Effect of Nitrogen to Phosphorus Ratios in Combination with Cytokinins on *in vitro* Growth and Development of *Dendrobium* Sonia 'Earsakul'

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ABSTRACT

Dendrobium Sonia 'Earsakul' is one of the most important tropical hybrid orchids in Thailand. It has only purple color and never been improved to develop other new colors. The possible new approach to improve is genetic engineering, which commonly needs *in vitro* culture for plant transformation and regeneration. In this study, we observed the effect of diverse nitrogen to phosphorus ratios, including 1:1, 0.25:1.5 and 0.25:3 relative to common amounts used in the basal MS medium, in combination with cytokinins, benzylaminopurine or BA (1, 2.5 and 5 mg/L) and thidiazuron or TDZ (0.5, 1 and 2 mg/L), on growth development of *D.* Sonia 'Earsakul' *in vitro* culture. All tested combinations induced shoot multiplication but no shoots underwent the transition to reproductive development in the 16-week period. The number of shoots generated from ½ MS medium supplemented with all N:P ratio and TDZ combinations were higher than all N:P ratio and BA combinations. However, the combinations of 0.25× N, 1.5× P and 1-2 mg/L of TDZ gave maximum shoot numbers and statistically significant difference from all N:P ratio and BA combinations. Most shoots generated from the media containing TDZ, particularly at high concentrations, were abnormal with dwarf shoots and short leaves. In contrast, most shoots derived from media supplemented with BA were normal.

Key words: BA, benzylaminopurine, *in vitro* culture, TDZ, thidiazuron

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INTRODUCTION

Dendrobium Sonia 'Earsakul' is one of the most important tropical hybrid orchids in Thailand. It has been popular, particularly as a cut flower, in the world markets due to its exotic flower, year-round availability and long vase life (Sagarik, 2002). Thailand exports *D.* Sonia 'Earsakul' cut flowers worldwide for many years. However, it has only purple color and never been improved to develop in other new vivid colors which always couple with new flower forms and shapes as other cross-pollinated plants (Fadelah, 2006). The possible new approach to improve only a color with no other modified characters is genetic engineering by alternating the anthocyanin biosynthetic pathway. *In vitro* culture is commonly a key tool of plant transformation and, therefore, it is required to be set up properly for both micropropagation and regeneration. *In vitro* flowering is also a subject of interest if the color modification is genetically engineered. This can shorten the flowering time for flower color determination in transgenic plants and also avoid the escape of transgenic plants to the environment (Teixeira da Silva *et al*., 2014).

Several *in vitro* propagation protocols for *Dendrobium* spp. and cultivars using various explant tissues and medium compositions have been successfully established (Teixeira da Silva *et al*., 2014). Nitrogen (N) and phosphorus (P) are ones of macronutrients required for plant cell or tissue growth. Tee *et al.* (2008) showed that applying low N (0.25×) and high P (1.25×) in Murashige and Skoog (MS) medium induced flowering in *D*. Sonia 17 at three-leaf stage plantlets. The different N: P ratios in tissue culture media as well as in field trials can play diverse roles in *Dendrobium* growth. Applying high N (100 and 200 mg/L) to *Dendrobium* cv. Red Emperor 'Prince' increased height of pseudobulbs whereas the flower number increased once no further N was applied. In contrast, applying P induced more flowering (Bichsel *et al*., 2008).

Cytokinins are plant growth regulators involved in cell division and cell differentiation. 6-Benzylaminopurine (BA) and 1-phenyl-3-(1, 2, 3-thiadiazol-5-yl)-urea or thidiazuron (TDZ) are ones of common cytokinins applied to culture media for induction of plant growth and differentiation. There are several reports of cytokinin applications in *Dendrobium* culture media for vegetative and reproductive growth development. Liao *et al*. (2011) reported the use of flower stalk nodes of *D.* Sonia cultured on MS medium supplemented with 4.43 μM BA to induce multiple shoots and protocorm-like bodies (PLBs) while half-strength MS medium (½ MS) supplemented with 2 mg/L TDZ induced the inflorescence of *D. wangliangii* (Dake *et al*., 2013). The ½ MS medium supplemented with 18.16 mM TDZ also induced embryogenesis of *D*. 'Chiengmai Pink' explants (Chung *et al*., 2005). There have also been reported that the combination of 0.04× N and 5× P adjustment and 2.5 mg/L TDZ can induce *in vitro* flowering of some orchids. Kostenyuk *et al*. (1999) demonstrated that *Cymbidium niveo-marginatum* Mak explants inoculated on basal MS medium added with the 0.04X N and 5X P ratio and 2.5 mg/L TDZ

could effectively induce flowers. Limsanguan *et al.* (2012) successfully induced flowers of *Oncidium* 'Jairak Rainbow' on MS medium added with 5X P and 10 mg/L BA.

In this research, we demonstrated the effect of diverse N:P ratios in combination with different BA or TDZ concentrations on shoot and flower development in *in vitro* culture of *D.* Sonia 'Earsakul'.

MATERIALS AND METHODS

Plant material

Three-leaf stage plantlets of *D.* Sonia 'Earsakul' grown on Vacin and Went (VW) basal solid medium and incubated at 25°C with 16-h photoperiod of 35 µmol m^{-2} s^{-1} from daylight fluorescent lamps for 3 months (Fig.1a) were used as initial experimental plants irrelevant to the experimental result.

Experimental design and statistical analysis

Roots of the 3-month-old plants (Figure 1c) were removed and inoculated on the experimental media. To determine the influence of the N:P ratio and cytokinin (BA or TDZ) on growth development of *D.* Sonia 'Earsakul', the diverse N:P ratios with cytokinin (BA or TDZ) concentrations (Table 1) were applied to the modified ½ MS medium supplemented with 30 g/L of sucrose and 7 g/L of agar. Media were adjusted to pH 5.7 before autoclaving at 1 kg cm^{-2} (121˚C) for 20 minutes. One plant was inoculated in an 8-ounce bottle containing 30 ml of medium for each treatment. Ten replicates of each treatment were employed in completely randomized designs (CRD). All cultures were incubated at 25˚C under a 16-h photoperiod of 35 µ$molm^{-2}$ s^{-1} from daylight fluorescent lamps and subcultured every 4 weeks until 16 weeks. The total culture period of experimental plants was 7 months.

Data on average shoot numbers were subject to analysis of variance (ANOVA). Means with a significant difference were subject further to Least Significant (LSD) using IBM SPSS Statistics Version 19 software with 0.05 alpha as an error value.

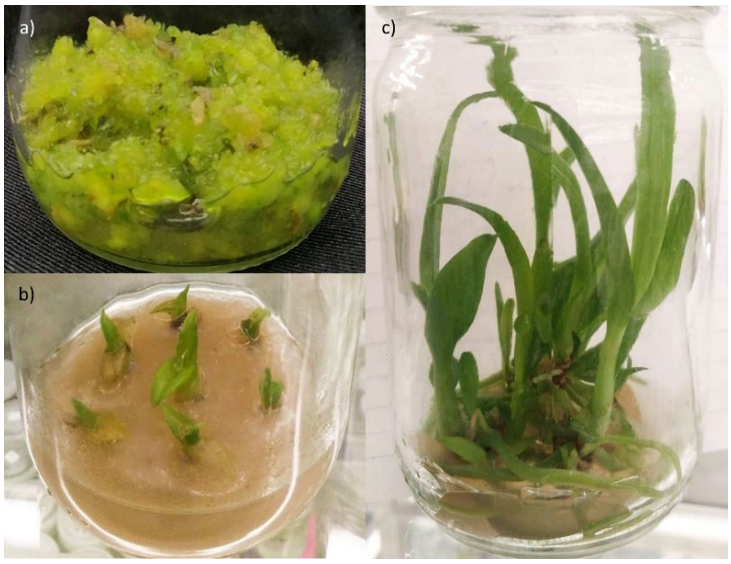

Figure 1 Irrelevant to the experiment, three-month old plants in VW medium.

Table 1 The combinations of the N:P ratios and cytokinins (BA or TDZ) applied in ½ MS medium for induction of *D*. Sonia 'Earsakul' growth and development.

Treatment	N (mg/L)	P (mg/L)	BA (mg/L)	TDZ (mg/L)
NP1BA1	1× (825 mg/L)	1× (85 mg/L)	1	-
NP1BA2			2.5	-
NP1BA3			5	-
NP1TDZ1			-	0.5
NP1TDZ2			-	1
NP1TDZ3			-	2
NP2BA1	0.25× (206.25 mg/L)	1.5× (127.5 mg/L)	1	-
NP2BA2			2.5	-
NP2BA3			5	-
NP2TDZ1			-	0.5
NP2TDZ2			-	1
NP2TDZ3			-	2
NP3BA1	0.25× (206.25 mg/L)	3× (255 mg/L)	1	-
NP3BA2			2.5	-
NP3BA3			5	-
NP3TDZ1			-	0.5
NP3TDZ2			-	1
NP3TDZ3			-	2

RESULTS AND DISCUSSION

Effect of the N:P ratios in combination with BA on growth development

During the 16-week period of observation, the experimental plants generated new shoots in all treatments of the N:P ratio and BA combinations but no any shoots underwent the transition from a vegetative to reproductive phase of development in the study period. The combination of treatment NP1BA3, which composted of 1× N, 1× P and 5 mg/L of BA, provided the highest shoot number (4.43 shoots) which was significantly different from other combinations except the NP3BA2 treatment (Table 2). Most of generated shoots had normal vegetative growth, which developed pseudobulbs and leaves (Fig. 2a). However, leaves turned brown and finally died after 2 months of culturing while new shoots were produced (Figure 2c). *Ricinus communis* L. seedlings cultured in MS medium supplemented with highest BA concentration (2 mg/L) gave a maximum number of shoots (Sujatha and Reddy, 1998). Pantipa *et al*. (2012) demonstrated that BA could induce multiple shoots in in vitro culture of *Oncidium* 'Jairak Rainbow', but shoots were small and no new root emerged. The 0.04× N, 5× P MS media

contained 0.05× N and 5× P in combination with 10 mg/L BA yielded highest shoots number whereas the decreased N and increased P without BA addition showed no significant difference on growth. Although it has been reported that the 0.25× N and high 1.25× P ratio combines with the 20μM BA concentration in MS medium induced *in vitro* flowering in 24 weeks (Tee *et al*., 2008), our given N:P ratios and BA concentrations may be not suitable for induction of *D*. Sonia 'Earsakul' flowering *in vitro*.

Effect of the N:P ratios in combination with TDZ on growth development

The experimental plants cultured in all treatments of the N:P ratio and TDZ combinations also produced new shoots and no transition to reproductive development during the 16-week period of observation. Although the number of shoots generated from ½ MS medium supplemented with all the N:P ratio and TDZ combinations were mostly higher than the N:P ratio and BA combinations, the NP1BA3 treatment gave the number of shoots with no statistically significant difference from the NP1TDZ3, NP2TDZ1, NP3TDZ1, NP3TDZ2 and NP3TDZ3. However, the maximum shoot numbers derived from the combinations of the treatments NP2TDZ2 (0.25× N, 1.5× P and 1 mg/L TDZ) and NP2TDZ3 (0.25× N, 1.5× P and 2 mg/L TDZ) were significantly different from all N:P ratio and BA combinations (Table 2). Although the number of shoots increased according to the increased TDZ level in the media (Table 2), most of generated shoots were abnormal with dwarf shoots and short leaves (Fig. 2b). Shirani *et al*. (2010) also reported that higher concentrations of TDZ hindered shoot elongation and resulted in high number of abnormal shoots in *Musa* spp. In apple trees, TDZ also causes greater proliferation than BA, but can inhibit elongation at high concentration (Huetteman and Preece, 1993; Marin *et al.,* 1993; van Nieuwkerk *et al*., 1986).

Table 2 Effect of the N:P ratio in combination with BA on shoot development of *D*. Sonia 'Earsakul' after treatment for 16 weeks.

Treatment	No. of shoot	No. of normal shoot	No. of abnormal shoot*	Average of leave number
NP1BA1	2.29±0.24^{f}	2.14±0.26^{ab}	0.14±0.14^{c}	2.45±0.23^{a}b
NP1BA2	2.29±0.52^{f}	2.14±0.55^{ab}	0.14±0.14^{c}	2.61±0.2^{a}b
NP1BA3	4.43±1^{cdef}	3.00±0.31^{a}	1.42±0.81^{c}	2.43±0.41^{ab}
NP1TDZ1	5.71±0.86^{abc}	1.57±0.48^{bc}	4.14±0.86^{b}	1.36±0.2^{cdef}
NP1TDZ2	5.28±0.71^{bcd}	0.86±0.4^{cd}	4.42±0.87^{b}	1.10±0.08^{ef}
NP1TDZ3	4.86±0.4^{cde}	0^{d}	4.86±0.4^{b}	0.96±0.26^{ef}

Table 2 (Continued)

Treatment	No. of shoot	No. of normal shoot	No. of abnormal shoot*	Average of leave number
NP2BA1	2.29±0.29 f	2.00±0.22 b	0.29±0.14 c	1.83±0.22 bcde
NP2BA2	2.14±0.26 f	2.14±0.26 ab	0±0 c	2.64±0.53 ab
NP2BA3	2.57±0.37 ef	2.14±0.4 ab	0.86±0. 2 c	1.79±0.17 bcde
NP2TDZ1	4.57±0.53 cdef	0.28±0.18 d	4.28±0.47 c	1.06±0.9 ef
NP2TDZ2	7.28±1.06 ab	0 d	7.28±1.06 a	0.75±0.21 f
NP2TDZ3	8.00±1.73 a	0 d	8.00±1.73 a	0.85±0.17 ef
NP3BA1	2.29±0.18 f	2.14±0.14 ab	0.14±0.14 c	2.86±0.55 a
NP3BA2	3.14±0.5 ef	2.14±0.4 ab	1.00±0.53 c	2.25±0.45 abc
NP3BA3	2.71±0.47 def	0.4±0.26 b	0..86±0.46 c	2.17±0.5 abcd
NP3TDZ1	4.28±0.52 cdef	0.14±0.14 d	4.14±0.51 b	1.25±0.17 def
NP3TDZ2	5.86±0.96 abc	0 d	5.86±0.96 ab	0.72±0.12 f
NP3TDZ3	5.86±0.74 abc	0 c	5.86±0.74 ab	1±0.19 ef

*The values followed by different letters within columns are significantly different from each other at 5% level. Data represent mean ± S.E.

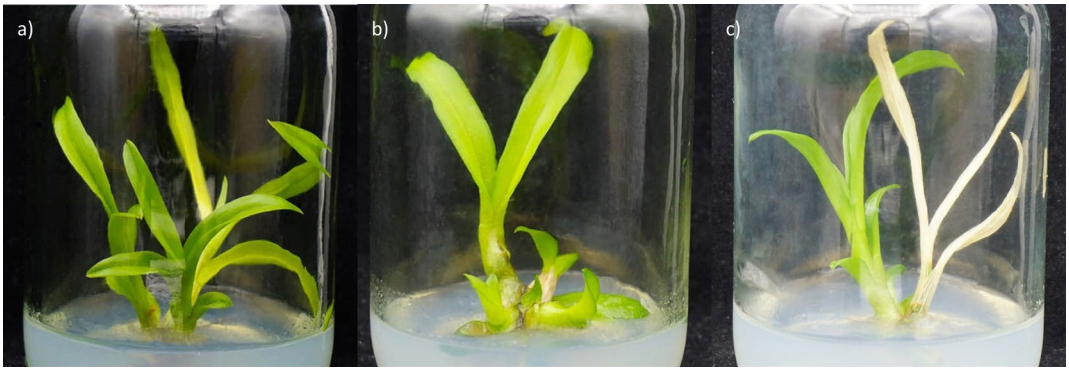

Figure 2 Four-month old plants of *D*. Sonia 'Earsakul' cultured on different modified MS media, (a) normal shoots under the NP1BA1 treatment, (b) abnormal shoots under the NP2TDZ3 treatment and (c) browning and dying shoot under the NP1BA1 treatment.

CONCLUSION

In this study, we observed that modification of low N and high P in 1/2 MS medium in combination with cytokinins (BA and TDZ) induced vegetative but not reproductive growth of *D*. Sonia 'Earsakul' during 16-week period. TDZ caused greater proliferation than BA but most shoots were no elongation and short leaves. In contrast, BA induced fewer shoots but most shoots were normal.

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