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OPTIMUM MICRONUTRIENT LEVEL FOR *PHALAENOPSIS DELICIOSA* ORCHID SEEDLING *IN VITRO* GROWTH

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ABSTRACT

There were many researches about the influence of micronutrient concentration toward growth of plants. However, there was no clear statement about the micronutrient level for growth of *Phalaenopsis* seedlings. Hence it was worth to investigate the optimum level of micronutrient for *Phalaenopsis*, an endangered orchid species. Using *Phalaenopsis deliciosa* as the subject, germinated seedlings were grown on defined culture media containing different MS micronutrient level. After 90 days with a subculture at day 45, fresh and dry weights of shoots and roots of the seedlings were measured. It was found that the optimum micronutrient level for *P. deliciosa* seedling growth was observed between 0.50× and 1.00× of MS micronutrient level. Higher micronutrient level caused roots and seedlings to deterioration except for a minority of seedling variants that grew exceptionally well, suggesting that high micronutrient level was selective for a small number of variants. The study demonstrated the importance of appropriate micronutrient level for supporting growth and development for wide range of genotypes in *P. deliciosa*. This micronutrient level may as well be optimum for other species under the genus *Phalaenopsis* and should be considered for maintaining genotype diversity *in vitro*.

Keywords: Genotype-Selectivity, Germinated Seedlings, *In Vitro* Nutrition, Moth Orchid

1. INTRODUCTION

Micronutrients are important for plant growth and morphogenesis (George and De Klerk, 2008). Micronutrients are essential elements required at minute amount and constitute less than 0.01% of plant tissue dry mass while beneficial elements are those that may enhance plant growth or required by certain plant species only (Barker and Pilbeam, 2007). Murashige and Skoog (1962) micronutrients consist of Iron (Fe), Manganese (Mn), Zinc (Zn), Boron (B), Iodine (I), Molybdenum (Mo), Copper (Cu) and Cobalt (Co), all of which are essential micronutrients except for Co and I, which are beneficial elements. The functions of these elements are described in detail elsewhere (George and De Klerk, 2008).

Non-optimum concentration of micronutrient may adversely affect plant growth. High concentration of B and Mn caused poor root development due to the enhancement of auxin destruction according to a research by Galston and Hillman (1957); the presence of Mn (II) decreased auxin level through degradation by Indole Acetic Acid (IAA)-oxidase (George and De Klerk, 2008). High B concentration resulted in decrease of number of roots formed, because high B concentration inhibited growth of roots through degradation of auxin (Jarvis, 1986). Excessive Zn concentration is inhibitory for root growth (George and De Klerk, 2008). Besides, some microelements like Cu are toxic to plant cells at high concentration (Kopsell and Kopsell, 2007).

Insufficient concentration of micronutrients also affects plants growth. Mo is essential and occurs in numerous oxido-reductase enzymes in plants (Hamlin, 2006). Zn deficient plants underwent low enzymatic activities and subsequently low production of protein,

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nucleic acid and chlorophyll (George and De Klerk, 2008). Insufficient B led to the cease of cell division and root elongation in plants via its effect on the metabolism of RNA (Ali and Jarvis, 1988).

Phalaenopsis species seedlings were found to grow well on (Choong *et al.*, 2013) medium which was shown to be non-genotype selective. However, optimum level of MS micronutrient in this medium is unknown; therefore the optimum MS micronutrient level for *P. deliciosa* seedling yield on CCT medium was investigated. *P. deliciosa* is an endangered orchid species listed in the Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2013) and therefore deserves to be propagated.

2. MATERIALS AND METHODS

Seedpods of *Phalaenopsis deliciosa* Rchb.f. were harvested 110 days after pollination. The seedpods were washed briefly under tap water before immersed into 5.25% sodium hypochlorite for 5 min and subsequently 70% ethanol for 1 min. This was for the sterilization of the seedpods. The seedpods were then flamed briefly until traces of ethanol burnt off, excised with a scalpel and seeds within the seedpods were inoculated onto CCT medium (Choong *et al*., 2013). Seedlings were maintained on the same medium with a 60-day subculturing regime.

In order to measure yield, seedlings with approximately 0.4±0.05 g were inoculated onto modified CCT media with different MS micronutrient level, which were 2.00, 1.00, 0.50 and 0.25× of MS micronutrient level. Each treatment had 23 replicates. CCT medium contained (mgL) NH_4NO_3 (190), $Ca(NO_3)_2{\cdot}4H_2O$ (470), $CaSO_4$ (77), $Mg(NO_3)_2{\cdot}6H_2O$ (255), $MgSO_4{\cdot}7H_2O$ (69), KNO_3 (370), KH_2PO_4 (800), NaCl (20), half strength of MS micro-nutrients, myo-inositol (100), niacin (0.5), pyridoxine HCl (0.5), thiamine HCl (0.1), glycine (2), sucrose (25,000) and gellan gum (2,200) at pH 5.2 (Choong *et al*., 2013). The amount of KNO_3 was reduced from 370 to 350 mg L^{-1} to achieve NPK ratio of 2.0:0.8:3.0. The group with 0.50× MS micronutrient level was assigned as the control medium. All treatments were incubated at 25°C and 16 h photoperiod with photon flux density of 30 µmol m^{-2} s^{-1} for 90 d, with a subculture done at day 45.

After that, roots and shoots of the seedlings were separated and respectively weighed to obtain the Shoot Fresh Weight (SFW) and Root Fresh Weight (RFW), subsequently dried at 70°C for 2 days before measuring the Shoot Dry Weight (SDW) and Root Dry Weight (RDW). Total Fresh Weight (TFW) and Total Dry Weight (TDW) of the seedlings were obtained by the addition of shoot and root fresh weight and dry weight respectively. Root to Shoot Ratio (R/S) was calculated by dividing RDW with SDW. Water content was calculated using on the formula (TFW-TDW)/TDW.

Data obtained from the measurements were tested for normality based on *z*-score of *kurtosis* and skewness at α = 0.05. Treatment means of the parameters mentioned above were analyzed by single-factor Analysis of Variance (ANOVA) and two-way pairwise comparisons between those treatment means were performed with Fisher's Least Significant Difference (LSD) test at α = 0.05.

3. RESULTS

Most of the data from the experiment were approved by normality tests in terms of *kurtosis* and skewness. Statistical analysis was only run with normal data, to prevent inaccuracy due to not normal data.

Analysis Of Variance (ANOVA) on various parameters of seedlings grown on different micronutrient levels showed that RFW, RDW, TFW, TDW, R/S ratio and WC were significantly different (ρ>0.995), while SFW and SDW not significantly different (**Table 1**).

The significance in ANOVA of RFW and RDW indicated that the micronutrient level affected the growth of roots. The significance in TFW and TDW indicated that micronutrient level also affected the total yield of seedlings. R/S ratio showed significant difference, which demonstrated that root development was also affected by micronutrient level. From ANOVA, there was no significant difference in SFW and SDW, which meant that shoot growth was not significantly influenced by micronutrient level tested in this experiment.

However, the ANOVA only indicated whether there were significant differences between the treatments but did not indicate differences among individual treatment means. Therefore multiple pairwise comparisons Fisher's LSD analysis was run after ANOVA (**Table 2**).

In terms of Fisher's LSD analysis, RFW, RDW, TFW, TDW and R/S ratio had a same distribution, which were resolved into two distinct groups where micronutrient level 0.25, 0.50 and 1.00× in group a, while 2.00× was in group b. For WC, there was three groups assigned, 0.25× and 0.50× in group a, 1.00× in group b and 2.00× in group c. SFW and SDW had no significance; all treatments were within one group.

Graph (**Fig. 1**) was used to represent the response of various parameters according to micronutrient level.

RFW (**Fig. 1c**) and TFW (**Fig. 1e**) had similar response pattern, which increased from 0.25× to 0.50× micronutrient level and steadily decreased from 0.50× to 2.00× micronutrient level; peaked when micronutrient level was at 0.50×. As it can be seen, root weight contributed mainly to total weight due to the fact that roots were at least 3.5 times heavier than shoots. R/S ratio (**Fig. 1g**) presented a similar trend as TFW. Apart from that, SDW (**Fig. 1b**), RDW (**Fig. 1d**) and TDW (**Fig. 1f**) had a peak between 0.50× and 1.0× micronutrient level, while SFW (**Fig. 1a**) had a maximum point between 0.5× to 0.75× micronutrient level. Graph for WC (**Fig. 1h**) had a same trend as RFW and R/S, which reflected that larger amount of fresh root and root development may contribute to the higher WC in seedlings. Thus, when RFW decreased, WC was observed to decrease as well.

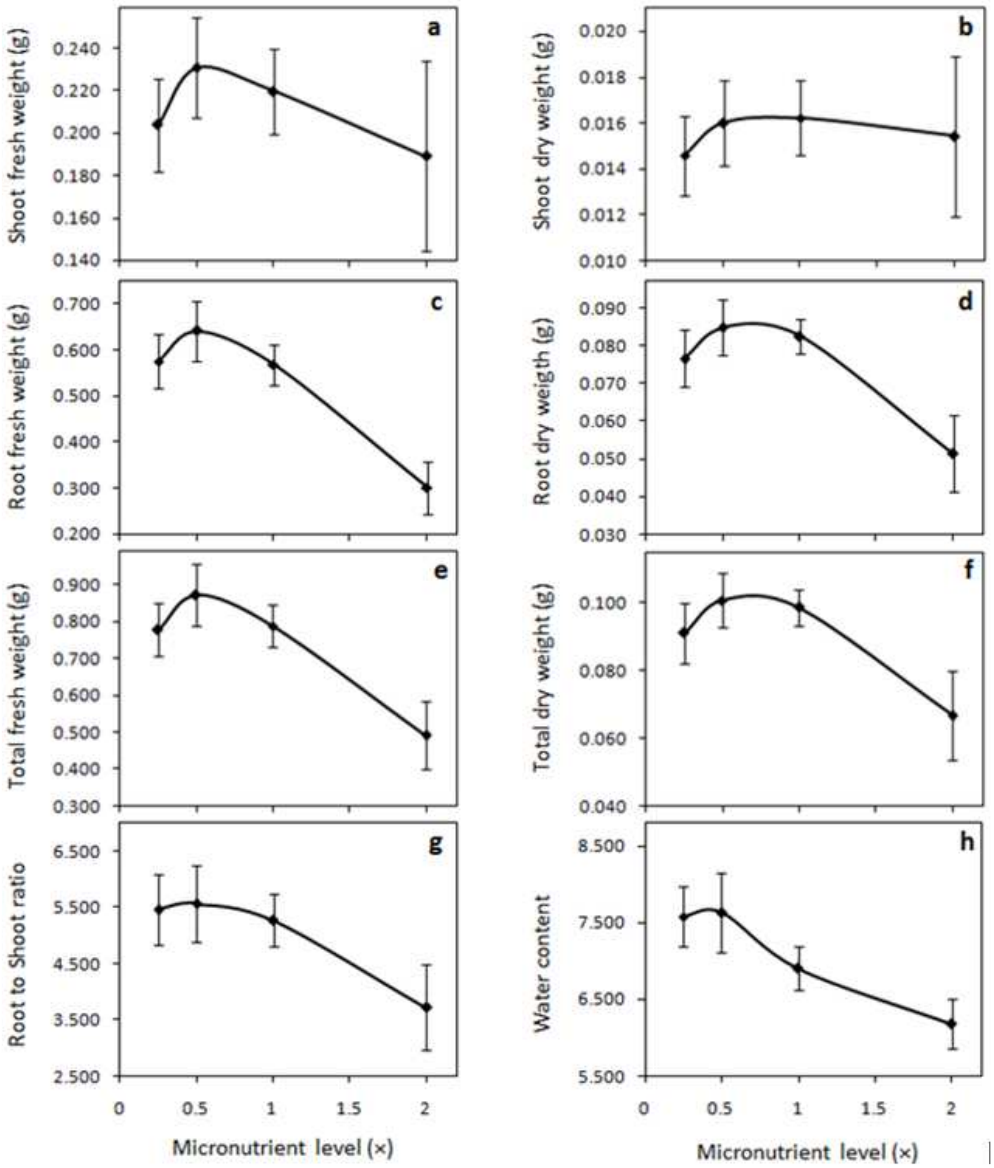

Fig. 1. Various parameters of *Phalaenopsis deliciosa in vitro* seedlings grown on different micronutrient levels. (a) Shoot Fresh Weight (SFW), (b) Shoot Dry Weight (SDW), (c) Root Fresh Weight (RFW), (d) Root Dry Weight (RDW), (e) Total Fresh Weight (TFW), (f) Total Dry Weight (TDW), (g) root to Shoot Ratio (R/S) and (h) Water Content (WC)

Table 1. Analysis Of Variance (ANOVA) on various parameters of seedlings grown on different micronutrient levels

Source of variation	Sum of Squares (SS)	Degree of Freedom (DF)	Mean Square (MS)	*F*-value
SFW	0.01900	3	0.006300	1.467^{ns}
Error	0.30700	72	0.004300	
SDW	0.00003	3	0.000010	0.387^{ns}
Error	0.00188	72	0.000026	
RFW	1.29000	3	0.430000	27.346*
Error	1.13200	72	0.016000	
RDW	0.01340	3	0.004500	15.219*
Error	0.02110	72	0.000300	
TFW	1.58100	3	0.527000	17.609*
Error	2.15500	72	0.030000	
TDW	0.01380	3	0.004600	10.969*
Error	0.03030	72	0.000400	
R/S	42.68200	3	14.227000	7.062*
Error	145.04200	72	2.014000	
WC	26.15400	3	8.718000	11.501*
Error	58.85200	72	0.817000	

*-99.5% significance (critical *F*-value = 4.73).

^{ns}-not significant.

Notes: SFW-shoot fresh weight, SDW-shoot dry weight, RFW-root fresh weight, RDW- root dry weight, TFW-total (seedling) fresh weight, TDW-total (seedling) dry weight, R/S-root to shoot ratio, WC-water content

Table 2. Treatment means of parameters of seedlings grown on different micronutrient level and resolved with Fisher's LSD analysis at $\alpha = 0.05$ into groups denoted by small caps superscript alphabets

Micronutrient level (×)	SFW	SDW	RFW	RDW	TFW	TDW	R/S	WC
0.25×	0.204±	0.015±	0.575±	0.077±	0.778±	0.091±	5.462±	7.579±
	0.022^{a}	0.002^{a}	0.058^{a}	0.008^{a}	0.074^{a}	0.009^{a}	0.616^{a}	0.394^{a}
0.50×	0.231±	0.016±	0.641±	0.085±	0.871±	0.101±	5.565±	7.635±
	0.024^{a}	0.002^{a}	0.065^{a}	0.007^{a}	0.085^{a}	0.008^{a}	0.674^{a}	0.523^{a}
1.00×	0.220±	0.016±	0.568±	0.082±	0.788±	0.099±	5.275±	6.906±
	0.020^{a}	0.002^{a}	0.044^{a}	0.004^{a}	0.056^{a}	0.005^{a}	0.456^{a}	0.291^{b}
2.00×	0.189±	0.015±	0.301±	0.051±	0.490±	0.067±	3.720±	6.181±
	0.045^{a}	0.003^{a}	0.057^{b}	0.010^{b}	0.092^{b}	0.013^{b}	0.766^{b}	0.328^{c}

Notes: SFW-shoot fresh weight, SDW-shoot dry weight, RFW-root fresh weight, RDW-root dry weight, TFW-total (seedling) fresh weight, TDW-total (seedling) dry weight, R/S-root to shoot ratio, WC-water content

4. DISCUSSION

Micronutrients are nutrients required in minute amount, which are components of many plant cell proteins involved in metabolic and physiological processes (George and De Klerk, 2008). In a previous study by (Murashige and Skoog, 1962), they introduced a medium called the MS medium that contained micronutrients (μM) Fe (100), B (100), Mn (100), Zn (30), I (5), Mo (1), Cu (0.2) and Co (0.2). Our experiment was based on the CCT medium containing different levels of MS micronutrients (2.00, 1.00, 0.50 and 0.25×). According to our observation, MS micronutrient level between 0.50× and 1.00× gave a better growth of the seedlings (**Fig. 2a**). Medium used for *Phalaenopsis* germination and growth *in vitro* often used 0.5× MS micronutrient level, such as those used for *Phalaenopsis bellina* (Khoddamzadeh *et al*., 2010) and *Phalaenopsis amabilis* (Chen and Chang, 2006).

In our observation, we found several replicates containing seedlings variants with very vigorous growth at 2.00× MS micronutrient level. The size of their leaves was larger than other treatments and the leaf color appeared as dark green; their roots also appeared unusually thick. These seedlings were variants with a phenotype that was tolerant to high concentration of micronutrients which was toxic to other seedlings. The appearance of these seedlings caused a second distribution which peaked at the higher end the first distribution. These data were removed, reducing the number of replicates from 23 to 19 for statistical analysis. This observation indicated that high concentration of micronutrient could select for several vigorous *P. deliciosa* seedlings and is undesirable for conservation purpose.

Loci responsible for tolerance to copper were identified in wheat (Bálint *et al*., 2007), supporting the existence of micronutrient tolerance trait in plant.

4.1. Micronutrient Excess

Micronutrient level higher than 1.00× affected the growth and development of roots and led to the decrease of yield. When micronutrient level was at 2.00×, the root tips became yellow and dark brown eventually (**Fig. 2b**). This revealed that micronutrient could be toxic to seedlings at high concentration. In a previous study done by (Sarkar *et al*., 2004) they found that excess of Mn supply was toxic for potato microplant and it caused stem streak necrosis as well as significant inhibition in seedling rooting and growth (Sarkar *et al*., 2004).

Mn^{2+} was one of the cofactors for IAA oxidases in plant cell (Galston and Hillman, 1957) as cited by (George and De Klerk, 2008). Therefore presence of Mn in culture medium could activate the IAA oxidases and promoted the degradation of auxin; thus it reduced the development of roots. In addition, high concentration of B could cause excessive IAA degradation as well, reducing root growth (Jarvis, 1986). B excess could cause yellowing of leaf margin and tip (Gupta, 2006), which occurred in some of the seedlings treated with 2.00× MS micronutrient.

Besides, high level of Zn was found to be inhibitory and prevent root growth, such as the result obtained by (Malik *et al*., 2011) in their experiment concerning effect of different levels of Zn on growth and yield of red amaranth and rice. Sedberry *et al*. (1988) found that Zn application resulted in a reduction of P concentration in rice plant tissue at the first joint and this may also be another reason for yield reduction on medium containing 2.00× MS micronutrient.

Figure 2 *Phalaenopsis deliciosa in vitro* seedlings. (a) Comparison of seedlings treated with different levels of MS micronutrients (0.25×, 0.50×, 1.00× and 2.00×). (b) *P. deliciosa* seedling grown on 2.00× MS micronutrient displayed yellow root tip (indicated by the arrowhead) that would eventually turn dark brown. Bar = 1 cm.

To compare, in Gamborg B5 medium used for general culturing, Zn concentration was 0.46 mg L^{-1} (Gamborg *et al*., 1968), which was less than half of 0.5× MS medium (0.96 mg L^{-1}) (Murashige and Skoog, 1962) (**Table 3**).

In XER medium (Ernst, 1994) and NDM medium (Tokuhara and Mii, 1993) used for culturing *Phalaenopsis*, the Zn concentration was even lower (0.11 mg L^{-1}). Thus, Zn concentration higher than 1.00× may be excessive and inhibitory to *P. deliciosa* seedling growth and development and this was probably due to Zn-induced P deficiency (Storey, 2007).

4.2. Micronutrient Deficiency

Regarding to our observation, we found that seedlings treated with 0.25× MS micronutrient had a lower growth rate but with no observable abnormality on root development. The leaves of these seedlings were lighter in green tone and the roots were thinner and shorter than roots of those seedlings from other treatments. This was probably due to lower than optimum concentration of micronutrients reduced metabolism of the seedlings. For example, manganese has similar chemical properties as magnesium in some enzymatic systems (Hewitt, 1948) and part of the structure of metalloproteins that participates in respiration and photosynthesis (Clarkson and Hanson, 1980). Therefore, deficient in Mn supply gave rise to low enzyme activity and subsequently may lead to reduction of protein, nucleic acid and chlorophyll synthesis.

Table 3. Comparison of the micronutrient content of 4 different culture media

	Medium			
Micronutrients (mg/L)	MS[1]	CCT[2] (0.5× MS)	NDM[3] and XER[4]	B5[5]
B	1.080	0.540	0.020	0.540
Mn	5.500	2.750	0.740	3.300
Zn	1.920	0.960	0.110	0.460
I	0.640	0.320	0.000	0.570
Cu	0.006	0.003	0.006	0.006
Mo	0.096	0.048	0.010	0.096
Co	0.006	0.003	0.006	0.006

Murashige and Skoog (1962), Choong *et al*. (2013), New Dogashima Medium (NGM)-Tokuhara and Mii (1993); Ernst (1994), B5 medium-Gamborg *et al*. (1968)

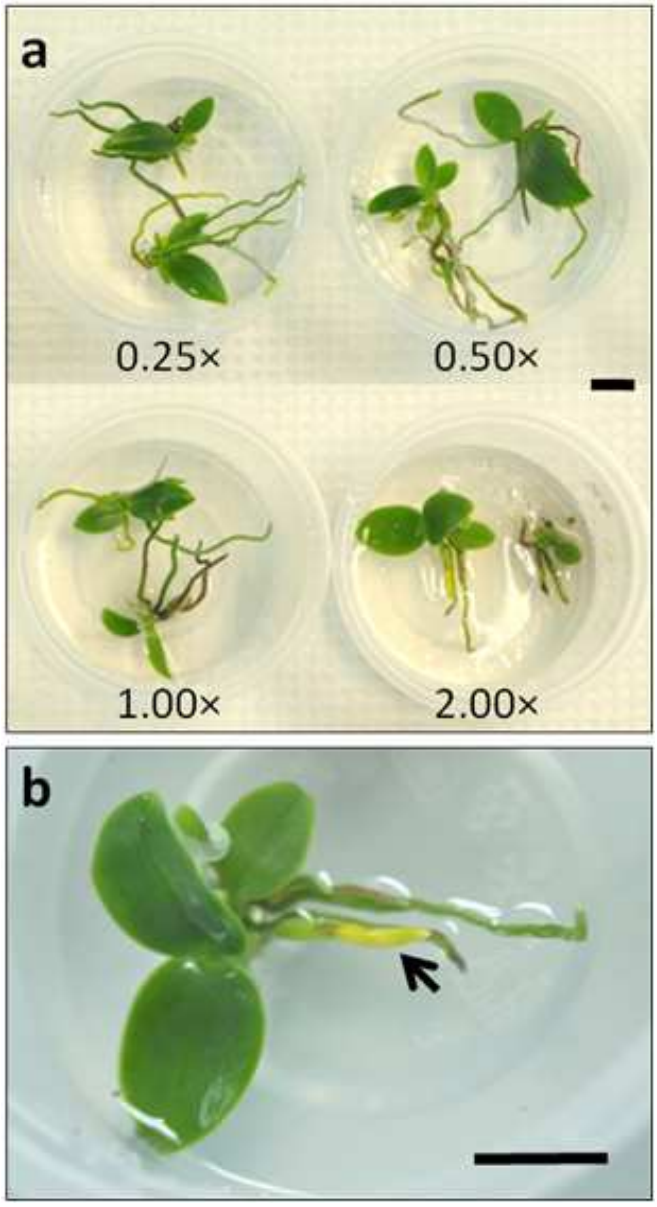

Fig. 2. *Phalaenopsis deliciosa in vitro* seedlings. (a) Comparison of seedlings treated with different levels of MS micronutrients (0.25, 0.50, 1.00 and 2.00×). (b) *P. deliciosa* seedling grown on 2.00× MS micronutrient displayed yellow root tip (indicated by the arrowhead) that would eventually turn dark brown. Bar = 1 cm

4.3. Comparisons Between Different Culture Media

When comparing the components of 0.50× MS micronutrient with other media, it was found that the B concentration was similar to the B5 medium (Gamborg *et al*., 1968) but much higher than NDM and XER media (Tokuhara and Mii, 1993; Ernst, 1994) (**Table 3**). The concentration of Cu and Co in 0.50× MS medium was half of the other 3 media. The amount of Cu and Co in the CCT medium could be doubled to achieve better seedling growth. In B5 medium, the Zn concentration was approximately half of 0.50× MS micronutrient and in NDM medium the concentration of Zn was even much lower than 0.50× MS micronutrient

(near one ninth). Hence, CCT medium could be better for seedling growth if the amount of Zn in the medium was reduced. Experiments involving different levels of individual micronutrient could be done to identify the optimum level of each micronutrient for *P. deliciosa*.

5. CONCLUSION

Based on our observation and data analysis, we found out that the optimum micronutrient level for *P. deliciosa* was between 0.50× to 1.00× MS micronutrient level in CCT medium. Micronutrient level between 0.50× to 1.00× gave the maximum development and growth of roots. High micronutrient level inhibited growth and development of roots probably due to excessive Mn-associated and B-associated IAA degradation and Zn-induced P deficiency. However some of variants had the capability of tolerating the high micronutrient level and grew vigorously. Low micronutrient level could cause deficiency of micronutrients and non-optimum growth. Besides, WC of the seedlings varied when grown on different micronutrient level; the roots of *P. deliciosa* contained most of the water, which predominantly contributed to total water content of whole seedlings. Micronutrient level did not have a significant influence on the growth of shoots. MS micronutrient of 0.50× or slightly higher is recommended for *in vitro* seedling growth and root development of *P. deliciosa*. Alternatively, a new micronutrient mixture can be designed based on literature or empirically determined by experimenting with individual micronutrient one at a time.

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