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# A Defined Culture Medium Suitable for Sensitive *Phalaenopsis* Species Seedlings

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**Abstract:** Half strength MS medium supplemented with peptone was found to inhibit growth of considerable number of *Phalaenopsis* species seedlings and this hamper conservation effort. It is therefore important to design a reproducible defined medium that is not selective and could support a wide range of genotypes. This could be achieved by adjusting the macronutrient concentrations. A new defined medium was designed with nitrate-nitrogen ($NO_3$-N) to ammonium-nitrogen ($NH_4$-N) ratio of 5 and N to S ratio of 15, buffered with 800 mg $dm^{-3}$ $KH_2PO_4$ and NPK ratio of 2.0:0.8:3.1. Mean fresh weight, dry weight and root to shoot ratio was compared with control medium. In contrary to control medium that stunted growth of significant number of *Phalaenopsis aphrodite*, *Phalaenopsis bellina* and *Phalaenopsis violacea* seedlings, the new medium was found to significantly support growth of the seedlings and improved root development. In addition, the new medium was not selective against sensitive seedlings as the data from new medium pass normality test while data from control medium was skewed in *P. bellina*. This new medium is recommended for maintaining genetic diversity in conservation programs by improving seedling survival and growth *in vitro*.

**Key words:** Moth orchid, germination, germinated seedlings, nitrate nutrition

## INTRODUCTION

*Phalaenopsis* culture suffers from the setback where exudation and oxidation of large amount of phenolics blacken culture medium and inhibiting growth (Chugh *et al*., 2009). In addition, it was observed that in some species, more than half of the seedlings were stunted with dark green leaves, necrotic spots and low root/shoot ratio, symptoms similar to ammonium excess and toxicity (Hinnen *et al*., 1989; Britto and Kronzucker, 2002). Concentration of ammonium in excess of 8 mM may be deleterious to cultures (Gamborg and Shyluk, 1970). Often complex supplements are added to enhance growth and buffer culture medium (Gnasekaran *et al*., 2010; Chen and Chang, 2006; Hinnen *et al*., 1989).

Ammonium is easier to assimilate but acidifies culture medium while nitrate alkalinizes culture medium. Co-presence of nitrate with ammonium stabilizes culture medium pH where assimilation of one creates pH shifts that favor assimilation of the other (George and de Klerk, 2008; Thorpe *et al*., 2008). Buffering can also be achieved by increasing the concentration of $KH_2PO_4$ in the medium but at a concentration inhibitory to growth (Vacin and Went, 1949; Thorpe *et al*., 2008).

Fertilizer with 75% nitrate-nitrogen ($NO_3$-N) to ammonium nitrogen ($NH_4$-N) ratio improved vegetative growth and flowering of a hybrid *Phalaenopsis* (Wang, 2008). A high nitrate to ammonium ratio was suggested as a possible mean to promote root growth *in vitro* while the opposite significantly reduced phosphorus, potassium, calcium and magnesium absorption in a hybrid *Phalaenopsis* (Kubota *et al*., 2000). To date, there is no study on the performance of *Phalaenopsis* species in response to different ratio of $NO_3$-N to $NH_4$-N nutrition *in vitro*. From the study on *Phalaenopsis* hybrids by Wang (2008) and Kubota *et al*. (2000), it is predicted that high $NO_3$-N to $NH_4$-N ratio would be suitable for *in vitro* culturing of *Phalaenopsis* species in general.

A new culture medium is desired to allow growth of sensitive *Phalaenopsis* species. This medium will be beneficial to conservation effort avoiding *in vitro* selection of vigorous genotypes thus narrowing genetic diversity. Defined medium can be reproduced consistently as opposed to medium containing complex supplements, which are commonly used for *Phalaenopsis* species culture but may vary between batches and sources. In addition, defined medium does not complicate experimental results with unknown growth-promoting

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factors present in complex supplements. Therefore the aim of this study was to design a defined medium suitable for a wide range of genotypes for *Phalaenopsis* species.

## MATERIALS AND METHODS

**Designing a new define medium:** The designed medium contained (mg $dm^{-3}$) $NH_4NO_3$ (190), $Ca(NO_3)\cdot 4H_2O$ (470), $CaSO_4$ (77), $Mg(NO_3)\cdot 6H_2O$ (255), $MgSO_4\cdot 7H_2O$ (69), $KNO_3$ (370), $KH_2PO_4$ (800), NaCl (20), half strength of MS (Murashige and Skoog, 1962) 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. The medium has NPK ratio of 2.0:0.8:3.1 (calculation for P is based on $P_2O_5$ and K is based on $K_2O$) and this ratio was chosen based on possible adjustment of the chemicals above and from literature (Wang, 2000).

This medium has $NO_3$-N to $NH_4$-N ratio of 5 due to previous findings (Wang, 2008; Kubota *et al*., 2000) and to effectively stabilize the pH of the culture medium (Britto and Kronzucker (2002). N to S ratio of the medium was adjusted to 15.0 because according to, this is the ratio of protein in general and also of balance crops. Low concentration of N was chosen (14.4 mM) so that $NH_4$-N was in low concentration (2.4 mM) to support growth of wide range of genotypes. This is because concentration of $NH_4$-N above 8 mM could be deleterious to cultures (Gamborg and Shyluk, 1970). Concentration of P was 5.9 mM and should not be toxic since concentration as high as 19.8 mM was used (George and de Klerk, 2008).

Control medium used was half-strength Murashige and Skoog 1962, MS) medium with (mg $dm^{-3}$) myo-inositol (100), niacin (0.5), pyridoxine HCl (0.5), thiamine HCl (0.1), glycine (2), peptone (1,000), $NaH_2PO_4$ (170), sucrose (25,000) and gellan gum (2,200) at pH 5.2. This medium has 10 mM of $NH_4$-N, $NO_3$-N to $NH_4$-N ratio of 2, N to S ratio of 26, 85 mg $dm^{-3}$ of $KH_2PO_4$ and 170 mg $dm^{-3}$ of $NaH_2PO_4$ (P concentration of 1.9 mM) without considering contribution from peptone.

**Germination of *Phalaenopsis* seedlings:** Mature green seedpods of 110 days after pollination (DAP) were obtained from *P. aphrodite* Rchb.f. and *P. pulcherrima* (Lindl.) J.J. Sm. while mature green seedpods of 150 DAP from *P. violacea* Witte and *P. bellina* (Rchb.f.) E.A. Christ. Each seedpod was cleaned under tapwater and then immersed in bleaching solution (5.25% sodium hypochlorite) for 5 min followed by 70% ethanol for 1 min. The seedpod was then aseptically flamed until all traces of ethanol burnt away. Scalpel was used to cut open the seedpod and seeds within were inoculated onto control medium. Seedlings were subcultured every 60 day.

**Growth measurement:** Seedlings of *P. aphrodite*, *P. bellina* and *P. violacea* were randomly cultured onto new and control media to initial weight of 1.2±0.05 g, 0.2±0.05 g and 0.4±0.05 g, respectively. Since *P. pulcherrima* were not sensitive to control medium, only root to shoot ratio was measured. To do this, 3 seedlings were randomly chosen and cultured onto new and control media.

The seedlings were allowed to grow at 25°C and 16 h photoperiod with photon flux density of 30 μmol $m^{-2}$ $sec^{-1}$. *P. aphrodite* and *P. pulcherrima* seedlings were cultured for 75 d while *P. bellina* and *P. violacea* seedlings for 90 day. After that, roots and shoots of the seedlings were separated and weighed to measure the fresh weight, then dried at 70°C for 2 day before measuring the dry weight. Plant Fresh Weight (FW) and Dry Weight (DW) were obtained by the addition of shoot and root fresh weight and dry weight, respectively. Data were tested for normality based on z-score of kurtosis and skewness at $\alpha = 0.05$. Treatment means of fresh weight, dry weight and root to shoot ratio (R/S; based on dry weights) were compared by two-way Student's t-test at $\alpha = 0.05$.

## RESULTS

***P. aphrodite*:** Significant difference ($p \geq 0.95$) in mean dry weight between healthy and unhealthy seedlings performance on the same medium was detected. On new medium, active growth of existing and new root was observed on both healthy and unhealthy seedlings. In contrast, both healthy and unhealthy seedlings initiated shoot growth when transferred onto control medium. When healthy and unhealthy seedlings were analyzed separately, data were found to pass normality test for both *kurtosis* and skewness. For healthy seedlings (n = 22), mean FW and DW were not significantly different between new and control media but mean R/S of seedlings grown on new medium was significantly higher ($p \geq 0.95$) than those on control medium. For unhealthy seedlings (n = 54), mean FW, DW and R/S ($p \geq 0.95$) of seedlings grown on new medium were significantly higher than those on control medium as shown in Fig. 1a.

***P. bellina*:** Active root growth was observed in the first two weeks after seedlings were subcultured onto new medium. Generally, plants in most of the replicates on new medium grew well. In contrast, significant number of the seedlings displayed stunted growth on control medium, with few growing exceptionally well, even better than those on new medium. Data from new medium (n = 24)

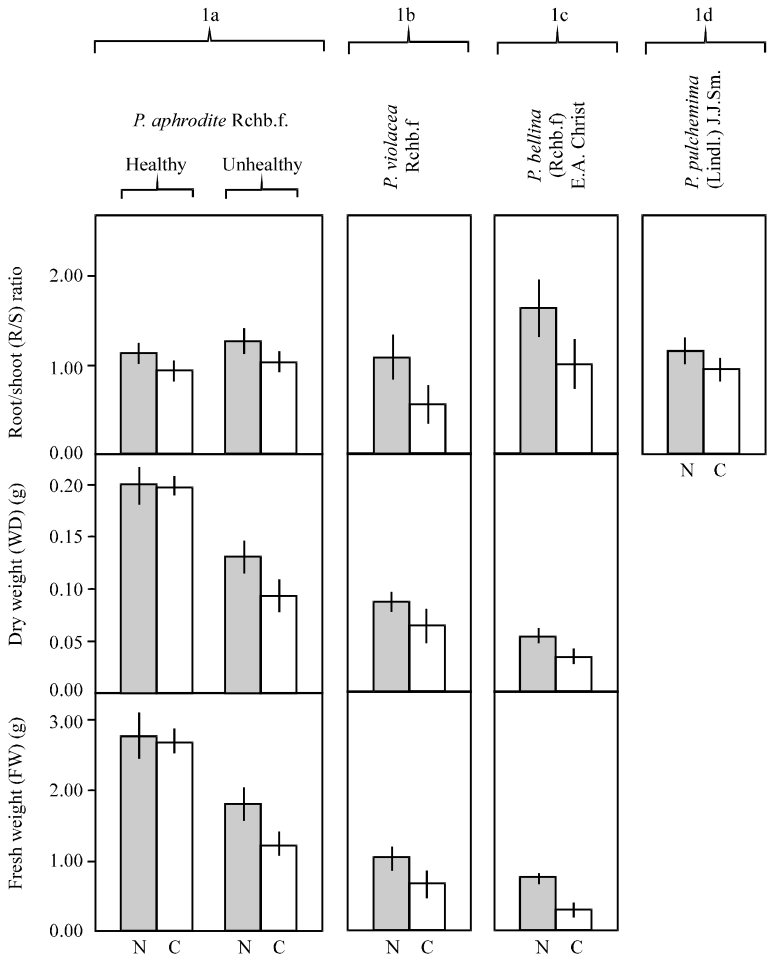


Fig. 1(a-d): Mean comparison of growth parameters between new and control medium. Mean comparison of fresh weight (FW), Dry Weight (DW) and root/shoot ratio (R/S) between seedlings grown *in vitro* on new (N) and control medium (C) of (a): *P. aphrodite* Rchb.f. healthy (n = 22) and unhealthy seedlings (n = 54), (b): *P. bellina* (Rchb.f.) E.A. Christ. (n = 42), (c): *P. violacea* Witte (n = 22) and (d) *P. pulcherrima* (Lindl.) J.J. Sm. (n = 47). Error bars indicate the lower and upper limits based on 95% confidence intervals

passed normality test for both kurtosis and skewness, indicating the non-selective nature of the new medium. Data of FW and DW from control medium (n = 24) failed normality test for skewness, supporting the observation that control medium was selective towards few vigorous seedlings. Six outliers were removed from the data from control medium to run the t-test (n = 42). Mean of FW, DW and R/S of seedlings grown on new medium were significantly higher ($p \geq 0.95$) than those on control medium as shown in Fig. 1b.

***P. violacea*:** Many seedlings grown on control medium developed mild to severe necrosis spots on their leaves. In addition, the leaves were thick and fleshy. Roots were short and stubby. In our experience, such seedlings would continue to be stunted or deteriorate and die. Data (n = 21) of FW, DW and R/S from both control and new media passed the normality test for both kurtosis and skewness. Mean of DW, FW and R/S of seedlings grown on new medium was significantly higher ($p \geq 0.95$) than those grown on control medium as shown in Fig. 1c.

***P. pulcherrima*:** Seedlings were not sensitive to control medium. However, it was observed that new medium produce plants with more roots. R/S from both control and new media(n = 47) passed the normality test for both kurtosis and skewness. Mean R/S of seedlings grown on new medium was significantly higher ($p \geq 0.95$) than those on control medium as shown in Fig. 1d.

## DISCUSSION

From the results, overall better growth was observed for *P. aphrodite*, *P. bellina* and *P. violacea* on new medium than control medium. Statistical result showed that control medium was selective towards vigorous seedlings and against sensitive seedlings. When seedlings were not separated prior to culturing, few vigorous seedlings skewed the data from *P. bellina*, indicating selection of vigorous seedlings on control medium. When sensitive and vigorous seedlings were analysed separately as for *P. aphrodite*, sensitive seedling growth on new medium were significantly higher than on control medium. Vigorous plants were normally selected *in vitro* and in cultivation (Christenson, 2001).

The reason for new medium to be less selective is probably due to better N (Kronzuker *et al*., 1999), cations (potassium, calcium and magnesium) and P assimilation (Kubota *et al*., 2000; Van Beusichem *et al*., 1988; Kirkby and Knight, 1977) at higher $NO_3$-N to $NH_4$-N ratio. Wang, 2008) found that fertilizer with $NO_3$-N to $NH_4$-N ratio of 4 improved growth of a hybrid *Phalaenopsis*. On top of that, pH stabilization at higher $NO_3$-N to $NH_4$-N ratio (Thorpe *et al*., 2008; Britto and Kronzucker, 2002), along with higher concentration of $KH_2PO_4$, may be another contributing factor to better growth. Improved phosphorus nutrition could be another reason for higher growth of seedlings on new medium, since it is beneficial to increase phosphate concentration in cultures (George and de Klerk, 2008).

The new medium also improved root development in all the four *Phalaenopsis* species (significantly higher R/S). High ratio of $NO_3$-N to $NH_4$-N was found to improve root development of a hybrid *Phalaenopsis in vitro* (Kubota *et al*., 2000). In *Phalaenopsis* species, the amount of roots and root development are probably better growth and health indicators than leaves. Many *Phalaenopsis* species are deciduous and only survive with roots during dryer months (Christenson, 2001).

## CONCLUSION

To our knowledge, a defined medium with such high $NO_3$-N to $NH_4$-N ratio and P concentration for *Phalaenopsis* species is not described elsewhere. The medium with $NO_3$-N to $NH_4$-N ratio of 5 and N to S ratio of 15, buffered with 800 mg $dm^{-3}$ $KH_2PO_4$, NPK ratio of 2.0:0.8:3.1, was found to be suitable for *in vitro* culturing of four *Phalaenopsis* species with improved growth and root development over a wider range of genotypes. This medium is recommended for conservation purpose where seedling survival is important for maintaining genetic diversity.

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