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Effects of Plant Growth Regulators and Sucrose on the Regeneration of *Paphiopedilum micranthum* var. North Vietnam

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Abstract

The objective of this study was to determine the effects of 2,4-Dichlorophenoxyacetic acid (2,4-D) combined with 6-Benzylaminopurine (BAP) and sucrose on the shoot and root regeneration of *Paph. micranthum* var. North Vietnam. Young fresh leaves were cut into small explants and then cultured on media containing 2,4-D (0.5, 1.0, or 2.0 ppm). Within each of the 2,4-D concentration, BAP was added at three different concentrations (0.5, 1.0, or 2.0 ppm) for callus induction after 4 weeks. Shoot tips (2-3 mm in length) from the leaf-derived calluses were then transferred vertically on half-strength MS medium (2,4-D combined with BAP (0.5, 1.0, or 2.0 ppm) for shooting, and rootless shoots with 2 leaves were transferred to the same MS medium for rooting after 4 months of culture. Rootless shoots with 2 leaves were also transferred vertically on half-strength MS medium with different sucrose concentrations (0, 10, 20, 30, or 40 g L^{-1}) to optimize the growth and development of the plantlets. The results indicated that the optimum degree of shoot and root regeneration occurred on half-strength MS medium containing 2.0 ppm 2,4-D combined with 2.0 ppm BAP. Approximately 86.6% of the cultures responded with 3.3 shoots and 3.0 leaves/explant. In addition, under the optimal concentrations, the number and length of roots per explant after 4 months of culture were 2.1 roots per explant and an average root length of 1.1 cm. MS medium supplemented with 30 g L^{-1} sucrose had the highest number of leaves and roots, and also the longest average length of roots, which gave 3.8 leaves, 3.3 roots/explant, and a root length of 1.8 cm after 4 months.

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Keywords

Paphiopedilum micranthum var. North Vietnam, regeneration, plant growth regulators (PGRs), half-strength MS medium, sucrose concentration

Introduction

Paphiopedilum micranthum var. North Vietnam, which belongs to the subfamily Cypripedioideae of the family Orchidaceae, is commonly known as the Silver Slipper Orchid. It is a new variety of the highly coveted *P. micranthum* from northern Vietnam and southwestern China, and has great value as cut flowers and indoor ornaments. The flower blooms during late spring and early summer with one flower per plant, and the flowers are aromatic.

In vitro plant regeneration is a highly effective method to conserve native, rare, and over-collected orchid species, thereby producing and maintaining large quantities of orchid populations (Teixeira, 2013). In particular, micro-propagation is the most frequently used and most convenient technique, enabling major trade with developed countries (Sagawa and Kunisaki, 1982). However, orchids of temperate terrestrial origin are difficult to propagate *in vitro* despite a relative wealth of literature, and exacerbating the situation is that explants from mature plants of orchid species are recalcitrant to shoot induction and plant regeneration (Arditti and Ernst, 1992). The propagation of *Paphiopedilum* through *in vitro* culture has been attempted using different explant sources, such as seed derived seedlings, buds, leaves, and roots (Yu *et al.*, 2011). However, the most effective propagation method for the *Paphiopedilum* species is dependent on many factors including habitat location, season, seed maturity, and growth stage (Zhang *et al.*, 2015). Conversely, many *in vitro* cultural conditions including physical and nutritional conditions can also affect the growth of *Paphiopedilum*. Yoon *et al.* (2006) demonstrated that medium types, plant growth regulators, carbohydrates, and other components, which cause impacts to the development of orchids.

Auxin and cytokinin are two important plant growth regulators (PGRs) that control plant growth and organize plant development. Akinyele (2010) observed that auxin causes cell growth expansion, modifies the cell wall and initiates cell division, promotes vascular differentiation, and plays a main role in root promotion; whereas cytokinins are involved in many plant processes, including cell division and growth of shoot buds. There is a need for the normalization of effective concentrations of PGRs for better growth of plants and orchids. Growth medium supplemented with PGRs alone or in combinations of two or more substances are better for growth regulation than medium without PGRs (Khatun *et al.*, 2010).

Because of difficulties in plant regeneration and plantlet formation, as all aforementioned procedures are inadequate for meeting the commercial needs of vegetative propagation. Therefore, the present study examined the effects of various concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D) combined with 6-Benzylaminopurine (BAP) and sucrose on plant regeneration from leaf explants of *P. micranthum* var. North Vietnam.

Materials and Methods

Plant material

P. micranthum var. North Vietnam was collected in April 2016, from In-Charm Orchid Laboratory, Taiwan, and grown to maturity from *in vitro* culture for 10 - 12 months. Randomly selected young fresh leaves (approximately 3 cm in length) were used for culture.

Effects of 2,4-D and BAP on shoot multiplication and root induction from leaves of *P. micranthum* var. North Vietnam

Callus induction

Young fresh leaves of *P. micranthum* var. North Vietnam were cut into small explants (0.5 x 0.5 cm) and were placed in glass tubes containing 15 mL of half strength MS medium (Murashige and Skoog, 1962) with either one of three concentrations of 2,4-D (0.5, 1.0, or 2.0 ppm) or without any PGRs (control medium). Within each of the 2,4-D concentrations, BAP was added at three different concentrations (0.5, 1.0, or 2.0 ppm) for callus induction after 4 weeks of culture.

Shoot multiplication and root induction

Shoot tips (2 - 3 mm in length) from the callus induction and rootless shoots with 2 leaves were transferred vertically on half-strength MS medium, either without PGRs or with 2,4-D (0.5, 1.0, and 2.0 ppm) combined with BAP (0.5, 1.0, and 2.0 ppm), after removing the brown part of the original explant to optimize shoot multiplication and root induction.

Shoot multiplication, number of shoots per explant, number of roots and leaves per explant, and average length of roots were calculated after 4 months of culture.

Effects of sucrose concentration on seedling development of *P. micranthum* var. North Vietnam

Rootless shoots with 2 leaves were transferred vertically on half strength MS medium supplemented with different sucrose concentrations (0, 10, 20, 30, or 40 g L^{-1}). The medium without sucrose was used as a control.

After 4 months of culture, the growth characteristics (number and length of roots per explant, number of leaves per explant, and length of roots) of young seedlings were calculated.

Requirements of culture media and culture conditions

Half-strength MS medium was used as a basal medium for this study. The medium was supplemented with 20 g L^{-1} sucrose (only for effects for PGRs experiment), the most suitable concentration of BAP combined with 2,4-D (for effect of sucrose experiment), and 10 g L^{-1} agar. The pH of the medium was adjusted to 5.8 with 0.1 N NaOH or HCl before being autoclaved at 1.2 kg/cm^2 pressure and 121^{o}C for 15 min.

Fifty-mL glass tubes were used and contained 15 mL of treatment medium per tube. Each experiment consisted of three replicates with 5 tubes per replicate, and one sample per tube. After culturing, the explants were incubated in darkness at 24 - 26^{o}C for 4 weeks to make calluses and then maintained at 24 - 26^{o}C under a 16/8h light/dark cycle with cool-white fluorescent lamps at 1000 lux.

Data analysis

All experiments were carried out in a completely randomized design (CRD). Analysis of variance (ANOVA) was conducted to test for significance at the 0.05 level using IRRISTART version 5.0. Fisher's $LSD_{0.05}$ (least significant difference at a 95% confidence level) was used in ANOVA to calculate the confidence intervals when comparing each pair of treatments and treatments were ranked via the superscripts (a, b, and c, etc.) with 'a' being the best result. Values with the same letter do not differ significantly at $P \leq 0.05$, and values with different letters are significantly different at $P \leq 0.05$.

Results and Discussion

The effects of 2,4-D and BAP on the induction of calluses from leaves of *P. micranthum* var. North Vietnam

Callus cultures are extremely important in plant biotechnology. They are defined as unorganized tissue masses growing on substrate. In this study, the medium containing the highest concentrations of 2,4-D (2.0 ppm) combined with the lowest concentrations of BAP (0.5 ppm) gave the highest callus formation (93.3%), when compared to the other treatments (*P<0.05*) (Table 1). The second highest callus formation was the 2.0 ppm 2,4-D mixed with 1.0 ppm BAP treatment which induced calluses at a rate of 86.7%. BAP did not stimulate the explants to grow and form a callus as compared to 2,4-D in this experiment. Nevertheless, a small amount of BAP (0.2 mg L^{-1}) had a positive effect on callus formation. BAP promotes RNA and protein synthesis, which activates enzyme activity for cell division and cell wall loosening (Kulaeva, 1980).

Moreover, the lowest level of 2,4-D (0.5 ppm) combined with the highest level of BAP (2.0 ppm) resulted in a lower callus induction rate than other concentrations (60.0%) (*P<0.05*). The control, containing no PGRs, did not produce any calluses (Table 1), probably due to the lack of auxin concentration required for callus induction.

The combinations of two PGRs had a positive effect on callus induction. Hasbullah *et*

Table 1. Effects of 2,4-D and BAP on induction of calluses from leaves of *P. micranthum* var. North Vietnam

Plant growth regulators (PGRs) (ppm)		Callus induction (%)
2,4-D	BAP	
0	0	0.0[g]
0.5	0.5	73.3[d]
0.5	1.0	66.7[e]
0.5	2.0	60.0[f]
1.0	0.5	82.5[bc]
1.0	1.0	80.0[c]
1.0	2.0	80.0[c]
2.0	0.5	93.3[a]
2.0	1.0	86.7[b]
2.0	2.0	80.0[c]
	$LSD_{(0.05)}$	4.3

Note: Values with the same letters do not differ significantly at P≤0.05.

al. (2011) demonstrated that 1.0 to 2.0 ppm 2,4-D with a small concentration of BAP produced calluses in *Gerbera jamesonii* when sub-cultured at 2-week intervals. Furthermore, Klaus (2007) observed the highest callus induction rates at higher levels of 2,4-D. Moreover, that study suggested that 2,4-D combined with the lower concentrations of BAP created the most favorable combination of growth regulators in order to produce calluses in grapevine.

The effects of 2,4-D and BAP on shoot multiplication from calluses of *P. micranthum* var. North Vietnam

By adding PGRs, shoot multiplication from calluses was promoted when cultured on half-strength MS medium. The results shown in Table 2 demonstrate that shoot tips on medium supplemented with the highest concentrations of BAP and 2,4-D together (2.0 and 2.0 ppm, respectively) gave the maximum shoot regeneration (86.6%). Also, it significantly enhanced shoot growth ($P<0.05$) at a rate of 3.3 shoots per explant and significantly increased the number of leaves (3.0 leaves per explant), when compared to the other treatments (Figure 1d). The promotion of growth on shoots and leaves resulted in increased plasticity of the cell walls, followed by the hydrolysis of starch to sugars, which decreased the water potential of the cells, resulting in the entry of water into the cells, and causing cell elongation (Kumar *et al.*, 2014).

However, as concentrations of 2,4-D combined with BAP decreased, shoot regeneration was observed to decrease; in addition, the lowest concentrations of PGRs (0.5 ppm BAP + 0.5 ppm 2,4-D) reduced the growth rate of shoots (53.3%), reduced the number of shoots (0.9/explant), and reduced the number of leaves (1.0/explant) (Figure 1b). Finally, the lowest shoot induction rate (46.7%) was from the control medium without any PGRs which also grew only 0.8 leaves per explant (Table 2, Figure 1a).

Ibanez *et al.* (2005) reported similar findings in that medium containing 1.0 or 2.0 mg L^{-1} BAP was suitable for the development of axillary buds and shoots in explants. Also, increases in the concentration of BAP in the basal MS medium increased the shoot multiplication rate of *in vitro* cultures of grape (Tehrim *et al.*, 2013). BAP gave the maximum number of shoots per explant (Asghar *et al.*, 2011) when propagated *in vitro* in orchid. Abido *et al.* (2013) reported that the maximum number of proliferated shoots of *Vitis vinifera* was found on MS medium containing 2.0 mg L^{-1} BAP and 0.2 mg L^{-1} 2,4-D.

Furthermore, cytokinins like BAP stimulate cell division and promote axillary shoot growth in tissue cultures of *Solanum tuberosum* L. (Kumar

Table 2. Effects of 2,4-D and BAP on the regeneration of shoots from calluses of *P. micranthum* var. North Vietnam after 4 months of culture

Plant growth regulators (PGRs) (ppm)		Shoot induction (%)	Avg. no. of shoots/explant	Avg. no. of leaves/explant
2,4-D	BAP			
0	0	46.7^{g}	0.7^{e}	0.8^{g}
0.5	0.5	53.3^{f}	0.9^{e}	1.0^{fg}
0.5	1.0	66.6^{d}	1.3^{d}	1.2^{ef}
0.5	2.0	73.3^{c}	2.4^{c}	2.1^{c}
1.0	0.5	60.0^{e}	1.4^{d}	1.5^{ef}
1.0	1.0	73.3^{c}	1.7^{d}	1.7^{de}
1.0	2.0	80.0^{b}	2.8^{b}	2.6^{b}
2.0	0.5	66.7^{d}	2.2^{c}	2.0^{cd}
2.0	1.0	80.0^{b}	2.5^{bc}	2.3^{bc}
2.0	2.0	86.6^{a}	3.3^{a}	3.0^{a}
LSD $_{(0.05)}$		2.1	0.40	0.3

Note: Values with the same letters do not differ significantly at P≤0.05.

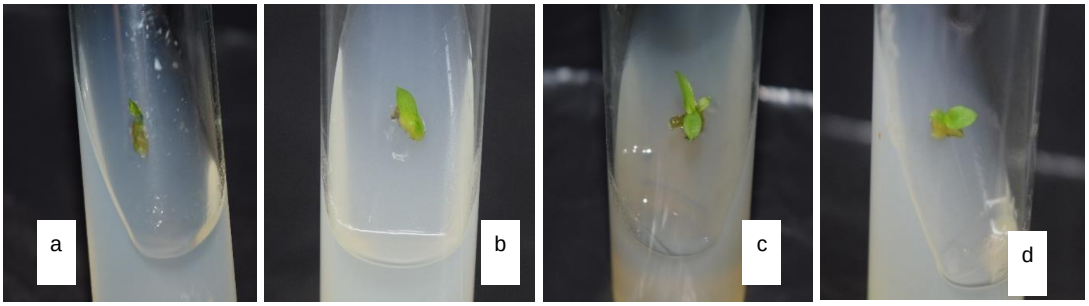

Figure 1. Number of leaves from shoot tips of *P. micranthum* var. North Vietnam with different concentrations of plant growth regulators after 4 months of culture

Note: (a) Medium without PGRs; (b) 0.5 ppm 2,4-D + 0.5 ppm BAP; (c) 1.0 ppm 2,4-D + 2.0 ppm BAP; (d) 2.0 ppm 2,4-D + 2.0 ppm BAP.

et al., 2014). The important role of BAP explained a better response in terms of shoots per explant, shoot length, and number of leaves and nodes in different potato varieties (Nasrin *et al.*, 2003). The results of Laboney *et al.* (2013) showed that the combination of 2.0 mg L^{-1} BAP and 1.0 mg L^{-1} 2,4-D resulted in the fewest days for shoot initiation (15 days), the highest shoot regeneration (93.33%), the highest number of shoots (4.67 shoots per explant) and leaves (3.67 leaves per explant), and the longest shoots (5.33 cm) from callus-derived explants.

The effects of 2,4-D and BAP on the regeneration of roots from calluses of *P. micranthum* var. North Vietnam

It can be seen in Table 3 that roots were induced on shoots in all treatments, except for the control. The best rooting was obtained on the medium supplemented with 2.0 ppm 2,4-D + 2.0 ppm BAP which resulted in the highest number ($P<0.05$) of roots per explant (2.1 roots per explant) and the highest average length of roots (1.1 cm) (Figure 2e). The combination of 2.0 ppm 2,4-D mixed with 1.0 ppm BAP induced the second greatest response with 1.9 roots per explant and an average root length of 0.8 cm. Media without any PGRs did not produce roots (Table 3; Figures 2a and 2b).

In this study, different effects of PGR combinations were determined because different concentrations were more effective for root formation at the specific concentrations. They were very effective for the rooting of orchids because auxin (2,4-D) combined with cytokinin (BAP) had an essential role in root initiation

(De Klerk, 2002) and root primordia formation. They also affect the cell wall, turgor, and osmotic pressures, and water permeability, which cause cell enlargement (Taiz and Zeiger,

Table 3. Effects of 2,4-D and BAP on the regeneration of roots from shoot-derived calluses of *P. micranthum* var. North Vietnam after 4 months of culture

Plant growth regulators (PGRs) (ppm)		Avg. no. of roots/explant	Avg. length of roots (cm)
2,4-D	BAP		
0	0	0.0^{f}	0.0^{f}
0.5	0.5	1.0^{e}	0.3^{e}
0.5	1.0	1.2^{de}	0.4^{de}
0.5	2.0	1.1^{de}	0.4^{de}
1.0	0.5	1.2^{de}	0.5^{cd}
1.0	1.0	1.5^{bc}	0.7^{bc}
1.0	2.0	1.3^{cd}	0.6^{c}
2.0	0.5	1.6^{b}	0.8^{b}
2.0	1.0	1.9^{a}	0.8^{b}
2.0	2.0	2.1^{a}	1.1^{a}
LSD $_{(0.05)}$		0.2	0.1

Note: Values with the same letters do not differ significantly at P≤0.05.

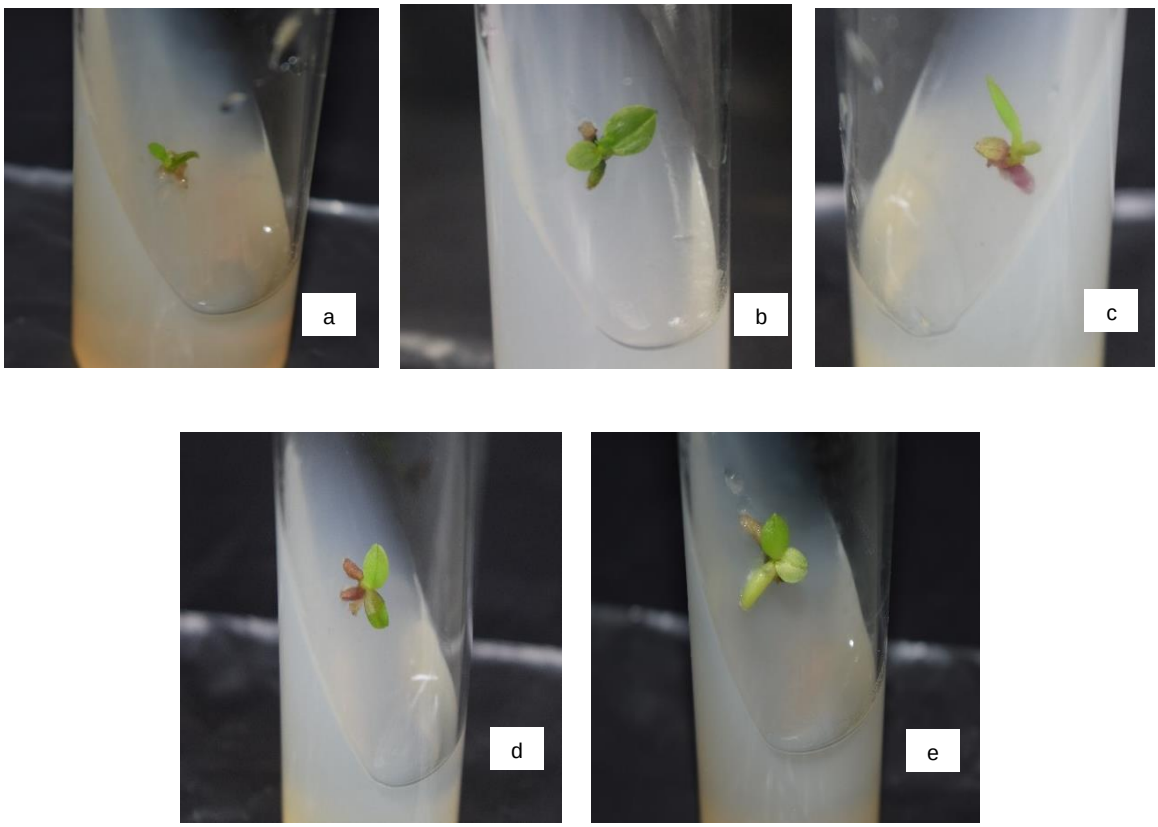

Figure 2. The growth of roots from shoot-derived calluses of *P. micranthum* var. North Vietnam with different concentrations of PGRs after 4 months of culture

Note: (a) Medium without PGRs; (b) 0.5 ppm 2,4-D + 0.5 ppm BAP; (c) 1.0 ppm 2,4-D + 2.0 ppm BAP; (d) 2.0 ppm 2,4-D + 1.0 ppm BAP; (e) 2.0 ppm 2,4-D + 2.0 ppm BAP.

2006). It has been observed that higher concentrations of auxin (2,4-D) cause rooting due to the stimulation of cell division. Han *et al.* (2009) reported that PGR combinations also enhance the growth of shoot buds and stimulate substances present in the roots for their better development.

The results from our trial indicate that auxin and cytokinin combinations produced higher numbers of roots than auxin alone. Similar observations were also reported by Khatun and Al-Amin (2006). This increase of rooting by application of 2,4-D combined with BAP may be due to the translocation of carbohydrates from the leaves which can contribute to root development (Bhatt and Tomar, 2010), although increases in rooting from application of auxin (2,4-D) is a common feature in many herbaceous perennial plants (Hartmann *et al.*, 2002). It has been reported that 2,4-D stimulates protein synthesis and RNA production, and enhances hydrolysis and the translocation of carbohydrates and nitrogenous substances at the base of cuttings to increase cell division for enhancing rooting of plants (Singh *et al.*, 2003).

The effects of sucrose concentration on seedling development of *P. micranthum* var. North Vietnam

Sucrose is the most widely used sugar in *in vitro* regeneration. The positive effects of sucrose on the growth of explants under *in vitro* conditions are linked with sucrose's high solubility in water, its electrical neutrality, and its lack of an inhibitory effect on the majority of biochemical processes (Smith *et al.*, 1995). Moreover, it is the main sugar translocated in the phloem (Konate *et al.*, 2013). Thus, in this experiment, rootless shoots were cultured on 0.5 MS medium supplemented with 2.0 ppm 2,4-D + 2.0 ppm BAP and mixed with different sucrose concentrations in order to measure the number of leaves and roots per explant, and the average length of the roots. The results are shown in Table 4 and Figure 3.

After 4 months of culture, sucrose concentrations from 10 to 40 g L^{-1} positively affected the growth and development of explants compared to the control. Specifically, the seedling development on medium supplemented with 30 g L^{-1} sucrose had the highest number of leaves (3.8 leaves per explant) and roots (3.3 roots per explant), and also the longest length of roots (1.8 cm) (Figure 3c). The second most effective concentration was the 20 g L^{-1} sucrose treatment (3.2 leaves per explant and 2.0 roots per explant).

At 40 g L^{-1} sucrose, seedling development was decreased, resulting in an average of 2.6 leaves per explant, 1.6 roots per explant, and 1.2 cm of root growth (Figure 3d). The lowest seedling development was observed on the medium without sucrose (Table 4 and Figure 3a), however the number of leaves and roots for the control were not statistically different in comparison with the 10 g L^{-1} and 40 g L^{-1} sucrose concentrations.

While carbohydrates serve as an energy source in the medium, they also act to supplement osmotic pressure in the culture of plant cells and affect the orchid seed germination and protocorm

Table 4. Effects of sucrose concentration on seedling development of *Paph. micranthum* var. North Vietnam after 4 months of culture

Sucrose concentration (g L^{-1})	Avg. no. of leaves/ explant	Avg. no. of roots/ explant	Avg. length of roots (cm)
0	2.2^{c}	1.0^{c}	0.5^{d}
10	2.4^{c}	1.3^{c}	1.0^{c}
20	3.2^{b}	2.0^{b}	1.5^{b}
30	3.8^{a}	3.3^{a}	1.8^{a}
40	2.6^{c}	1.6^{bc}	1.2^{c}
$LSD_{(0.05)}$	0.5	1.0	0.2

Note: Values with the same letters did not differ significantly at P≤0.05.

development significantly (Yoon *et al.*, 2016). In particular, sucrose is considered as an important carbon and energy source because it is the most common carbohydrate in phloem sap and is involved in controlling the developmental processes (Gibson, 2000). Sucrose could provide a balanced carbon source for cell growth with the release of hexoses that could directly participate in the glycolytic and pentose phosphate pathways (Zha *et al.*, 2007). Sucrose supports the growth of plant cells in culture. Many studies have shown that soluble sugars in media are usually added in concentrations between 10 and 30 g L^{-1} of glucose, fructose, or sucrose (Rasmussen, 1995). Mongomake *et al.* (2015) reported that increasing the concentration of sugar from 1 to 3% had a positive effect on the height of plants and biomass production. Generally, using 3% sucrose in the medium as per the recommendations of Murashige and Skoog (1962) results in optimum performance. Other results have demonstrated that sucrose concentrations between 20 - 30 g L^{-1} are the most commonly used in orchid tissue culture, and that shoots can be regenerated from shoot tips on MS medium (Nipawan *et al.*, 2013).

Nevertheless, excess sucrose concentrations could delay the development of cultured cells by ending the cell cycle when nutrients were limited (Wu *et al.*, 2006). Deb and Pongener (2011) reported that the different concentrations of sucrose affected seed germination of *Cymbidum aloifolium*, and Johnson *et al.* (2011) commented that increasing the sucrose concentration from 40 to 50 mM resulted in poorer germination and development of *Bletia purpurea*. Other researchers have also found that higher concentrations of sucrose added to the culture medium could have an inhibitory effect on nutrient uptake by lowering water potential of the medium (Shim *et al.*, 2003), and inducing the osmotic stress (Shohael *et al.*, 2006).

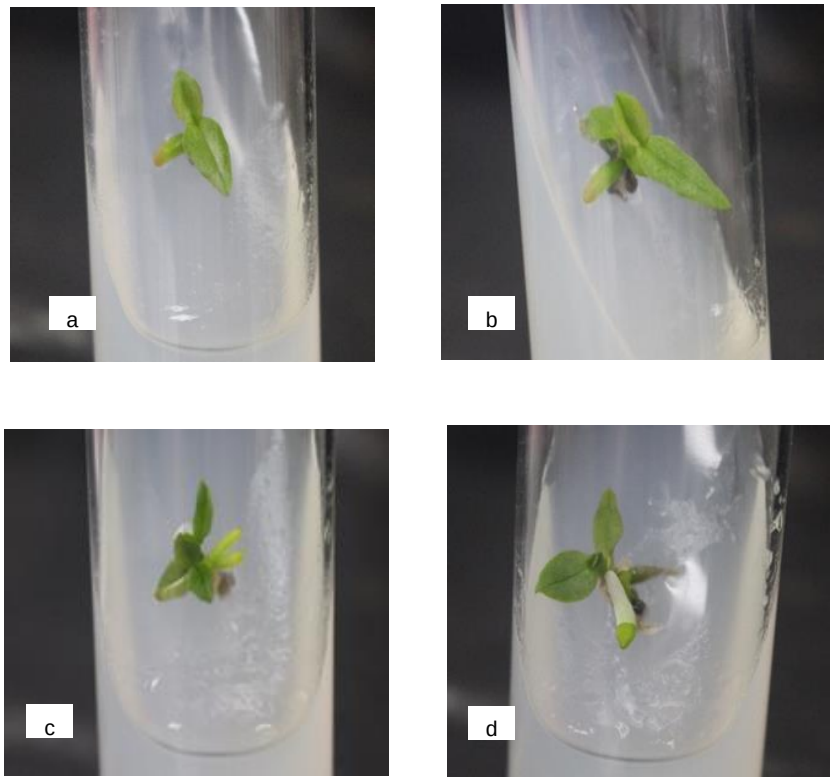

Figure 3. Characteristics of seedling development from shoot-derived calluses of *P. micranthum* var. North Vietnam depending on the sucrose concentration after 4 months of culture

Note: (a) Medium without sucrose; (b) 10 g L^{-1} sucrose; (c) 30 g L^{-1} sucrose; (d) 40 g L^{-1} sucrose.

However, other results have shown that increasing the concentration of sucrose in the medium could lead to increases in number of shoots and roots. Mongomake *et al.* (2015) reported that the concentrations of 4%, 5%, and 6% sucrose increased plant height, number of leaves, root length, and biomass of *Bambara groundnut* at a similar rate when compared to the control. In another study, medium supplemented with 60 g L^{-1} sucrose was the most efficient for increasing the height and fresh weight of *Dendrobium nobile* in *in vitro* culture (Faria, 2004).

Conclusions

Half-strength MS medium supplemented with the combination of 2,4-D and BAP induced greater shoot and root growth of *P. micranthum* var. North Vietnam than medium without PGRs. The medium supplemented with 2.0 ppm 2,4-D combined with 0.5 ppm BAP resulted in the highest callus induction rate (92.8%). The most effective medium for shooting and rooting of *P. micranthum* var. North Vietnam was half-strength MS medium supplemented with 2.0 ppm 2,4-D and 2.0 ppm BAP, which resulted in a 90.2% shoot regeneration rate, 3.3 shoots/explant, 3.0 leaves/explant, and produced the greatest number of roots (2.1 roots/explant) and an average root length of 1.1 cm.

Sucrose had a positive effect on the seedling development of *P. micranthum* var. North Vietnam. The half-strength MS medium supplemented with 2.0 ppm 2,4-D and 2.0 ppm BAP, and mixed with 30 g L^{-1} sucrose was the optimal concentration for the growth of seedlings, which produced 3.8 leaves/explant, 3.3 roots/explant, and an average root length of 1.8 cm.

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