OnLine Journal of Biological Sciences

Original Research Paper

Effect of *In Vitro* Nitrogen Nutrition to *Phalaenopsis deliciosa* Seedling Growth

[1]Chieh Wean Choong, [1]Chin Hao Lim, [1]Zherui Xiong and [2]Siew Mee Choong

[1]*Faculty of Science, Technology, Engineering and Mathematics (FOSTEM), INTI International University, Persiaran Perdana BBN, Putra Nilai, 71800 Nilai, Negeri Sembilan, Malaysia*
[2]*HELP University, BZ-2 Pusat Bandar Damansara, 50490 Kuala Lumpur, Malaysia*

Article history
Received: 30-06-2015
Revised: 08-08-2015
Accepted: 31-10-2015

Corresponding Author:
Chieh Wean Choong
Faculty of Science, Technology, Engineering and Mathematics (FOSTEM), INTI International University, Persiaran Perdana BBN, Putra Nilai, 71800 Nilai, Negeri Sembilan, Malaysia

Tel: +606 798 2000
Fax: +606 799 7513
Email: chiehwean.choong@newinti.edu.my

Abstract: In a continuous effort to conserve *Phalaenopsis* species, the optimization of a previously designed defined medium was carried out to understand the effect of *in vitro* nitrogen nutrition on *Phalaenopsis deliciosa* Rchb.f. In order not to alter the nutrient balance, macronutrient level instead of solely Nitrogen (N) was adjusted. Seedlings were obtained from *in vitro* germination and randomly inoculated on media with various macronutrient levels (0.3×, 0.5×, 1×, 2× and 3×). Maximum shoot yield was observed at 2× macronutrient level. However, the best macronutrient level, which was 1× macronutrient level, resulted in the maximum root and seedling yield. This macronutrient level was applied on the subsequent experiment where seedlings were inoculated on media with various nitrate-nitrogen (NO_3-N) to ammonium-nitrogen (NH_4-N) ratios. Seedling yield was the highest at NO_3-N/NH_4-N ratio of 2.0 while Root to Shoot ratio (R/S) was the highest at NO_3-N/NH_4-N ratio of 5.0. NO_3-N/NH_4-N ratio significantly affected Water Content (WC) of seedlings and the response to NO_3-N/NH_4-N ratio was similar to R/S, correlating better root development to higher WC. By altering NO_3-N/NH_4-N ratio, plant quality in terms of yield and organ development could be encouraged.

Keywords: Ammonium-Nitrate, *In Vitro* Nutrition, Macronutrient, Moth Orchid, Nitrogen, *Phalaenopsis deliciosa*

Introduction

Wild orchids, including *Phalaenopsis* species are listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2013) as endangered. *P. deliciosa* is a miniature *Phalaenopsis* that grows fast and grouped in the same subgenus (Christenson, 2001) as its more well-known moth orchid relatives, such as *Phalaenopsis amabilis* that is extensively used for ornamental breeding. *P. deliciosa* is therefore a suitable model plant for experimentation.

Phalaenopsis species *in vitro* nutritional requirement for seedling growth was not extensively studied. Often, complex organic supplements such as peptone (Chen and Chang, 2006), banana extract (Khoddamzadeh *et al*., 2010) and others (Ichihashi and Islam, 1999), were added to the existing basal media to promote seedling growth *in vitro*. Recently, a defined medium which was non-genotype-selective was found to be suitable for growing four different *Phalaenopsis* species (Choong *et al*., 2013) without complex organic supplement. This medium was designed based on literature and optimization of the medium is necessary, of which at the same time, understanding on the nutritional requirement for *Phalaenopsis* species seedling growth and development could be achieved.

One nutrient of interest is N, which is the most important macronutrient for plants, notably as the component of protein and nucleic acids. Protein in leaves predominantly accumulated in chloroplast where in N deficiency, chloroplast structure is severely affected accompanied by loss of chlorophyll (Barker and Bryson, 2007). N nutrition is often balanced with Phosphorus (P) and Potassium (K), thus N is often expressed as ratio to other macronutrients to indicate these macronutrients' relative amount to N. N is known to interact with Sulfur (S), where they affect assimilation of one another (Jamal *et al*., 2010).

N is usually supplied as ammonium and nitrate, the former being the more readily absorbed and metabolized form. Ammonium assimilation is efficient in alkali condition, discharge proton and thus acidify culture medium (George and De Klerk, 2008) where at sufficient concentration, causes ammonium toxicity (Britto and Kronzucker, 2002). Ammonium was also thought to affect endogenous growth regulators metabolism, resulting in the increase of shoot to root ratio (Britto and Kronzucker, 2002). At high concentration, it was also shown to induce production of ethylene as stress signal (Barker and Corey, 1991).

In contrast, nitrate assimilation is efficient in acidic condition, alkalinize culture medium when assimilated (George and De Klerk, 2008) and require conversion to ammonium prior to metabolism (Britto and Kronzucker, 2002), thus slower growth response. Co-presence of ammonium and nitrate however balances medium pH (George and De Klerk, 2008) and plant development (Britto and Kronzucker, 2002) in addition to more efficient assimilation of other nutrients (Kubota *et al*., 2000; Van Beusichem, 1988). For example, nitrate increases while ammonium reduces assimilation of Magnesium (Mg) (Merhaut, 2007). The optimum ratio depends on the species or even the cultivar of a species (Duan *et al*., 2007).

In this study, the optimum nitrogen concentration will be determined by measuring growth of seedlings on medium with increasing and decreasing macronutrient level. Macronutrient was modified instead of N to ensure that the nutrient balance of N with other macronutrients was not altered. After that, using the identified optimum N concentration, response of seedlings to various NO_3-N/NH_4-N ratios was observed. Thus the aim of this study was to identify optimum nitrogen concentration and NO_3-N/NH_4-N ratio for growth and development of *P. deliciosa*.

Materials and Methods

Seed Germination

Seedpods of *Phalaenopsis deliciosa* Rchb.f. was cleaned briefly under the flow of tap water and then immersed in 5.25% sodium hypochlorite for 5 min in a laminar air flow cabinet. Then, the seedpods were transferred into 70% ethanol and immersed for 1 min. The seedpods were flamed briefly until traces of ethanol evaporated. By using a scalpel, seedpods were excised and the seeds within were inoculated on a modified Choong *et al*. (2013) defined medium, where KNO_3 was reduced from 370 to 350 mg L^{-1}. This medium had 1× macronutrient level and NO_3-N/NH_4-N ratio of 5.0. Germinated seedlings were transferred onto the same medium. Thereafter, subculture was done every 90 days.

Medium Preparation

Basal medium used for macronutrient level study was the modified CCT medium as described above except for the macronutrient level that was altered to 1/3×, 1/2×, 1×, 2× and 3×. Macronutrients altered were NH_4NO_3, KNO_3, $Ca(NO_3)_2·4H_2O$, $Mg(NO_3)_2·6H_2O$, KH_2PO_4, $CaSO_4·2H_2O$ and $MgSO_4·7H_2O$. Basal medium used for NO_3-N/NH_4-N ratio study was the modified CCT medium as described above except that the NO_3-N/NH_4-N ratios were altered to 5.0, 4.0, 3.0 and 2.0. This was achieved by increasing NH_4NO_3 and decreasing KNO_3 and $Ca(NO_3)_2·4H_2O$. Potassium and calcium final concentrations were maintained with KCl and $CaCl_2·2H_2O$. Media were autoclaved at 121°C and 15 psi for 15 min. After that, media were poured approximately 20 ml per sterile 100-mL polypropylene food grade container in a laminar air flow cabinet.

Experimental Procedure

Approximately 0.3±0.05 g of seedlings was randomly inoculated onto the prepared medium in 8 replications for macronutrient level study and 9 replications for NO_3-N/NH_4-N ratio study. Seedlings were incubated at 25°C with 16 hours photoperiod at 30 µmol m^{-2} s^{-1} photon flux density for 90 days. In between, seedlings were subcultured onto the same media after 45 days of incubation. After the incubation period, seedlings from each flask/subject were washed to remove gel traces and blotted dry on tissue paper before dissected into shoots and roots. Shoot Fresh Weight (SFW) and Root Fresh Weight (RFW) for each subject were measured by weighing the freshly dissected shoots and roots respectively. Then those shoots and roots were dried at 70°C for 2 days. Shoot Dry Weight (SDW) and Root Dry Weight (RDW) for each subject were measured by weighing the dried shoots and roots respectively.

Calculations

Total (seedling) Fresh Weight (TFW) for each subject was calculated by the addition of SFW and RFW. Total (seedling) Dry Weight (TDW) for each subject was calculated by the addition of SDW and RDW. Root to Shoot Ratio (R/S) for each subject was calculated by dividing RDW with SDW. Water Content (WC) for each subject was calculated with the formula (TFW-TDW)/TDW.

Statistical Analysis

Measured and computed variables for each of the parameter mentioned above were subjected to normality test in terms of *z*-score of *kurtosis* and skewness at α = 0.05. Normally-distributed data was analyzed with single-factor Analysis of Variance (ANOVA) to test for significant difference between the groups/treatments. After that, two-way pairwise comparisons were performed with Fisher's Least Significant Difference (LSD) to test for significant difference between pairs of treatment means at α = 0.05. Treatment means were assigned into groups represented by lowercase alphabets, with the highest mean values assigned to group a while the next significantly lower mean values assigned to group b and so on.

Results

Data passed normality test in terms of *kurtosis* and skewness. Only normal data would be used for statistical analysis to prevent bias that may arise due to outliers.

Macronutrient Level

Single-factor between-subject ANOVA on the measured parameters showed that SFW, SDW and R/S were significantly different (ρ>0.995) between seedlings treated with different macronutrient level (Table 1). Other parameters, RFW, RDW, TFW, TDW and WC were not significantly different (ρ<0.95) between seedlings treated with different macronutrient level. Macronutrient level significantly affected shoot growth/yield, thus affecting R/S ratio of seedlings.

ANOVA only indicated whether there were significant differences between the treatments but did not indicate differences among individual treatment means. After multiple pairwise comparisons with Fisher's LSD, it was found that RFW, RDW, TFW and TDW did produce significant difference between seedlings treated with different macronutrient level (Table 2). The treatment means of these parameters were separated into two distinct groups, a and b. Parameters that exhibited significant differences earlier with ANOVA, i.e., SFW, SDW and R/S, were separated into three distinct groups denoted by a, b and c. WC was found to be not significantly different across the macronutrient levels for both ANOVA and Fisher's LSD analysis.

Table 1. Single-factor between-subjects ANOVA on measured parameters of seedlings treated with different macronutrient level. Significance (ρ) is indicated for each of the parameter

Source of variation	Sum of Square (SS)	Degree of Freedom (DF)	Mean Square (MS)	F value	Critical F value (significance)
SFW	0.16300	4	0.04100	5.369	4.62 (ρ>0.995)
Error	0.26600	35	0.00800		
SDW	0.00092	4	0.00023	6.162	4.62 (ρ>0.995)
Error	0.00130	35	0.00004		
RFW	0.17600	4	0.04400	1.242	2.69 (ρ<0.95)
Error	1.24000	35	0.03500		(not significant)
RDW	0.00169	4	0.00042	1.238	2.69 (ρ<0.95)
Error	0.01191	35	0.00034		(not significant)
TFW	0.48700	4	0.12200	1.814	2.69 (ρ<0.95)
Error	2.34800	35	0.06700		(not significant)
TDW	0.00317	4	0.00079	1.466	2.69 (ρ<0.95)
Error	0.01891	35	0.00054		(not significant)
R/S	110.61700	4	27.65400	7.881	4.62 (ρ>0.995)
Error	122.80900	35	3.50900		
WC	1.49000	4	0.37200	0.756	2.69 (ρ<0.95)
Error	17.24500	35	0.49300		(not significant)

Table 2. Mean values of parameters with 95% confidence intervals of seedlings treated with different macronutrient level. Multiple pairwise comparisons with Fisher's LSD assigned mean values into different groups and is shown by the superscript alphabets

Macronutrient level (×) (Nitrogen concentration)	SFW (g)	SDW (g)	RFW (g)	RDW (g)	TFW (g)	TDW (g)	R/S	WC
1/3 × (4.7 mM)	0.183± 0.040^{c}	0.011± 0.003^{c}	0.701± 0.083^{b}	0.077± 0.010^{b}	0.884± 0.109^{b}	0.089± 0.013^{b}	7.976± 2.526^{a}	9.060± 0.342^{a}
1/2 × (7.1 mM)	0.243± 0.041^{bc}	0.016± 0.003^{bc}	0.781± 0.063^{ab}	0.088± 0.011^{ab}	1.024± 0.095^{ab}	0.103± 0.014^{ab}	6.055± 1.255^{b}	9.062± 0.557^{a}
1 × (14.2 mM)	0.312± 0.031^{ab}	0.021± 0.002^{ab}	0.903± 0.130^{a}	0.096± 0.011^{a}	1.215± 0.150^{a}	0.116± 0.013^{a}	4.700± 0.305^{bc}	9.429± 0.496^{a}
2 × (28.4 mM)	0.360± 0.099^{a}	0.025± 0.007^{a}	0.766± 0.176^{ab}	0.081± 0.017^{ab}	1.126± 0.258^{ab}	0.106± 0.022^{ab}	3.470± 0.596^{c}	9.542± 0.430^{a}
3 × (42.6 mM)	0.328± 0.064^{ab}	0.022± 0.004^{a}	0.755± 0.162^{ab}	0.082± 0.014^{ab}	1.084± 0.226^{ab}	0.104± 0.018^{ab}	3.734± 0.150^{c}	9.278± 0.569^{a}

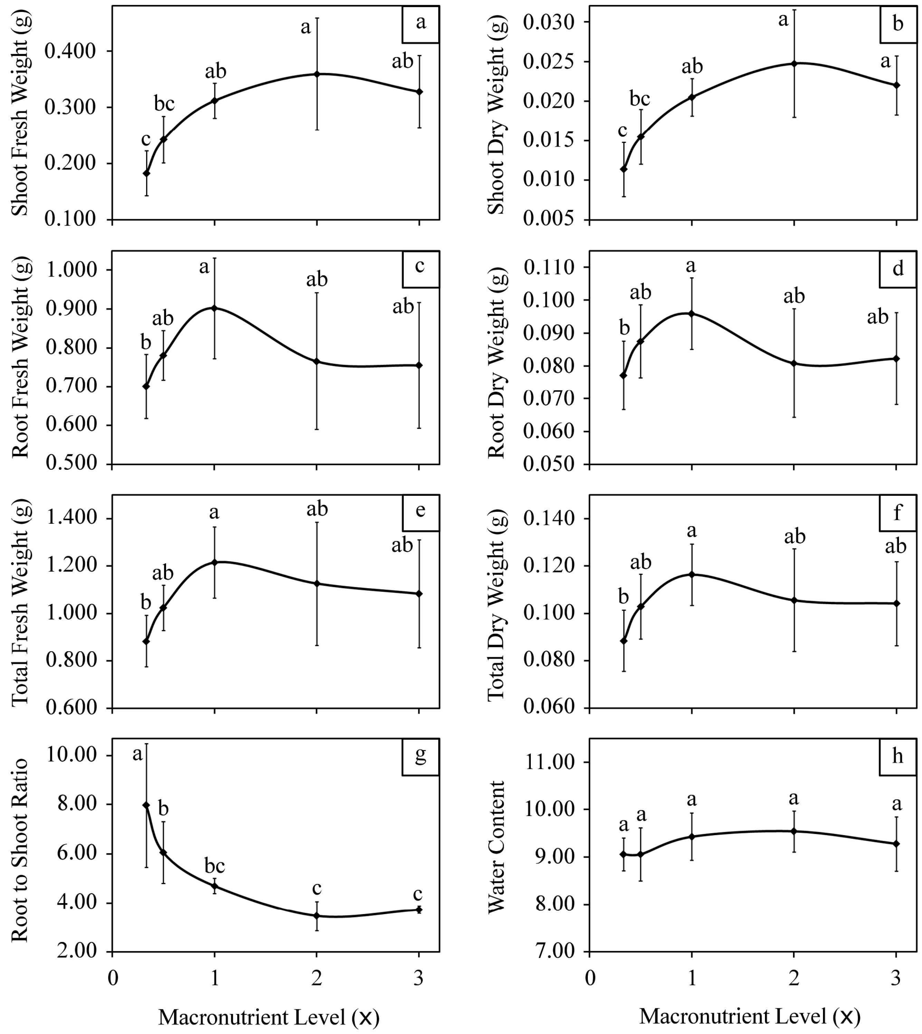

Fig. 1. Changes of treatment means of parameters, (a) shoot fresh weight (SFW), (b) shoot dry weight (SDW), (c) root fresh weight (RFW), (d) root dry weight (RDW), (e) total fresh weight (TFW), (f) total dry weight (TDW), (g) root/shoot ratio (R/S) and (h) water content (WC), with macronutrient level. Error bars indicate 95% confidence intervals. Different groups are shown by the lowercase alphabets above the error bars

Graph was used to represent the treatment means of various parameters. SFW and SDW were at maximum at 2× macronutrient level (Fig. 1A and 1B). RFW and RDW however were at maximum at 1× macronutrient level (Fig. 1C and 1D). Since root weight was much heavier than shoot weight, root weight contributed more weight to the seedlings, thus TFW and TDW were also maximum at 1× macronutrient level (Fig. 1E and 1F). R/S reduced with macronutrient level and was at minimum at 2× macronutrient level (Fig. 1G) that was mentioned above to produce maximum shoot yield. WC was not significantly different with macronutrient level (Fig. 1H).

NO_3-N/NH_4-N Ratio

As shown in Table 3, SDW, RDW, TDW, R/S and WC were significantly different (ρ>0.995) between seedlings treated with different NO_3-N/NH_4-N ratio. Parameters SFW, RFW and TFW were not significantly different (ρ<0.95) between seedlings treated with different NO_3-N/NH_4-N ratio. WC was significantly different between treatments causing significant differences to occur with dry weight parameters but not fresh weight parameters.

As shown in Table 4, Fisher's LSD analysis found that seedlings treated with different NO_3-N/NH_4-N ratio supported the result of the ANOVA analysis, with SFW, RFW and TFW were not significantly different, thus all the treatment means were assigned in group a. Treatment means of the rest of the parameters could be clearly assigned into two distinct groups, a and b, except for the treatment means of SDW which could be assigned into 3 distinct groups, a, b and c.

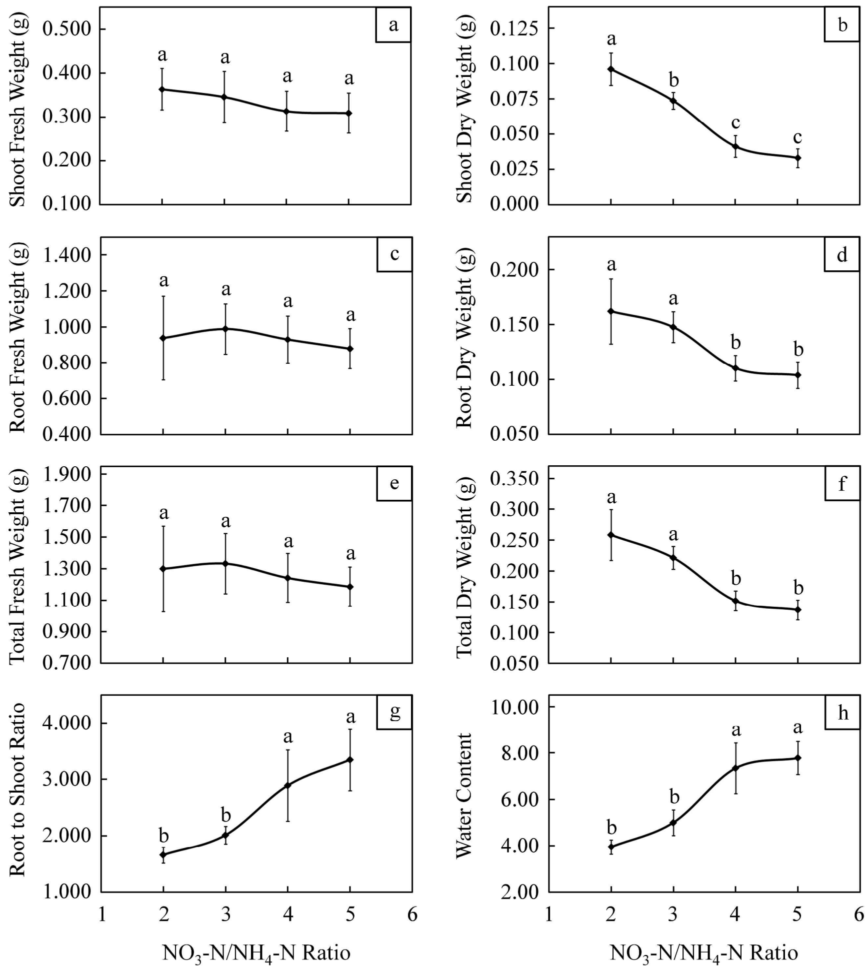

Fig. 2. Changes of treatment means of parameters, (a) shoot fresh weight (SFW), (b) shoot dry weight (SDW), (c) root fresh weight (RFW), (d) root dry weight (RDW), (e) total fresh weight (TFW), (f) total dry weight (TDW), (g) root/shoot ratio (R/S) and (h) water content (WC), with NO_3-N/NH_4-N ratio. Error bars indicate 95% confidence intervals. Different groups are shown by the lowercase alphabets above the error bars

Table 3. Single-factor between-subjects ANOVA on measured parameters of seedlings treated with different NO_3-N/NH_4-N ratio. Significance (ρ) is indicated for each of the parameter

Source of variation	Sum of Square (SS)	Degree of Freedom (DF)	Mean square (MS)	F value	Critical F value (significance)
SFW	0.019	3	0.0060	1.0790	2.92 (ρ<0.995)
Error	0.184	32	0.0060		(not significant)
SDW	0.023	3	0.0077	48.4180	5.24 (ρ>0.995)
Error	0.005	32	0.0002		
RFW	0.054	3	0.0180	0.2940	2.92 (ρ<0.995)
Error	1.947	32	0.0610		(not significant)
RDW	0.022	3	0.0072	0.0008	5.24 (ρ>0.995)
Error	0.026	32	9.0900		
TFW	0.112	3	0.0370	0.4270	2.92 (ρ<0.995)
Error	2.788	32	0.0870		(not significant)
TDW	0.089	3	0.0300	19.9960	5.24 (ρ>0.995)
Error	0.048	32	0.0010		
R/S	16.303	3	5.4340	12.5990	5.24 (ρ>0.995)
Error	13.802	32	0.4310		
WC	91.467	3	30.4890	24.7040	5.24 (ρ>0.995)
Error	39.493	32	1.2340		

DOI: 10.3844/ojbsci.2016.17.25

Table 4. Mean values of parameters with 95% confidence intervals of seedlings treated with different NO_3-N/NH_4-N ratio. Multiple pairwise comparisons with Fisher's LSD assigned mean values into different groups and is shown by the superscript alphabets

NO_3-N/NH_4-N ratio	SFW (g)	SDW (g)	RFW (g)	RDW (g)	TFW (g)	TDW (g)	R/S	WC
2.0	0.363± 0.047[a]	0.096± 0.012[a]	0.937± 0.234[a]	0.162± 0.030[a]	1.300± 0.271[a]	0.258± 0.041[a]	1.656± 0.137[b]	3.942± 0.310[b]
3.0	0.346± 0.059[a]	0.074± 0.006[b]	0.988± 0.141[a]	0.148± 0.014[a]	1.334± 0.191[a]	0.221± 0.018[a]	2.013± 0.155[b]	4.997± 0.552[b]
4.0	0.313± 0.045[a]	0.041± 0.008[c]	0.930± 0.131[a]	0.110± 0.012[b]	1.243± 0.154[a]	0.151± 0.016[b]	2.892± 0.632[a]	7.335± 1.096[a]
5.0	0.309± 0.045[a]	0.033± 0.007[c]	0.879± 0.111[a]	0.104± 0.012[b]	1.188± 0.124[a]	0.137± 0.016[b]	3.343± 0.542[a]	7.772± 0.711[a]

Figure 2 shows the changes of treatment means of various parameters with NO_3-N/NH_4-N ratio. SFW, RFW and TFW were not significantly different with NO_3-N/NH_4-N ratio (Fig. 2A, 2C and 2E). SDW, RDW and TDW were the highest at NO_3-N/NH_4-N ratio of 2.0 and reduced all the way to NO_3-N/NH_4-N ratio of 5.0 (Fig. 2B, 2D and 2F). However, the largest difference occurred between NO_3-N/NH_4-N ratio 3.0 and 4.0. R/S and WC were the lowest at NO_3-N/NH_4-N ratio of 2.0 and increased all the way to NO_3-N/NH_4-N ratio of 5.0 (Fig. 2G and 2H). Similarly, the largest difference occurred between NO_3-N/NH_4-N ratio 3.0 and 4.0.

Discussion

Data collected was normal in terms of *kurtosis* and skewness. Previously, it was found that MS medium supplemented with peptone and 0.17 g of NaH_2PO_4 selectively favor the growth of several *Phalaenopsis bellina* seedlings but majority of other seedlings experience poor growth, producing not normal data (Choong *et al*., 2013). Such result supported that certain genotypes of (vigorous) *Phalaenopsis* was thought to be selected *in vitro* (Christenson, 2001). Our result in this study further confirmed the non-genotype-selective nature of CCT medium, even after macronutrient level and NO_3-N/NH_4-N ratio modifications.

Macronutrient level was found to affect shoot yield, root yield, seedling yield and R/S but not WC of *P. deliciosa* seedlings. Maximum shoot yield was observed at 2× macronutrient level while maximum root and seedling yield was obtained at 1× macronutrient level. Root weight was far greater than shoot weight, hence contributed more to the seedling yield than shoot weight. However, in term of significance, shoot yield (ρ>0.995) was more significantly affected by macronutrient than root yield (ρ>0.95). This is not surprising given the fact that N, a component of macronutrient, greatly affect normal chloroplast and chlorophyll formation in leaves (Barker and Bryson, 2007), thus its importance in shoot growth. R/S ratio was higher at lower macronutrient level and reduced to a minimum at 2× macronutrient level, which coincide with maximum shoot yield. As N increases, crop yield increases as well. However deficiency of N will reduce leaf to stem ratio, indicating positive effect of N to leaf yield (Lawlor, 2002).

Nitrogen is the most important element in macronutrient. The effect of macronutrient level to orchid development was similar to response of *P. amabilis* to N, a species in the same subgenus as *P. deliciosa* (Christenson, 2001), where R/S ratio was found to reduce with N concentration (Tavares *et al*., 2012). In addition, we observed maximum seedling yield at 1× macronutrient level, or 14.2 mM or 200 ppm of N. This result was contradicting with work on *P. amabilis* and *Phalaenopsis* hybrid, where maximum yield was observed at 7.5 mM (Tavares *et al*., 2012) or 100 ppm (Poole and Seeley, 1978) of N respectively. This could be due to the higher amount of phosphorus in the media used in this study, which was 5.9 mM or 180 ppm compared to 1.25 mM (Tavares *et al*., 2012) and 20 ppm (Poole and Seeley, 1978) in other studies.

In all the curves involving yield *versus* macronutrient level, there appeared a maximum value where higher or lower macronutrient level from this point both reduced yield. In contrast, theoretically, increase of N will increase yield until it reaches asymptote where further increase of N will not increase yield anymore (Lawlor, 2002). Similarly to Tavares *et al*. (2012), our results did not exactly fit the N response curve probably because of another factor at work, the genetic factor, since all the plants used for the study were of different genotypes raised from different seeds.

In addition, our strategy was to increase macronutrient level, thus preserving the nutrient balance of all the macronutrients in the media. This was different with the approach used by other authors (Tavares *et al*., 2012; Poole and Seeley, 1978) where only nitrogen was altered but other macronutrients were not. This experiment was done with the assumption that nutrient balance could be another factor contributing to the differences observed, especially for important nutrient such as N. For example, fertilizers are normally applied in N:P:K ratio. In addition, N:S ratio was shown to affect *P. bellina* yield (Choong and Choong, 2013).

At higher level of macronutrient, nutrients such as P and S may be at concentrations that were inhibitory to growth, thus lowering yield rather than asymptotic. P excess is known to reduce micronutrient availability to crops (Sanchez, 2007). High concentration of S *in vitro* was shown to significantly reduce *P. bellina* seedling yield (Choong and Choong, 2013). We believed that ammonium toxicity probably was not the contributing factor to yield reduction at high macronutrient level since NO_3-N/NH_4-N ratio was maintained at 5.0. At this ratio, pH would be balanced as these N sources were assimilated (George and De Klerk, 2008). In addition, NH_4-N concentration at 3× macronutrient level was 7.1 mM, lower than the 8 mM critical NH_4-N concentration (Gamborg *et al*., 1976) that was suggested to possibly cause ammonium toxicity *in vitro*.

Different NO_3-N/NH_4-N ratio produced significant differences with seedling WC, thus significant differences between treatments were observed with dry weight parameters but not fresh weight parameters. Higher NO_3-N/NH_4-N ratio increased R/S ratio, favoring root development relative to shoot development that also coincided with higher WC in this study and *vice versa*. On the other hand, lower NO_3-N/NH_4-N ratio increased seedling yield or biomass and *vice versa*.

As NO_3-N/NH_4-N ratio increased, R/S also increased (Britto and Kronzucker, 2002) and this was also as observed in *Phalaenopsis* hybrid (Kubota *et al*., 2000) and other species such as coniferous trees (Cumming and Brown, 1994; Van Dijk *et al*., 1990). There is no literature information stating that NO_3-N/NH_4-N ratio affects WC. However, in this present study, the response curve of WC *versus* NO_3-N/NH_4-N ratio resembled the response curve of R/S *versus* NO_3-N/NH_4-N ratio. Differences in R/S caused by NO_3-N/NH_4-N ratio could be the reason for the differences in WC observed in those seedlings.

Although seedling fresh weights were not significantly different, seedlings grown on lower NO_3-N/NH_4-N ratio had higher dry weight or biomass (lower water content) than those grown on higher NO_3-N/NH_4-N ratio. *Phalaenopsis* was found to absorb solely ammonium, nitrate and even urea (Trépanier *et al*., 2009), tolerating wide range of nitrogen sources but preferring solely ammonium to solely nitrate. This could be the reason for higher biomass yield observed with lower NO_3-N/NH_4-N ratio or higher concentration of ammonium.

However, using solely ammonium to grow *P. deliciosa* seedling *in vitro* may not be practical due to acidification of medium as ammonium is assimilated (George and De Klerk, 2008; Britto and Kronzucker, 2002). This would require buffer or glutamate addition to balance medium pH (George and De Klerk, 2008). In addition, if only ammonium is used, then the concentration of ammonium in the medium would be 14.2 mM, more than the critical ammonium concentration of 8 mM (Gamborg *et al*., 1976) and this may cause ammonium toxicity. Potted *Phalaenopsis* was found not to grow well at 100% ammonium fertilizer (Wang, 2008). Co-presence of nitrate is therefore important to balance medium pH (George and De Klerk, 2008) and alleviate ammonium toxicity (Britto and Kronzucker, 2002).

In wheat, co-presence of nitrate and ammonium was found to have better nutrient absorption and grain yield than nitrate alone (Heberer and Below 1989). Similarly, sunflower leaf biomass and total nitrogen was higher with co-presence of nitrate and ammonium than nitrate or ammonium alone (Weissman, 1964). Study done on *Phalaenopsis* hybrid by Kubota *et al*. (2000) showed that the best yield and nutrient absorption occurred at high NO_3-N/NH_4-N ratio, ranging from 6:4 (1.5) to 10:0 (solely nitrate). *Phalaenopsis species* was shown to grow well on medium with NO_3-N/NH_4-N ratio of 5 (Choong *et al*., 2013) and it is of interest to study the response to lower NO_3-N/NH_4-N ratio as ammonium is preferred by *Phalaenopsis* to nitrate (Trépanier *et al*., 2009).

This study identified that NO_3-N/NH_4-N ratio of 2.0 produced highest biomass yield. Two other basal media commonly used for culturing *Phalaenopsis* were Murashige and Skoog (1962) medium and New Dogashima Medium (Tokuhara and Mii, 1993), both of which had NO_3-N/NH_4-N ratio of 2. *P. deliciosa* grown on NO_3-N/NH_4-N ratio of 2.0 had low R/S ratio (meaning relatively lesser root development). However, roots may be more important than shoots for *Phalaenopsis* orchids, where some species are known to shed all their leaves during dry season and rely on their green roots to photosynthesis (Christenson, 2001).

Therefore in order to produce higher fresh weight with reasonable amount of roots, *P. deliciosa* can be grown on medium with NO_3-N/NH_4-N ratio of 3. Potted *Phalaenopsis* was found to grow well preferably with fertilizer with NO_3-N/NH_4-N ratio of 3 (Wang, 2008). In order to produce *P. deliciosa* with higher R/S and WC for slower dehydration during acclimatization, seedlings can be grown on medium with NO_3-N/NH_4-N ratio of 4 or higher. Kubota *et al*. (2000) even suggested seedlings to be grown on medium with 100% nitrate to accelerate root growth. It was thought that NO_3-N/NH_4-N ratio affects morphogenesis by regulating plant growth regulators metabolism (George and De Klerk, 2008), possibly the reason for the observed effect on R/S at different NO_3-N/NH_4-N ratio.

Conclusion

The study identified that CCT medium was non-genotype-selective even after macronutrient or NO_3-N/NH_4-N ratio adjustments. Shoot yield was maximum at 2× macronutrient level (28.4 mM of N) while root and seedling yield were maximum at 1× macronutrient level (14.2 mM of N). Macronutrient was found to be the most significantly affecting shoot yield, as indicated by SFW and SDW and shoot development, as indicated by R/S. Macronutrient level did not significantly affect WC of *P. deliciosa* seedlings. NO_3-N/NH_4-N ratio affected shoot yield the most significantly followed by root yield, as indicated by SDW and RDW respectively. There was also significant difference with R/S ratio where the value increased with NO_3-N/NH_4-N ratio increase. Higher R/S caused higher WC in seedlings and *vice versa*. Adjustment of N to 1× level or 14.2 mM could increase seedling yield. When NO_3-N/NH_4-N ratio was adjusted to 3.0, higher seedling yield with reasonable amount of roots could be obtained. When NO_3-N/NH_4-N ratio was adjusted to 4.0, higher amount of roots with reasonable seedling yield could be achieved.

Acknowledgement

The research was carried out at and financially supported by INTI International University, Malaysia, part of the Laureate International Universities network.

Author's Contributions

Chieh Wean Choong: First author of the manuscript and supervisory role of the project.

Chin Hao Lim: Experimental design, conducted the experiment on macronutrient level, data collection and reviewed the manuscript.

Zherui Xiong: Experimental design, conducted the experiment on nitrate-ammonium ratio, data collection and reviewed the manuscript.

Siew Mee Choong: Statistical analysis of data and reviewed the manuscript.

Ethics

Seedlings were derived from cultivated parents that are not collected from the wild.

References

Barker, A.V. and K.A. Corey, 1991. Interrelations of ammonium toxicity and ethylene action in tomato. HortSci., 26: 177-180.

Barker, A.V. and G.M. Bryson, 2007. Nitrogen. In: Handbook of Plant Nutrition, Barker, A.V. and D.J. Pilbeam (Eds.), CRC Press, Boca Raton, ISBN-10: 9781439881972, pp 21-50.

Britto, D.V. and H.J. Kronzucker, 2002. NH_4^+ toxicity in higher plants: a critical review. J. Plant Physiol., 159: 567-584. DOI: 10.1078/0176-1617-0774

Chen, J.T. and W.C. Chang, 2006. Direct somatic embryogenesis and plant regeneration from leaf explants of *Phalaenopsis amabilis*. Biol. Plant, 50: 169-173. DOI: 10.1007/s10535-006-0002-8

Choong, C.W. and S.M. Choong, 2013. Optimum nitrogen to sulfur ratio to increase *in vitro* seedling yield of *Phalaenopsis bellina*. Int. J. Chem. Environ. Biol. Sci., 1: 796-797.

Choong, C.W., S.M. Choong and W.H. Thong, 2013. A defined culture medium suitable for sensitive *Phalaenopsis* species seedlings. Biotechnology, 12: 163-167. DOI: 10.3923/biotech.2013.163.167

Christenson, E.A., 2001. *Phalaenopsis*: A Monograph. 1st Edn., Timber Press, Portland, ISBN-10: 0881924946, pp: 330.

CITES, 2013. Appendices I, II and III. Convention of International Trade in Endangered Species of Wild Fauna and Flora.

Cumming, J.R. and S.M. Brown, 1994. Effects of elevated nitrate and aluminum on the growth and nutrition of red spruce (*Picea rubens*) seedlings. Tree Physiol., 14: 589-599. PMID: 14967676

Duan, Y.H., Y.L. Zhang, L.T. Ye, X.R. Fan and G.H. Xu *et al*., 2007. Responses of rice cultivars with different nitrogen use efficiency to partial nitrate nutrition. Annals Bot., 99: 1153-1160. DOI: 10.1093/aob/mcm051

Gamborg, O.L., T. Murashige, T.A. Thorpe and I.K. Vasil, 1976. Plant tissue culture media. Vitro, 12: 473-478. DOI: 10.1007/BF02796489

George, E.F. and G.J. De Klerk, 2008. The Components of Plant Tissue Culture Media I: Macro-and Micro-Nutrients. In: Plant Propagation by Tissue Culture, George, E.F., M.A. Hall and G.J. De Klerk (Eds.), Springer, Dordrecht, ISBN-10: 978-1-4020-5005-3, pp 65-113.

Heberer, J.A. and F.E. Below, 1989. Mixed nitrogen nutrition and productivity of wheat grown in hydroponics. Annals Bot., 63: 643-649.

Ichihashi, S. and M.O. Islam, 1999. Effects of complex organic additives on callus growth in three orchid genera, *Phalaenopsis, Doritaenopsis* and *Neofinetia*. J. Japan Society Horticultural Sci., 68: 269-274. DOI: 10.2503/jjshs.68.269

Jamal, A., Y.S. Moon and M.Z. Abdin, 2010. Sulphur-a general overview and interaction with nitrogen. Aus. J. Crop Sci., 4: 523-529.

Khoddamzadeh, A.A., U.R. Sinniah, M.A. Kadir, S.B. Kadzimin and M. Mahmood *et al*., 2010. Detection of somaclonal variation by random amplified polymorphic DNA analysis during micropropagation of *Phalaenopsis bellina* (Rchb.f.) Christenson. Afr. J. Biotechnol., 9: 6632-6639.

Kubota, S., K. Yoneda and Y. Suzuki, 2000. Effects of ammonium to nitrate ratio in culture medium on growth and nutrient absorption of *Phalaenopsis* seedlings *in vitro*. Environ. Control Biol., 38: 281-284. DOI: 10.2525/ecb1963.38.281

Lawlor, D.W., 2002. Carbon and nitrogen assimilation in relation to yield: Mechanisms are the key to understanding production systems. J. Exp. Bot., 53: 773-787. DOI: 10.1093/jexbot/53.370.773

Merhaut, D.J., 2007. Magnesium. In: Handbook of Plant Nutrition, Barker, A.V. and D.J. Pilbeam (Eds.), CRC Press, Boca Raton, ISBN-10: 9781439881972, pp: 145-182.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant, 15: 473-497. DOI: 10.1111/j.1399-3054.1962.tb08052.x

Poole, H.A. and J.G. Seeley, 1978. Nitrogen, potassium and magnesium nutrition of three orchid genera. Cornell University.

Sanchez, C.A., 2007. Phosphorus. In: Handbook of Plant Nutrition, Barker, A.V. and D.J. Pilbeam (Eds.), CRC Press, Boca Raton, ISBN-10: 9781439881972, pp: 51-90.

Tavares, A.R., J.L.M. Young, S.S. Ori, S. Kanashiro and G.P. Lima *et al*., 2012. Orchid *in vitro* growth as affected by nitrogen levels in the culture medium. Hortic. Bras., 30: 119-124. DOI: 10.1590/S0102-05362012000100020

Tokuhara, K. and M. Mii, 1993. Micropropagation of *Phalaenopsis* and *Doritaenopsis* by culturing shoot tips of flower stalk buds. Plant Cell Report, 13: 7-11. DOI: 10.1007/BF00232306

Trépanier, M., M.P. Lamy and B. Dansereau, 2009. *Phalaenopsis* can absorb urea directly through their roots. Plant Soil, 319: 95-100. DOI: 10.1007/s11104-008-9852-5

Van Beusichem, M.L., E.A. Kirkby and R. Baas, 1988. Influence of nitrate and ammonium Nutrition on the uptake, assimilation and distribution of nutrients in *Ricinus communis*. Plant Physiol., 86: 914-921. DOI: 10.1104/pp.86.3.914

Van Dijk, H.F.G., M.H.J. de Louw, J.G.M. Roelofs and J.J. Verburgh, 1990. Impact of artificial, ammonium-enriched rainwater on soils and young coniferous trees in a greenhouse. Part II-Effects on the trees. Environ. Pollut., 63: 41-59. DOI: 10.1016/0269-7491(90)90102-I

Wang, Y.T., 2008. High NO_3-N to NH_4-N ratios promote growth and flowering of a hybrid *Phalaenopsis* grown in two root substrates. HortSci., 43: 350-353.

Weissman, G.S., 1964. Effect of ammonium and nitrate nutrition on protein level and exudate composition. Plant Physiol., 39: 947-952. PMID: 16656039