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An Effective Nutrient Medium for Asymbiotic Seed Germination and *In Vitro* Seedling Development of *Phalaenopsis* 'Bahia Blanca'

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Abstract

The objective of the present study was to develop a rapid and successful method for promoting asymbiotic seed germination and growth and development of protocorm-like bodies (PLBs) and subsequent plantlets of *Phalaenopsis* orchid. Four basal media, i.e. Murashige and Skoog (MS), half-strength MS (½MS), Knudson C (KC), and Vacin and Went (VW) supplemented with coconut water and peptone, were examined for their effectiveness in large-scale multiplication by asymbiotic seed germination. Seed germination, leaf number, root number, root length, seedling length, and fresh and dry weight were scored. Well-rooted plantlets 4-cm in height were transferred to a greenhouse in trays containing expanded clay, charcoal, cocopeat and perlite (4:2:1:1) or sphagnum moss. The seed germination frequency and seedling development were found to be significantly higher in organic growth supplement-enriched MS and ½MS media. The results indicated that the highest seed germination (94%) was obtained in MS medium supplemented with 100 mg/L coconut water or 1 g/L peptone and ½MS containing 1 or 2 g/L peptone. However, the longest seedling was found in MS or ½MS media supplemented with 100 mg/L coconut water or MS containing 1 g/L peptone. Ninety-seven percent of plantlets survived after 1 month in mixture media expanded clay: charcoal: cocopeat: perlite.

Keywords: Coconut water, Media, Orchid, Peptone, Protocorm-like bodies.

INTRODUCTION

Phalaenopsis orchid, belonging to the Orchidaceae family, is one of the most important orchids and most popular epiphytic monopodial orchid, which is grown for commercial production of cut flowers and potted plants. It is known for its beautiful flowers in terms of large size, form, color and known to be originated from the island of Borneo (Gnasekaran *et al.*, 2010). *Phalaenopsis* grew in popularity and in the 1980s, it displaced Cattleya as the most popular orchid. It is widely recognized that potted *Phalaenopsis* production has increased tremendously in last few years (Griesbach, 2002).

It is difficult to propagate *Phalaenopsis* asexually under natural conditions (Tsai and Chu, 2001). Also, tissue culture techniques that were commercially successful for many orchids could not be used with *Phalaenopsis* (Griesbach, 2002). In *Phalaenopsis*, tissue culture propagation has produced too much genetic variation. In some instances, over 50% of the propagated plants have produced flowers that were significantly different than the mother plant (Griesbach, 2002). Propagation by seedling is the main method for mass production at present. Seeds of orchid are incredibly small and contain an undifferentiated embryo that lacks enzymes to metabolize polysaccharides and lipid and does not have endosperm to store nutrients for germination and early growth. They utilize a mycorrhizal relationship with compatible fungi during germination and early development. Sowing *in vitro* is the most efficient way for the growth and development of orchid seeds and seedlings (Tsai and Chu, 2001; Kauth *et al.*, 2008). The efficiency of the tissue culture propagation is strongly influenced by the mineral composition of culture medium and its interaction with organic nutrient additives (Chen *et al.*, 2015; Fallahpour *et al.*, 2015). The media and its formulation are very important to maximize orchid's vigor in the tissue culture conditions, but it is known that responses of orchid seeds to different nutrient media vary among species (Gnasekaran *et al.*, 2010; Paul *et al.*, 2012). Balanced nutrient availability for quality medium and low cost is required to attain sustainable protocols in orchids (Gnasekaran *et al.*, 2010). The technique of asymbiotic seed germination by *in vitro* culture was first introduced by Knudson (1946). Since then, *in vitro* seed germination protocols have been established for many orchid species, and a number of media and salts have been used for germination and propagation (Paul *et al.*, 2012; Teixeira da Silva, 2013). An in-depth review on i*n vitro* orchid seed germination was provided by Arditti (1967), Kauth *et al.* (2008), Yan and Arditti (2009), and Teixeira da Silva (2013). The roles of nitrogen form and concentration, carbohydrate source, vitamins, and plant growth regulators (PGRs) in asymbiotic germination were examined in earlier studies. More recently, the role of individual media components has not been extensively investigated (Kauth *et al.*, 2008). A large number of organic growth supplements like taro extract, coconut water, casein hydrolysate, banana pulp, potato extract, corn extract, peptone, tomato juice, slap honey, apple juice, yeast extract, and beef extract can be very effective in providing undefined mixture of organic nutrients and have the ability to influence *in vitro* multiplication of PLBs and growth of orchid seedlings (Aktar *et al.*, 2008; Kaur and Bhutani, 2012).

Therefore, the present study was conducted to evaluate asymbiotic seed germination of *Phalaenopsis* and test the efficacy of some media and growth supple¬ments on early development of PLBs and plantlets without the use of any growth regulators.

MATERIALS AND METHODS

The plants of *Phalaenopsis* 'Bahia Blanca' (*Ph.* 'Memoria Natalie Wood' × *Ph.* 'Zuma Urchin') grown in a greenhouse were artificially self-pollinated by transferring pollen onto the stigma of the same flower as they became fully opened. Undehisced 4-month-old capsules were harvested and kept in self-sealing plastic sacs for carrying and pre-sterilization temporary storing to curtail dehydration (Balilashaki *et al.*, 2015). The capsules were washed under running tap water for 20 min, and surface-sterilized by dipping into 70% (v/v) ethanol for 2 min followed by twice rinsing thoroughly with sterilized distilled water. The sterilized capsules were cut vertically with

a sterile scalpel, and the seeds were gently distributed directly onto the four basal nutrient media, i.e. MS (Murashige and Skoog, 1962), half-strength MS (½MS), KC (Knudson, 1946), and VW (Vacin and Went, 1949) containing peptone (0, 1 and 2 g/L) and filter-sterilized CW (coconut water) (0, 30, 60 and 100 mg/L). Basal media were supplemented with 25 g/L sucrose and 7.8 g/L agar to solidify the media. The pH was adjusted to 5.7 with 0.1 N NaOH or 0.1 N HCl before autoclaving at 121°C for 20 min. The cultures were maintained in a growth chamber and allowed to grow at 25±1°C under 16 h photoperiod of 2500 lux provided by cool white fluorescent tubes.

The subculture was done twice in three-month intervals. Percentage seed germination (swollen and not-swollen seeds) and PLBs development were determined under binocular microscope at 20x magnification after two months of culture and calculated by using the formula:

$$\text{Seed germination (\%)} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \times 100$$

After 6 months, the growth of seedlings was monitored for leaf number, root number, root length, seedling length, and fresh and dry weight of seedlings.

Plantlets with 2-3 leaves and well-defined roots were obtained after 9 months. They were removed from the culture vessels and washed thoroughly with tap water to remove adhering medium without damaging the roots before transplanting into trays containing expanded clay, charcoal, cocopeat, and perlite (4:2:1:1) or sphagnum moss. Plantlets were sprayed with water to inhibit dehydration and were moved to a greenhouse for growing under mean temperature of 28°C, 80-90% relative humidity, and an illumination of 24000 lux. After three months, the adapted plants were transplanted in 6-cm plastic pots containing pine bark and kept in a greenhouse at 80% relative humidity. The pots were watered at intervals of 5 days for 12 weeks when flowering was observed.

The experiment was established as factorial in a completely randomized design with 10 replications. Data were analyzed using one-way ANOVA and the differences were contrasted by Duncan's multiple range test at the 5% level of probability using SAS software.

RESULTS

The seed germination and PLBs and seedlings development varied with the different media and organic additives used. Coconut water and peptone proved optimum for profuse development of PLBs and their early growth into plantlets. The seeds of *Phalaenopsis* germinated after two months and developed into greenish PLBs. The PLBs eventually differentiated into shoots and root (Fig. 1A); no intervening callus stage was observed. The roots were lengthy, thick, and covered all over the surface by root hairs (Fig. 1B). Maximum germination percentage and the appearance of PLBs were achieved on MS medium supplemented with 100 mg/L CW (94.0%) or 1 g/L peptone (94.7%) and ½MS containing 1 or 2 g/L peptone (90.0 and 91.7%, respectively) (Fig. 2). Leaf number was the highest in MS + 100 mg/L CW (3.4 per explant) or ½MS + 30 mg/L CW + 1 g/L peptone (3.8 per explant). However, these media were not significantly different compared to MS + 60 mg/L CW or 1 g/L peptone (Fig. 3). Root number and root length on MS + 100 mg/L CW or 1 g/L peptone were considerably greater compared with the other media (Fig. 4 and 5). Seedling length was significantly higher in MS + 100 mg/L CW or 1 g/L peptone (4.9 and 4.8 cm, respectively) and ½MS + 100 mg/L CW (4.9 cm) compared to other treatments (Fig. 6). The highest fresh and dry weight of seedlings were obtained from MS + 1 g/L peptone followed by ½MS + 30 mg/L CW + 1 g/L peptone (Fig. 7 and 8).

Of the two substrates used, the substrate made up of expanded clay, charcoal, cocopeat, and perlite (4:2:1:1) was found to be more suitable for the survival of the transferred plantlets. This approach supported 97% survival after one month of hardening under greenhouse conditions (Fig. 1C). The *Phalaenopsis* plants begin to flower about six months after transplanting (Fig. 1D).

Fig. 1. Protocorms developed shoot and root on MS containing 100 mg/L CW after 3 (A) and 6 (B) months. (C) Acclimatized plantlets in clay, charcoal, cocopeat, and perlite (4:2:1:1) after one month. (D) flowering of six-month-old acclimatized plants in pots.

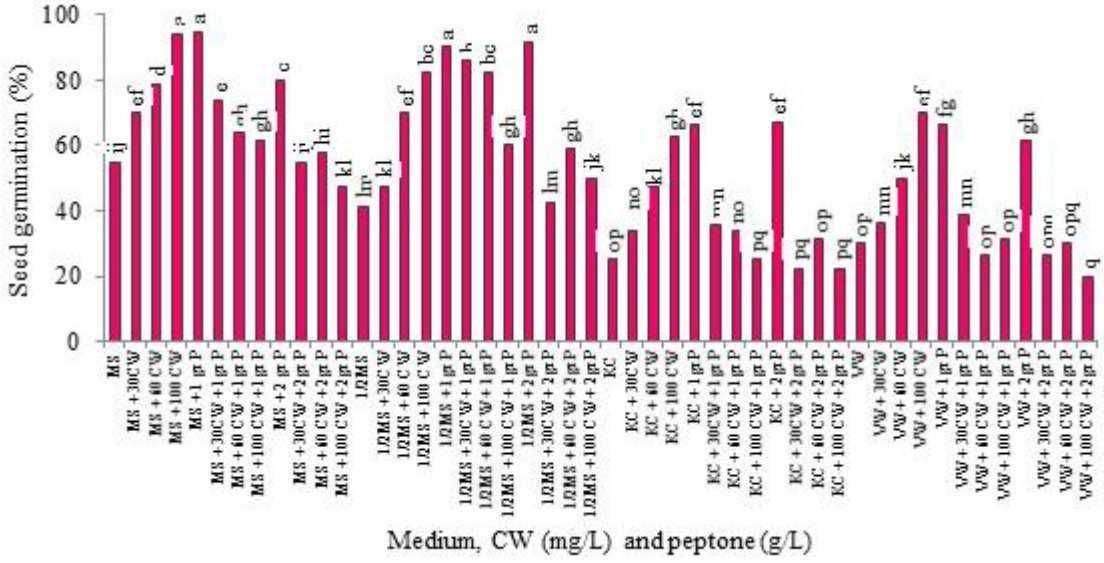

Fig. 2. Effect of media, coconut water, and peptone on seed germination of *Phalaenopsis* after 30 days of culture. Mean values with the same letter(s) are not significant at P<0.05 according to Duncan's test.

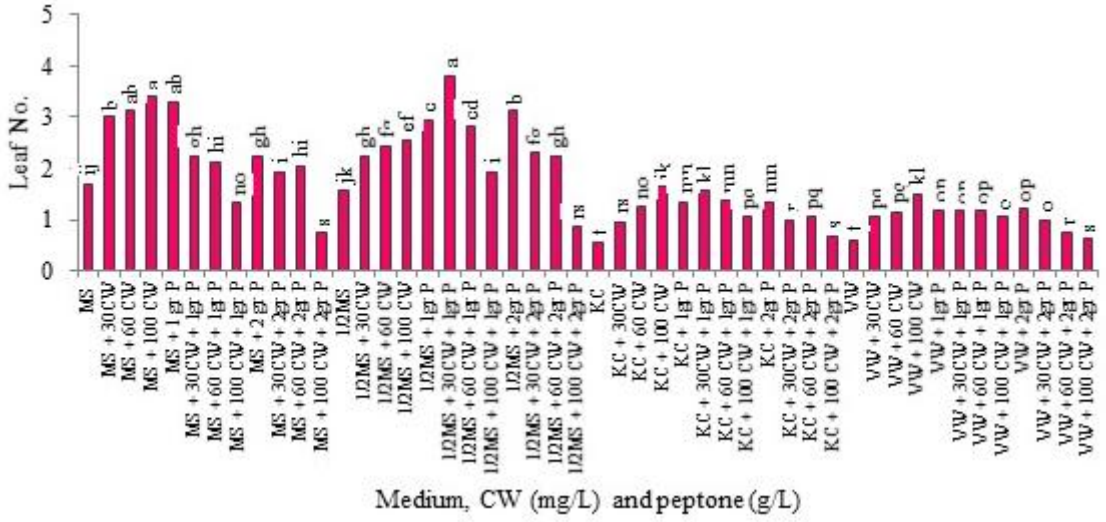

Fig. 3. Effect of media, coconut water, and peptone on leaf number of *Phalaenopsis* after 60 days of culture. Mean values with the same letter(s) are not significant at P<0.05 according to Duncan's test.

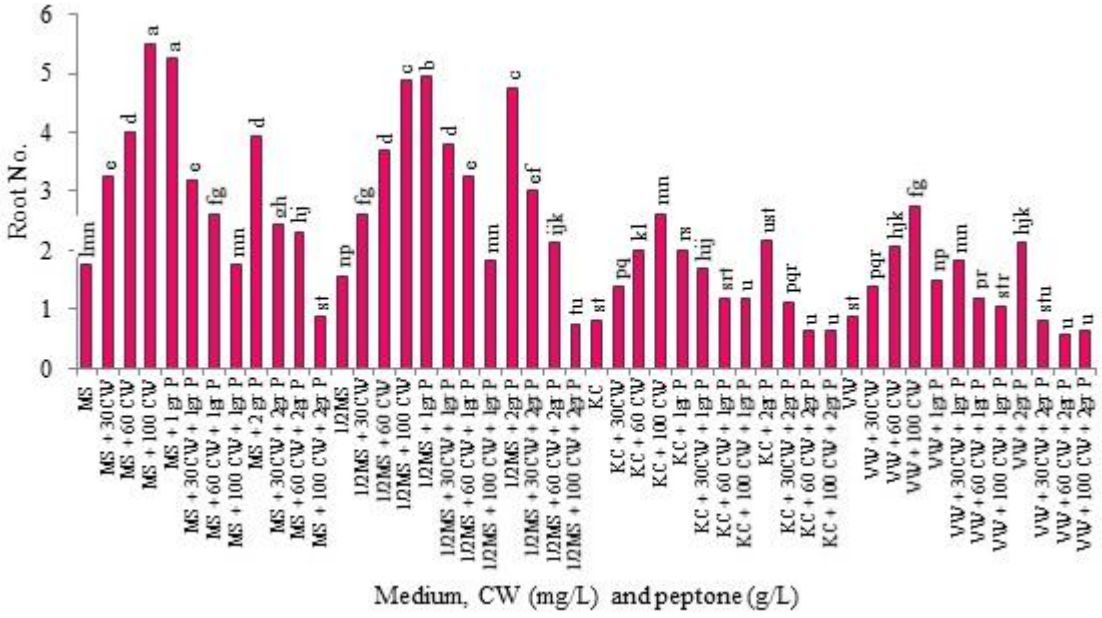

Fig. 4. Effect of media, coconut water, and peptone on root number of *Phalaenopsis* after 60 days of culture. Mean values with the same letter(s) are not significant at P<0.05 according to Duncan's test.

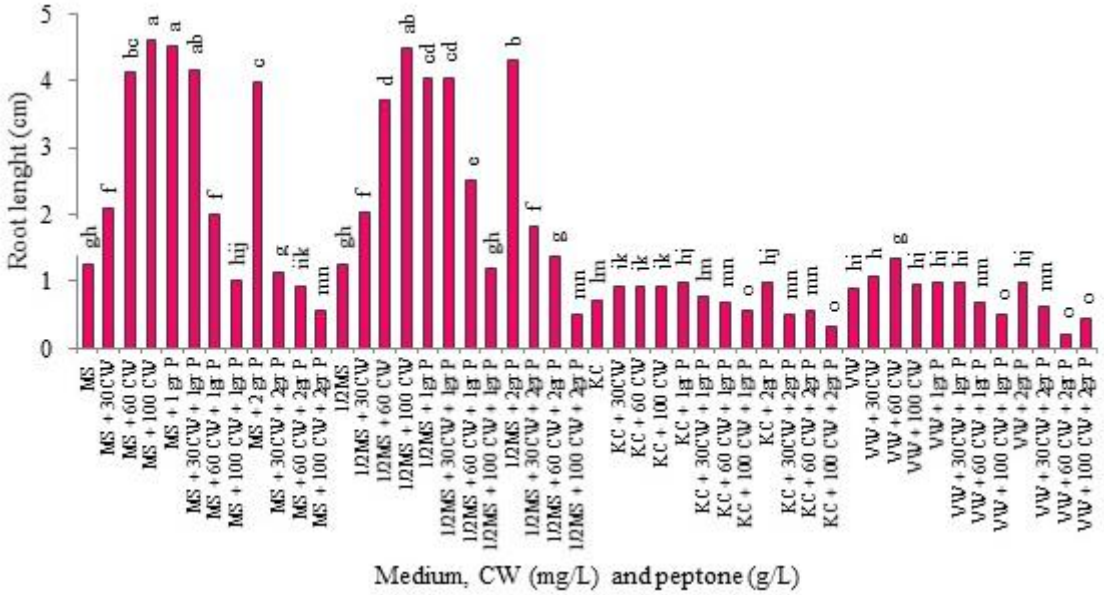

Fig. 5. Effect of media, coconut water, and peptone on root length of *Phalaenopsis* after 60 days of culture. Mean values with the same letter(s) are not significant at P<0.05 according to Duncan's test.

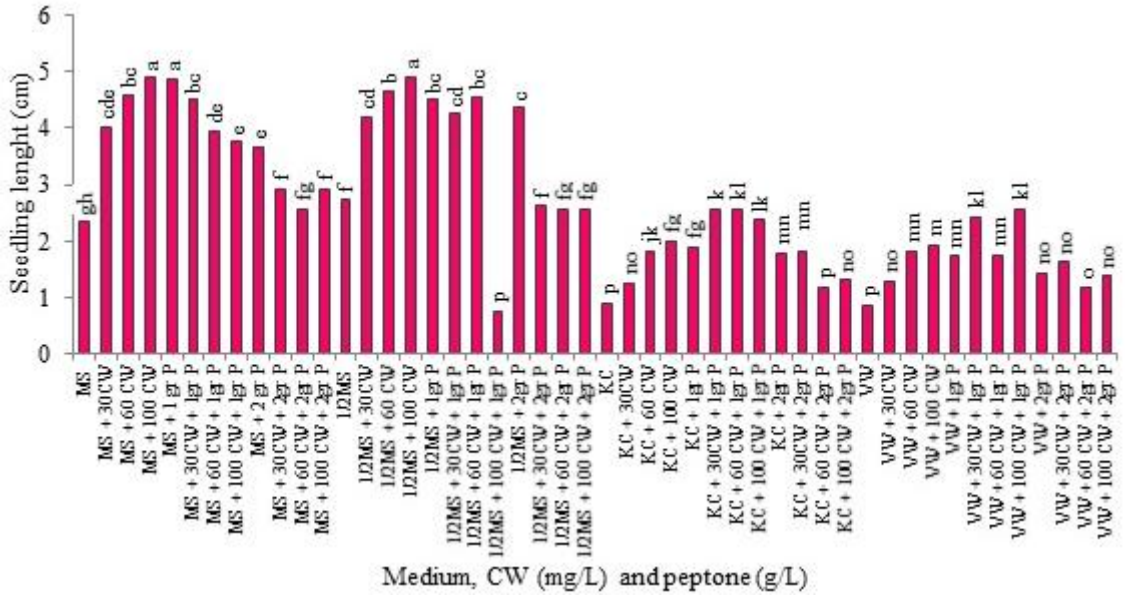

Fig. 6. Effect of media, coconut water, and peptone on seedling length of *Phalaenopsis* after 60 days of culture. Mean values with the same letter(s) are not significant at P<0.05 according to Duncan's test.

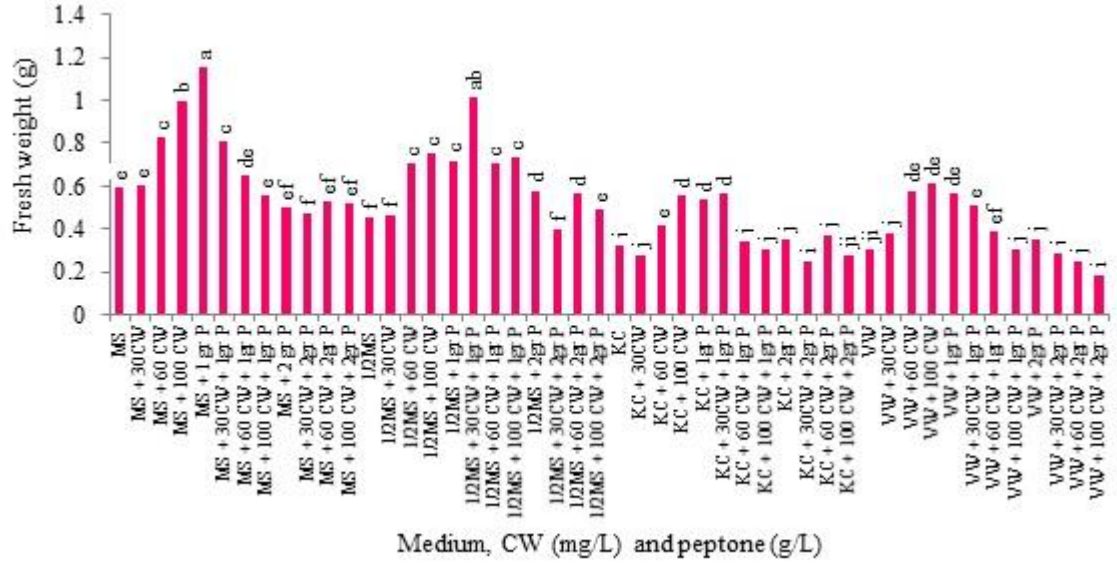

Fig. 7. Effect of media, coconut water, and peptone on seedling fresh weight of *Phalaenopsis* after 60 days of culture. Mean values with the same letter(s) are not significant at P<0.05 according to Duncan's test.

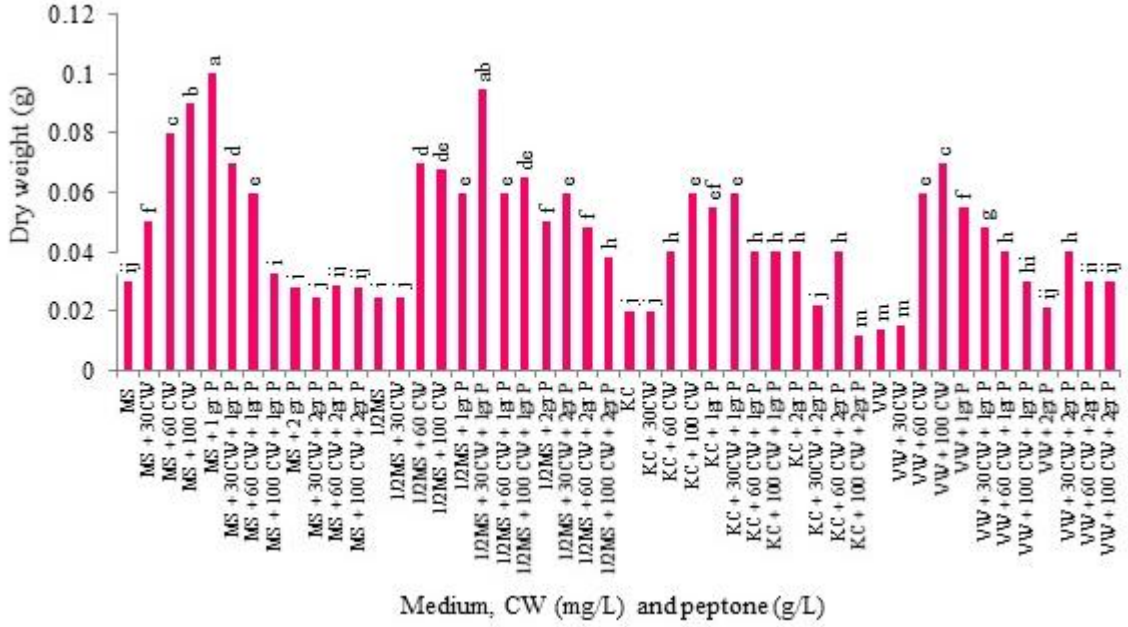

Fig. 8. Effect of media, coconut water, and peptone on seedling dry weight of *Phalaenopsis* after 60 days of culture. Mean values with the same letter(s) are not significant at P<0.05 according to Duncan's test.

DISCUSSION

The choice of culture medium strongly affected germination, presumably because of differences in the balance and supply of organic and inorganic (Paul *et al.*, 2012). The high germination and strong further development in MS and ½MS media could be attributed to the fact that these media are especially rich in both macro- (especially nitrogen) and micro-nutrients. The presence of ammonium nitrate in MS medium may explain the high germination rate because NH^{4+} is readily assimilated during the initial stages of development and greatly influences growth and development of PLBs (Paul *et al.*, 2012). Furthermore, MS contains an increased Ca^{2+} concentration that is required for cell wall synthesis and membrane function as well as cell signaling (Erfani *et al.*, 2017). Although some micro salt concentrations of MS and ½MS (manganese, zinc, boron, iodine, molybdenum, copper and cobalt) are higher than that of KC and VW (Paul *et al.*, 2012; Shekarriz *et al.*, 2014), these differences in salt concentration might have resulted in the differences of the efficiency of seed germination and PLBs growth. A high seed germination and seedling development on MS or ½MS media has also been reported for *Phalaenopsis* (Gnasekaran *et al.*, 2010; Shekarriz *et al.*, 2014) or other orchids (Paul *et al.*, 2012; Zeng *et al.*, 2012) despite KC or VW media being suitable for *Geodorum densiflorum* (Roy and Banerjee, 2001), *Hygrochilus parishii* (Shadang *et al.*, 2007). On the other hand, Zeng *et al.* (2012) reported lower seed germination of *Paphiopedilum wardii* on MS than on ½MS medium possibly because of the high salt concentration of MS. Also, Chen *et al.* (2015) found that the seeds of *Paphiopedilum spicerianum* imbibed in ¼MS medium retained their integrity while in MS medium, seeds showed shrinkage.

They indicated that the MS medium solute concentration is too high for *P. spicerianum* leading to solute leakage from the seed. Therefore, the nutritional requirements of germinating orchid seeds vary due to their physiological state and this may be species specific (Paul *et al.*, 2012). Furthermore, due to variations within the hybrids, even for the same species, different media have been reported to give the best conditions for germination (Chen *et al.*, 2015). Thongpukdee *et al.* (2010) found that seeds from a 3-month-old capsule of *Phalaenopsis* 'Silky Moon' provided the highest percentage of germination (98.7%) on modified stationary-liquid Hyponex medium. Also, Zahara *et al.* (2017) indicated that *Phalaenopsis* hybrid 'Pink' cultured on a combination of sucrose (20 g/L) and carrot juice (10%) supplemented with either MS or VW media can be used for plantlet growth of this species.

Coconut water and peptone, at an efficient concentration, were found to be effective in seed germination, growth of PLBs and early plantlet development. Similar growth promoting effects of these growth supplements in *Dendrobium pendulum* (Kaur and Bhutani, 2012), *Paphiopedilum wardii* (Zeng *et al.*, 2012) and *Phalaenopsis* hybrid (Shekarriz *et al.*, 2014) are earlier reported. Growth promontory nature of CW is related to its ability to induce cell divisions in non-dividing cells, thereby promoting early protocorm differentia¬tion. Also, beneficial effect of CW is correlated with the fact that the presence of amino acids, vitamins, sugars, and plant growth regulators such as cytokinin (kinetin), as well as various inorganic ions such as phosphorus, magnesium, potassium, and sodium in coconut water is the probable cause of orchid seed germination (Kaur and Bhutani, 2012; Zeng *et al.*, 2012). The addition of CW (15%) to the basal culture media increased the formation of root and leaf in *Hygrochilus parishii* (Shadang *et al.*, 2007). On the other hand, Zahara *et al.* (2017) reported an inhibitory effect on the production of rooting, leaf, fresh weight, dry weight, plant height, and survival frequency *Phalaenopsis* in ½MS and VW media with higher concentrations of CW (20–30%). The inhibitory effects of higher concentration of CW on the plantlet growth could be due to the use of phytohormones above the optimum concentration.

Peptone, as an organic nitrogen source, may have contributed to the increased seedling development by supplying auxin-like compounds or various amino acids. Increased uniformity of seedling development was also observed in seedlings cultured in the presence of peptone (Kauth *et al.*, 2008). Similar beneficial effects of peptone were observed in *Cymbidium pendulum* cultures

(Kaur and Bhutani, 2012). In *Peristeria elata*, peptone favored early and healthy growth of seedlings (Bejoy *et al.*, 2004). Supplementations of organic growth adjuncts in orchid culture medium is simple, practical, beneficial and conventional method to improve media used for commercial production (Kaur and Bhutani, 2012).

Ex vitro survival of orchid seedlings is often low, and improving the survival of orchid seedlings ex vitro is essential for reintroduction programs. To increase seedling survival during *ex vitro* transfer, seedling acclimatization may be necessary. Since *in vitro* grown seedlings often have low or no stomatal activity (due to continually high humidity) and are grown in a high nutrient environment, acclimatization procedures are used to gradually decrease relative humidity levels, increase photosynthetic capacity, and acclimate seedlings to low nutrient environments (Kauth *et al.*, 2008). In our experiment, the survival rate of the transferred plantlets in a substrate mixture made up of expanded clay, charcoal, cocopeat, and perlite was 97%. Paul *et al.* (2012) reported that the survival rate of the *Dendrobium hookerianum* plantlets transferred to a compost mixture made up of broken brick and charcoal pieces with a covering layer of moss was 90%. The expanded clay, charcoal and perlite provided good drainage and aeration to the roots, and the cocopeat is thought to retain moisture content at an optimal level (Kauth *et al.*, 2008).

CONCLUSION

Our results indicated that MS or ½MS media were the most sufficient for maximum seed germination and early PLB and seedling development. Also, organic growth supplements (coconut water and peptone) proved beneficial for asymbiotic seed germination besides enhancing growth of *Phalaenopsis* PLBs and plantlets. This is a simple and low-cost medium for *Phalaenopsis in vitro* culture without the use of plant growth regulators and it can be used for large-scale propagation of this orchid species.

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