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Effect of Cytokinins (BAP and TDZ) and Auxin (2,4-D) on Growth and Development of *Paphiopedilum callosum*

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ABSTRACT

Paphiopedilum callosum seedlings were grown on half strength macro- and micro-elements of Murashige and Skoog (1962) ($^1/_2$ MS) medium supplemented with cytokinins 6-benzylaminopurine (BAP) and 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ) alone or in combination with auxin, 2,4-dichlorophenoxyacetic acid (2,4-D). Three months after incubation, shoots in the medium with 0.5 μM TDZ produced 1.6 ± 0.40 shoots/explant. In addition, it was found that $^1/_2$ MS medium without plant growth regulators produced more roots (1.80 ± 0.20) per shoot and longer roots (30.40 ± 8.04 mm) than $^1/_2$ MS with 0.5 μM TDZ combined with 50 μM 2,4-D. The $^1/_2$ MS medium with BAP at 10 and 50 μM resulted in 2.30 ± 1.42 and 2.20 ± 1.03 shoots/explant, while $^1/_2$ MS medium without plant growth regulators resulted in 100% root induction with an average 3.70 ± 0.62 roots/explant and mean root length 34.01 ± 4.87 mm. Overall, BAP appeared to elicit the best shoot multiplication in response with *P. callosum* shoot explants compared with either 2,4-D or TDZ. The combined effects of TDZ and BAP may be worthwhile investigating in future shoot proliferation experiments. Root induction appeared to be restorable if TDZ is removed and BAP reduced, as the presence of BAP at the lower concentrations tested did not appear to completely inhibit root induction (unlike TDZ). Hopefully, the current study will assist with future development of *ex situ* conservation methods with endangered Thai orchids.

Keywords: *Paphiopedilum callosum*, *in vitro* culture, TDZ, 2, 4-D

INTRODUCTION

Orchids are some of the most important ornamental plants in Thailand. They exhibit an incredible range of diversity in size, shape and flower colour. At present, many species are endangered due to a variety of factors, including deforestation for agriculture and biofuels, over-harvesting to service the wild orchid trade and potentially climate change. Therefore, there is an urgent need for orchid conservation in Thailand to reduce the danger of local extinction of some species and an overall loss of biodiversity in general.

The genus *Paphiopedilum* (slipper orchids), subtribe *Paphiopedilum*, is native to Tropical Asia and its members are clearly distinguishable from others by the leathery,

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sometimes mottled, foliage which persists for more than one season and the unique 'slipper-like' flower form (hence the common epithet). There are approximately 100 species of *Paphiopedilum* ranging from Sri Lanka to Hong Kong through India, Malaysia, Borneo, Thailand, Java Myanmar, China, New Guinea and the Philippines (Cribb, 1987). Most species are terrestrials, but there are a few lithophytes. Leaves are closely set at ground level and are either tessellated and alternately dark and pale green, or in some cases predominantly yellow-green. The sympodial growth produces new pairs of leaves each season, resulting in dense leaf formation. A single terminal flower stem arises from the center of the leaves bearing one flower, and rarely, two or more (Cho and Valmayor, 1988; Mark, 1988) (Figure 1).

The use of micropropagation techniques has revolutionized the commercial orchid industry. Orchids are one of the few flowering plants of commercial value to be mass propagated *in vitro* both through seed and tissue culture (Morel, 1974; Bajaj, 1992; Polthampitak, 1997). Aseptic germination of seed is the most commonly utilized *in vitro* method, as orchid seeds have no endosperm, no cotyledons and no root initials and do not have the capacity to directly utilize substrates available in nature (Savina, 1974; Stewart and Button, 1975; Singh, 1981; Sheehan, 1983; Singh, 1988).

The aim of this research was to establish efficient *in vitro* propagation techniques for Thai slipper orchid conservation, based on investigation of a range of plant growth regulators (PGRs) for maximizing shoot multiplication from seedling explants incubated on defined basal nutrient media.

MATERIALS AND METHODS

Explant type

Flasks of aseptically germinated seedlings of *Paphiopedilum callosum* were obtained from a commercial orchid company (http://www.paphanaticsunlimited.wordpress.com). Each flask contained up to 15 plants and each plant consisted of a single, shoot 10-25 mm in length. Plants were removed from the flasks and replated on MS basal medium (Murashige and Skoog, 1962) with 60 mM sucrose, 555 μM myo-

Figure 1 a) Stem, b) flower, c) pod, and d) seed of *Paphiopedilum callosum*.

inositol, 4 μM nicotinic acid, 2.5 μM pyridoxine HCL, 1 μM thiamine HCL, pH 6, 0.7 % (w/v) agar) without hormone supplements. All cultures were incubated under a 16/8-hour photoperiod at 30 μmol $m^{-2}s^{-1}$ (white fluorescent lamps; Philips TLD 36W/54) at 25 ± 2°C for 1 month before use in the following experiments.

Nutrient media

Experiment 1**:** Seedling explants were placed on half strength macro- and micro-elements of Murashige and Skoog (1962) ($^1/_2$ MS) media supplemented with 100 μM myo-inositol, 0.5 μM nicotinic acid, 2 μM glycine, 0.5 μM pyridoxine HCL, 0.1 μM thiamine HCL, 1,000 μM peptone, 170 μM NaH_2PO_4, 20 g/L sucrose, 2.5 g/L phytagel. PGRs were added as various combinations of auxin and cytokinin concentration, 0 (control treatment) and 0.5 μM 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ) combined with 2,4-dichlorophenoxyacetic acid (2,4-D) at 0, 5 and 50 μM. The media were adjusted to pH 6. Explants were incubated in darkness at 25 ± 2°C for a 3-month period with the aim of inducing callus tissue and/or regeneration of adventitious shoots. Explant growth/regeneration was assessed after the 3-month incubation period.

Experiment 2: Explants were placed on $^1/_2$ MS media supplemented with 100 μM myo-inositol, 0.5 μM nicotinic acid, 2 μM glycine, 0.5 μM pyridoxine HCl, 0.1 μM thiamine HCl , 20 g/l sucrose, 2.5 g/l phytagel and the plant growth regulator BAP was added at 0 (control), 10, 20, 50 and 100 μM. The media were adjusted to pH 6. Explants were incubated under a 16/8-hour photoperiod at 30 m mol $m^{-2}s^{-1}$ (white fluorescent lamps; Philips TLD 36W/54) at 25±2°C. Shoot multiplication was assessed after incubation for a 3-month period.

Experimental design and data analysis

Experiments were based on a complete randomized design (CRD) with eight replicates per treatment. Statistical analysis was provided by ANOVA with treatment means separated using Duncan's multiple range test (DMRT) with significance determined at the 5% level ($p < 0.05$) (Duncan, 1955).

RESULTS AND DISCUSSION

Paphiopedilum callosum seedlings cultured on $^1/_2$ MS medium supplemented with 0 and 0.5 μM TDZ combined with 0, 5 and 50 μM 2,4-D revealed that multiplication of shoots was very slow on all treatments. Neither callus production nor regeneration of adventitious shoots was forthcoming. After a 3-month period, the medium treatment with 0.5 μM TDZ had the highest shoot number (1.6 ± 0.40 shoots/explant), however this was not significantly different from other medium treatments including the control. The length of shoots, although exhibiting some empirical differences between some treatments, was highly variable, but all TDZ-treatments were not significantly different from the control, despite the control treatment having the greatest mean shoot length of 37.00 ± 4.36 mm (Table 1, Figure. 2).

While the control treatment ($^1/_2$ MS media without PGR) induced 1.80 ± 0.20 roots per shoot with 30.40 ± 8.04 mm length (Table 2, Figure 2), media with TDZ or combined with 2,4-D completely inhibited root growth during the 3-month incubation period.

P. callosum seedling cultured on modified $^1/_2$ MS media supplemented with 0 (control), 10, 20, 50, and 100 μM BAP exhibited increased shoot multiplication compared with the control treatment, (Table 3, Figure 3). After 90 d of incubation, the results showed that media supplemented with BAP 10 or 50 μM produced the highest number of shoots per explant (2.3 ± 0.45 and 2.2 ± 0.33, respectively) and these were significantly higher than the control treatment (1.0

± 0.0). This finding is similar to those of Long *et al*. (2010) showing a relatively high N6-benzyladenine (BA) concentration increased the shoot number in *P. villosum* var. *densissimum* and *P. armeniacum*. The 100 μM BAP treatment produced significantly fewer shoots per explant (1.40 ± 0.16) than the 10 μM BAP treatment. It would appear that 100 μM BAP may be supra-optimal. Shoot length was highest in the control treatment, but there were no significant differences among treatments. (Table 3, Figure 3).

Media with only BAP did not completely inhibit root development except at the highest concentration of 100 μM. The control treatment ($^1/_2$ MS medium minus PGR) exhibited root lengths averaging 34.01 mm, compared to significantly shorter roots on media with BA at 10, 20 or 50 μM (Table 4, Figure 3).

Orchids are typically propagated from seed or seedling explants and slipper orchids are no exception (Flamee, 1978; Tay *et al*., 1988; Tochareon, 1996; Chen *et al*., 2002; Chang *et al*., 2005). In the present study, tissue cultured shoots of *P. callosum* were utilized to test the efficacy of TDZ and 2,4-D on enhancement of callus induction and/or adventitious shoot regeneration from explants and to investigate the effects of BAP on multiple shoot proliferation. The results suggested that explants were more responsive to PGR stimulation, with 10 μM BAP producing a better than two-fold shoot multiplication after a 90-day incubation period. Increasing the concentration of BAP beyond 10 μM would appear to have no particular advantage based on the results of the present study. The results with TDZ and 2,4-D or a combination of both indicated that shoot explants were not responsive to the medium or to high auxin:cytokinin ratios with these two

Table 1 Average shoot number and shoot length of *P. callosum* shoots cultured on $^1/_2$ MS media supplemented with TDZ and 2,4-D for a 3-month period.

2,4-D (μM)	TDZ (μM)	Number of shoots/explant ± standard error	Shoot length (mm) ± standard error
0	0	1.00 ± 0.00	37.00 ± 4.36
0	0.5	1.60 ± 0.40	28.00 ± 5.83
5	0	1.00± 0.00	20.00 ± 3.54
5	0.5	1.40 ± 0.40	24.40 ± 3.98
50	0	1.00 ± 0.00	21.00 ± 4.00
50	0.5	1.00 ± 0.00	21.00 ± 2.45

Means followed by different letters within a column are not significantly different at $p < 0.05$ (Duncan, 1955).

Table 2 Average root number/shoot and root length of *P. callosum* shoots after culture on $^1/_2$ MS media supplemented with TDZ and 2,4-D for a 3-month period.

2,4-D (μM)	TDZ (μM)	Number of roots/shoot ± standard error	Root length (mm) ± standard error
0	0	1.80 ± 0.20 a	30.40 ± 8.04 a
0	0.5	0.00 ± 0.00 b	0.00 ± 0.00 b
5	0	0.00 ± 0.00 b	0.00 ± 0.00 b
5	0.5	0.00 ± 0.00 b	0.00 ± 0.00 b
50	0	0.00 ± 0.00 b	0.00 ± 0.00 b
50	0.5	0.00 ± 0.00 b	0.00 ± 0.00 b

Means followed by different letters within a column are significantly different at $p < 0.05$ (Duncan, 1955).

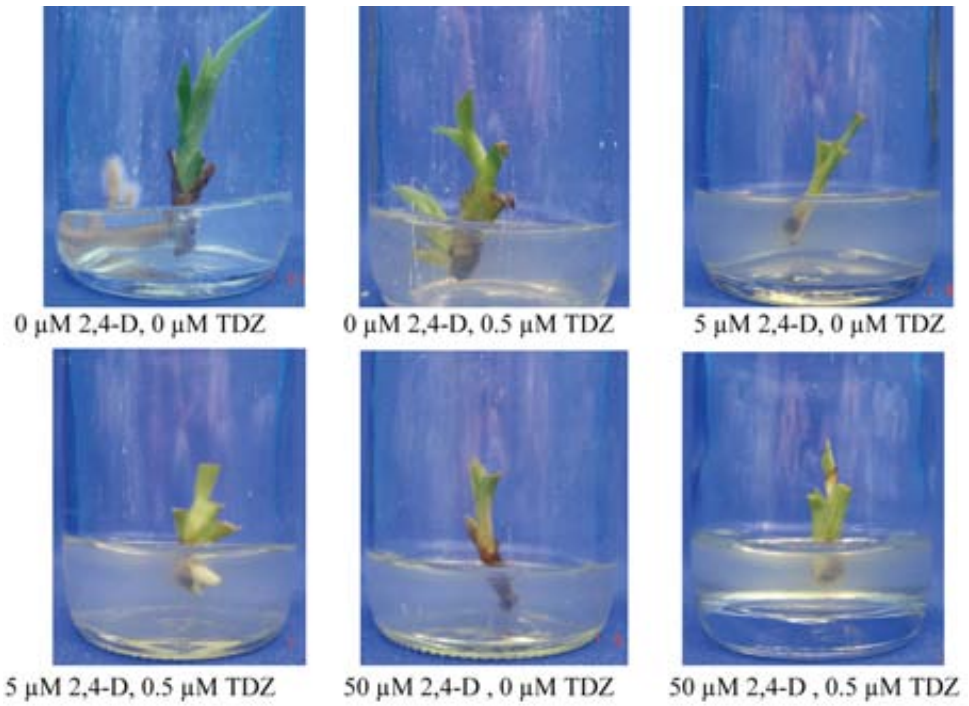

Figure 2 Shoots of *P. callosum* cultured for a 3-month period on $^1/_2$ MS media supplemented with 0 and 0.5 µM TDZ combined with 0, 5 and 50 µM 2,4-D.

Table 3 Average shoot number and shoot length of *P. callosum* of shoot explants cultured for a 3-month period on $^1/_2$ MS medium supplemented with BAP (0-100 µM).

BAP (µM)	Number of shoots/explant ± standard error	Shoot length (mm) ± standard error
0	1.00 ± 0.00 c	24.00 ± 5.62
10	2.30 ± 0.45 a	21.25 ± 4.72
20	1.50 ± 0.22 abc	21.05 ± 2.39
50	2.20 ± 0.33 ab	18.29 ± 2.11
100	1.40 ± 0.16 bc	20.50 ± 1.89

Means followed by different letters within a column are significantly different at $p < 0.05$ (Duncan, 1955).

Table 4 Average number of roots, root length and percentage root induction of *P. callosum* shoots after culture for a 3-month period on $^1/_2$ MS media supplemented with BAP (0-100 µM).

BAP (µM)	Number of roots/shoot ± standard error	Root length (mm) ± standard error
0	3.70 ± 0.62 a	34.01 ± 4.87 a
10	0.80 ± 0.29 b	9.25 ± 3.61 b
20	0.70 ± 0.42 b	6.50 ± 3.80 b
50	0.10 ± 0.10 b	1.00 ± 1.00 b
100	0.00 ± 0.00 b	0.00 ± 0.00 b

Means followed by different letters within a column are significantly different at $p < 0.05$ (Duncan, 1955).

normally highly phyto-active PGRs. Huang *et al.* (2001) also reported that TDZ inhibited shoot proliferation and rooting in *Paphiopedilum*. However, Chen *et al.* (2002) reported that either TDZ alone or in combination with 2,4-D were effective in multiple shoot induction from stem nodal explants of *Paphiopedilum*.

The implications for future work are that BAP appears to elicit the best shoot multiplication response with *P. callosum* shoot explants. It is suggested that it may be worthwhile investigating the combined effects of TDZ and BAP (perhaps beginning with 10 μM BAP ± 0.5 μM TDZ) in future shoot proliferation experiments, in order to see whether this combination enhances shoot multiplication. Root induction should be restorable if TDZ is removed and BAP reduced (as the presence of BAP does not appear to completely inhibit root induction, unlike TDZ).

CONCLUSION

This study investigated *in vitro* propagation techniques with *Paphiopedalum callosum* to assist with the conservation of endangered Thai slipper orchids. *P. callosum* seedling explants produced more shoots when cultured for a 3-month period on $^1/_2$ MS medium supplemented with 0.5 μM TDZ alone, as well as with 10 or 50 μM BAP compared with the control treatment (no PGR) in respective experiments. There was 100% root induction of shoots after transferring for a further a 3-month period onto $^1/_2$ MS medium without PGR (control) compared

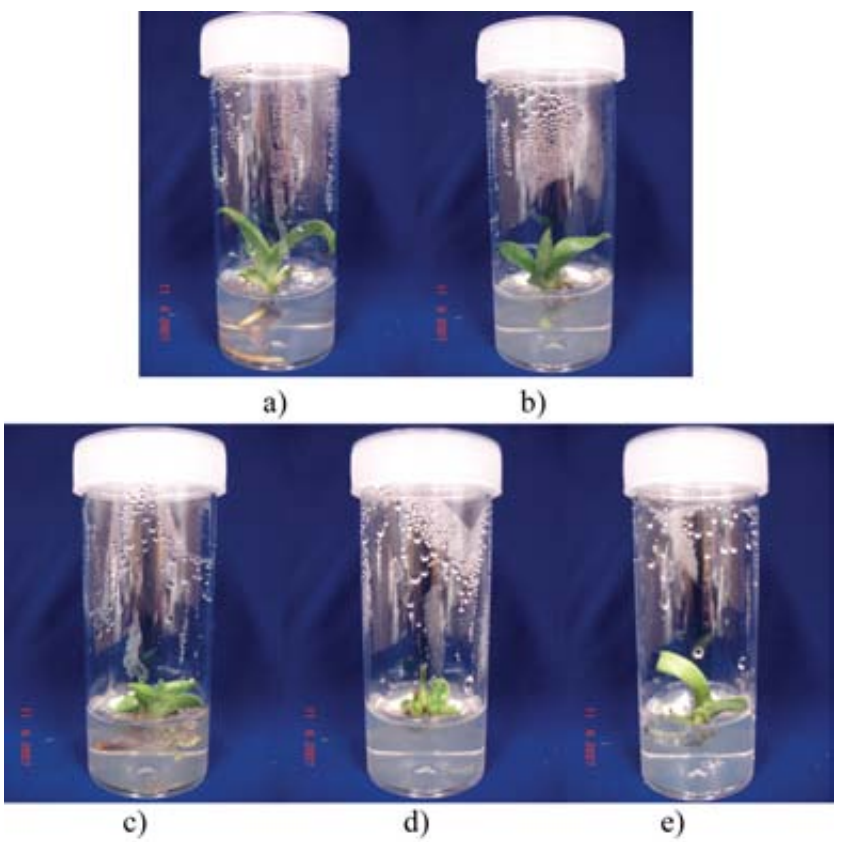

Figure 3 Shoots of *P. callosum* cultured on $^1/_2$ MS media supplemented with a) BA, b) 10, c) 20, d) 50, and e) 100 μM.

to the treatment with 2,4-D alone or combined with TDZ. Shoots cultured on $^1/_2$ MS medium without PGR also produced more roots per shoot and longer roots than on $^1/_2$ MS containing BAP. It is hoped that these results will assist with further in vitro research for the future development of *ex situ* conservation methods with endangered Thai slipper orchids.

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