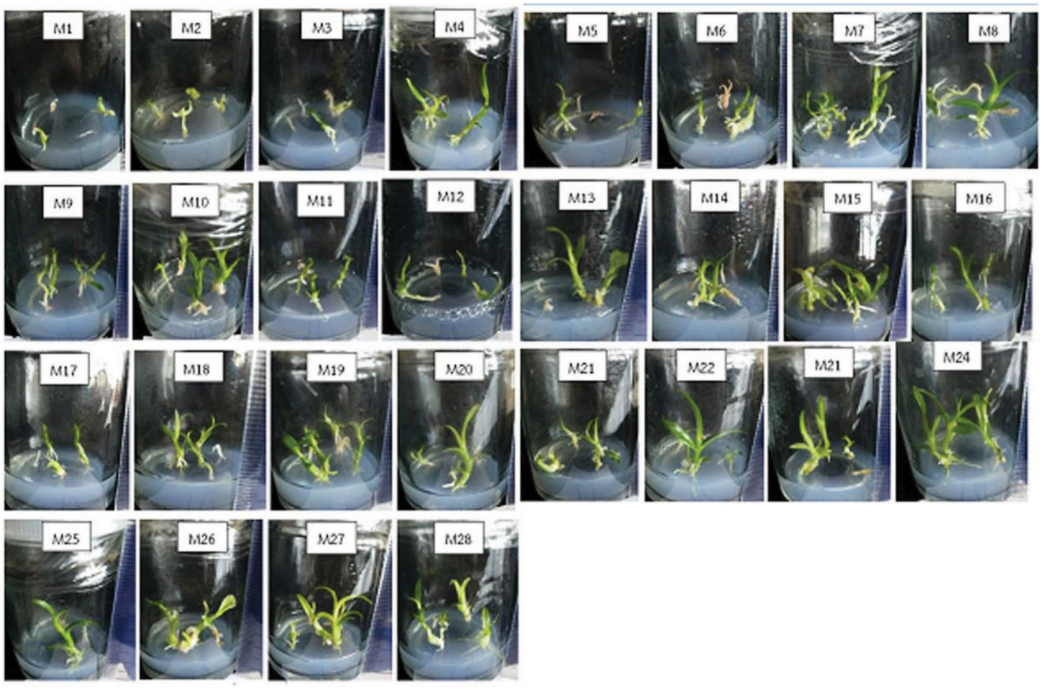

Figure 2. Shoot regeneration of *D. signatum* when cultured in MS and ½MS medium supplemented with BA, Kinetin and TDZ at 0, 1 and 2 mg/l, and 0 and 0.5 mg/l of NAA for three months

M1 = MS
M2 = MS + 1 mg/l BA
M3 = MS + 2 mg/l BA
M4 = MS + 1 mg/l Kinetin
M5 = MS + 2 mg/l Kinetin
M6 = MS + 1 mg/l TDZ
M7 = MS + 2 mg/l TDZ
M8 = MS + 0.5 mg/l NAA
M9 = MS + 1 mg/l BA + 0.5 mg/l NAA
M10 = MS + 2 mg/l BA + 0.5 mg/l NAA
M11 = MS + 1 mg/l Kinetin + 0.5 mg/l NAA
M12 = MS + 2 mg/l Kinetin + 0.5 mg/l NAA
M13 = MS + 1 mg/l TDZ + 0.5 mg/l NAA
M14 = MS + 2 mg/l TDZ + 0.5 mg/l NAA

M15 = ½MS
M16 = ½MS + 1 mg/l BA
M17 = ½MS + 2 mg/l BA
M18 = ½MS + 1 mg/l Kinetin
M19 = ½MS + 2 mg/l Kinetin
M20 = ½MS + 1 mg/l TDZ
M21 = ½MS + 2 mg/l TDZ
M22 = ½MS + 0.5 mg/l NAA
M23 = ½MS + 1 mg/l BA + 0.5 mg/l NAA
M24 = ½MS + 2 mg/l BA + 0.5 mg/l NAA
M25 = ½MS + 1 mg/l Kinetin + 0.5 mg/l NAA
M26 = ½MS + 2 mg/l Kinetin + 0.5 mg/l NAA
M27 = ½MS + 1 mg/l TDZ + 0.5 mg/l NAA
M28 = ½MS + 2 mg/l TDZ + 0.5 mg/l NAA

CONCLUSION

MS added to 10% potato extract, ½MS supplemented with 10% potato extract and MS added to 5% mashed banana can improve seed germination. In addition, the most suitable medium for improving shoot proliferation observed in this study was the ½MS medium added to 2 mg/l BA combined with 0.5 mg/l NAA. However, there was no significant difference in percentage of shoot proliferation and average number of leaves between media with and without plant growth regulators. Therefore, to save cost, some would choose to use MS media without plant growth regulators or use them at lower concentrations. As mentioned above, this experiment is simple and efficient and provides mass propagation in a short period of time as well as natural conservation of a rare orchid species.

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