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In Vitro Propagation is an Optimal Method for the Production of Orchid *Phalaenopsis schilleriana* 'Karen Rockwell'

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Abstract

Phalaenopsis is an orchid genus of high economic value in world floriculture used as a pot plant and cut flowers. High genetic variation and the lack of uniformity in vegetative and reproductive propagation make the production of this orchid economically uninteresting. *In vitro* proliferation is the only large-scale feasible method for *Phalaenopsis* propagation. The purpose of the present study was to evaluate the effect of types and concentrations of α-naphthaleneacetic acid (NAA) and 6-benzyladenine (BA) (both at the concentrations of 0.0, 0.5, 1.0, 1.5 and 3.0 mg l^{-1}, individually or in combination), as a completely randomized design, on the *in vitro* propagation of *Phalaenopsis schilleriana* 'Karen Rockwell'. Activated charcoal (AC; 0.0, 0.5 and 1.0 mg l^{-1}) was added to the media for prevention of the browning of the media and tissues. Murashige and Skoog (MS), and protocorm were used as culture medium and explant, respectively. The results showed that the highest leaf number was obtained in medium enriched with 1.0 mg l^{-1} NAA together with 1.5 mg l^{-1} BA along with 1.0 mg l^{-1} AC. The treatment containing 1.5 mg l^{-1} NAA together with 0.5 mg l^{-1} BA along with 1.0 mg l^{-1} AC induced the highest number of roots. Fully *in vitro*-produced plantlets were transferred to pots containing a mixture of LECA (Light Expanded Clay Aggregate), peat moss, coco peat, charcoal soil, coco chips and perlite, and acclimatized in greenhouse conditions with 100% survival rate.

Keywords: Activated charcoal, Orchidaceae, Plant growth regulators, Protocorm-like bodies, Tissue culture.

INTRODUCTION

Orchids from family Orchidaceae are one of the most diverse flowering plant families with 800 genera, 25000 species, and thousands of hybrids from different regions of the world (Chugh *et al.,* 2009; Christenhusz and Byng, 2016). Orchids, including *Phalaenopsis*, cultivated as cut flowers and pot plants, are commercially important plants in world floriculture because of their medicinal and exotic values, such as variety in colors, sizes, shapes, and fragrances, as well as high durability of their flowers (Khoddamzadeh *et al.,* 2011; Park *et al.,* 2018; Cardoso *et al.,* 2020). *Phalaenopsis schilleriana* is a hybrid orchid. The genus *Phalaenopsis* (as epiphytic plants) comprises approximately 60 species native to tropical rainforests of South and South-East Asia, Australia and New Guinea (Winkelmann *et al.,* 2006).

The characteristics of seedlings propagated by vegetative means are not uniform, also propagation by seeds results in high genetic variability and the production of heterozygous plants; therefore, lots of tissue culture protocols have been developed in this genus (Murthy *et al.,* 2018; Asa and Kaviani, 2020). Natural clonal propagation of orchids is a slow process, which results in traits segregation and is, therefore, not possible for *Phalaenopsis*. Although, the micropropagation of genus *Phalaenopsis* has been demonstrated good development, the wide spread application of micropropagation is still limited due to some problems such as contamination, the exudation of phenolic compounds and somaclonal variation (Zahara, 2017). *In vitro* culture of *Phalaenopsis* could be considered reliable for guaranteeing the uniformity of flowers (Lee *et al.,* 2013; Zanello *et al.,* 2022). *In vitro* propagation is an extremely important and useful technique for clonal propagation of many species, particularly ornamental plants like orchids (Guo *et al.,* 2024).

Different procedures have been established for *in vitro* proliferation of orchids species, including *Phalaenopsis*, by various explants such as seeds, node, shoot tips, floral stalks, protocorm, protocorm-like bodies (PLBs), leaf, root, inflorescence, tuber, and rhizome, as well as somatic embryos, callus, thin cell layer, and plantlets obtained from seed (Roy *et al.,* 2011; Panwar *et al.,* 2012; Baker *et al.,* 2014; Mahendran, 2014; Chen *et al.,* 2015; Bhattacharyya *et al.,* 2016; Kaviani *et al.,* 2017; Yam and Arditti, 2018; Zakizadeh *et al.,* 2019; Mohammadi *et al.,* 2019; Asa and Kaviani, 2020). Many studies have shown that the optimization of medium composition was an important approach to improve the micropropagation process of orchids by culturing PLBs that is species-specific (Shimura and Koda, 2004; Luo *et al.,* 2009; Guo *et al.,* 2024). Protocorms and PLBs are tuberous embryonic masses of cells that are developed from seeds and vegetative tissues, respectively; they can grow into new plantlets and be applied in commercial micropropagation (Cui *et al.,* 2014; Lo *et al.,* 2022). PLBs derived from different types of somatic tissues represent a clonal method applied to produce large numbers of plantlets from few mother plants and explants (Zanello *et al.,* 2022). PLBs are similar to protocorms in morphology and biological characteristics (Lee *et al.,* 2013; Cardoso *et al.,* 2020). The main difference between protocorms and PLBs is basically the origin of the tissue. Induction of PLBs facilitate the micropropagation of orchids (Chen *et al.,* 2019). In orchids, the formation of protocorms and PLBs is regulated by various factors, and plant growth regulators (PGRs) are among the most important ones (Cardoso *et al.,* 2020).

Cytokinins are the most important factors to improve the plant regeneration from PLBs (Luo *et al.,* 2009). α-naphthaleneacetic acid (NAA) and 6-benzyladenine (BA) are widely applied for the regeneration of shoots from protocorms or PLBs in many *Phalaenopsis* species or hybrids (Park *et al.,* 2002; Paek *et al.,* 2011; Bali Lashaki *et al.,* 2014; Zanello *et al.,* 2022). Another PGRs such as indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), N-phenyl-N′-1,2,3-thiadiazol-5-yl-urea (TDZ), and 6-furfurylaminopurine or kinetin (Kin)

have been used for tissue culture of some orchids like *Cymbidium*, *Phalaenopsis*, *Dendrobium*, and *Paphiopedilum* and others (Roy *et al.,* 2011; Panwar *et al.,* 2012; Zeng *et al.,* 2012; Baker *et al.,* 2014; Bhattacharyya *et al.,* 2016; Kaviani *et al.,* 2017; Zakizadeh *et al.,* 2019; Mohammadi *et al.,* 2019; Asa and Kaviani, 2020; Cardoso *et al.,* 2020; Guo *et al.,* 2024).

Internal factors (such as genotype) as well as external factors (such as culture media, PGRs, and growing conditions) play a crucial role in enhancing the multiplication rate to improve the efficiency of micropropagation and plantlet production in *Phalaenopsis* (Khatun *et al.,* 2020; Zanello *et al.,* 2022). Therefore, the purpose of the present study was to evaluate the effect of different concentrations of NAA and BA, individually and in combination, on *in vitro* propagation of *Phalaenopsis schilleriana* 'Karen Rockwell' *via* organogenesis using protocorm explant.

MATERIALS AND METHODS

Plant material

Experiments were carried out on orchid *Phalaenopsis schilleriana* 'Karen Rockwell' (Fig. 1A) in June 2020 in tissue culture laboratory and greenhouse of the Hyrcan Agricultural Sciences and Biotechnology Research Institute, Amol, Mazandaran, Iran. The geographical coordinates of Amol are as follows: Latitude: 36°28′10″ N, longitude: 52°21′02″ E, and elevation above sea level: 96 m (314 ft). Healthy and sterilized PLBs prepared from a plant tissue culture in Austria was used as explant. *P. schilleriana* as Moth Orchid and the most popular species in this genus has relatively oblong leaves and the pink flowers.

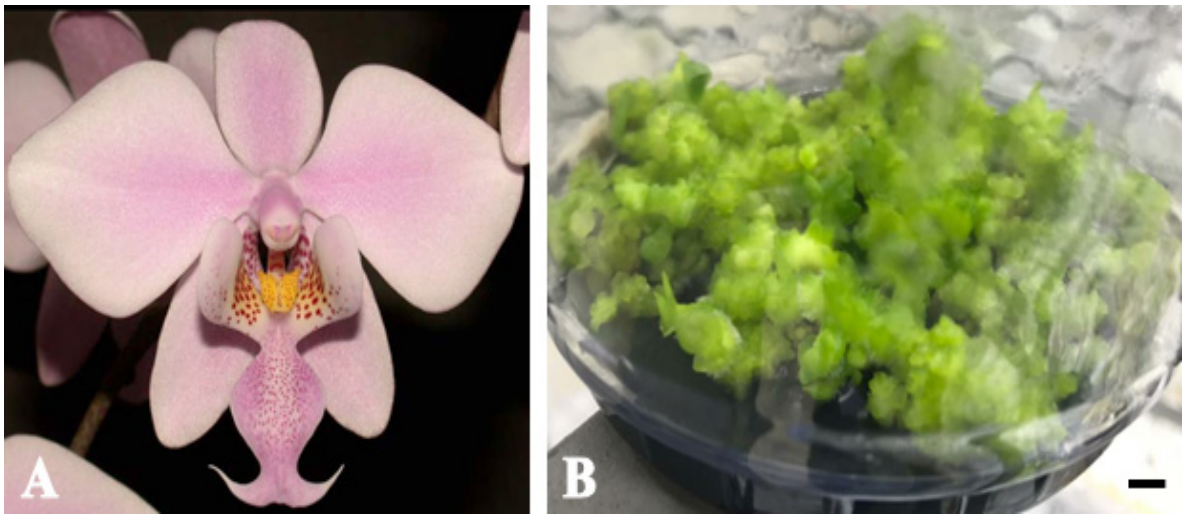

Fig. 1. Effect of NAA and BA on PLBs mass proliferation of *Phalaenopsis schilleriana*. A) In flowering stage; B) Protocorms produced from *in vitro* culture of seeds (scale bar = 10 mm).

Culture media and treatments

The explants (seed-originated protocorms) (Fig. 1B) were cultured on MS (Murashige and Skoog, 1962) medium containing 3% sucrose and 0.8% agar. The pH of the media was adjusted to 5.6–5.8 with 0.1 N NaOH or HCl prior to autoclaving. All media contained in culture bottles were autoclaved at 105 kPa and 121°C for 20 min. The media were enriched with different concentrations of BA and NAA both at the concentrations of 0.0, 0.5, 1.0, 1.5 and 3.0 mg l^{-1}, individually or in combination. Explants secrete phenolic compounds into the media; therefore, activated charcoal (AC; 0.0, 0.5 and 1.0 mg l^{-1}) was added to the media for prevention of the browning of the media. AC absorbs phenolic compound. All the cultures were

incubated at 24 ± 2 °C, 70−80% RH, and 16-h photoperiod of 50–60 µmol m^{-2} s^{-1} irradiance provided by cool−white fluorescent tubes.

Measured traits

Observations on leaf length, leaf width, leaf number, root length, root number, callus number and viability percentage were recorded 60 days after the culture initiation. Leaf length and width, as well as root length were measured with a ruler. The number of organs was counted with the naked eye.

Plantlets acclimatization

For *ex vitro* establishment, *in vitro*-rooted plantlets were taken out from culture vessels and washed with sterile distilled water to remove adherent medium from the plantlet body and transferred to plastic pots (18 cm height × 12 cm diameter) filled with a potting mixture of LECA (Light Expanded Clay Aggregate), peat moss, coco peat, charcoal soil, coco chips and perlite in the proportion of 15:10:20:5:30:20%. All the pots were then transferred to a greenhouse with temperature of 24 ± 2 ºC to 20 ± 2 ºC day/night (light intensity of 3,500 lux, RH of 80−90% and a 14h/10h day/night photoperiod) for acclimatization. The pots were covered with another plastic pots (18 cm height × 12 cm diameter) to retain moisture. Plantlets were exposed gradually to external environment. Thus, these pots were removed after two weeks. The plantlets were watered every five days. The number of surviving plants was recorded after two months of transfer.

Experimental design and data analysis

The experiments were established in a completely randomized design. For each treatment, three replicates and for each replicate, three specimens (explants) were taken (in total 75 treatments, 225 replicates, and 675 explants). PGR-free MS medium was used as control in the experiments. Data were subjected to analysis of variance (ANOVA) and means were compared by the LSD test at $P < 0.05$ using the SPSS ver. 17 (SPSS Inc., USA).

RESULTS

The results of Kolmogorov-Smirnov's test showed that the significance level was greater than 0.05 in all the traits measured in relation to shoot proliferation and there was no significant difference between the data and the data had a normal distribution (data not shown). Also, the results of Levene's homogeneity test showed that in all measured traits related to shoot proliferation, the significance level was greater than 0.05 and there was no significant difference between the data. Therefore, the data had a homogeneous distribution (data not shown). Analysis of variance (ANOVA) showed significant differences among different concentrations of NAA and BA for leaf length, root length, root number and viability percentage (all at $P<0.05$), as well as leaf number, leaf width and callus number (all at $P<0.01$) (data not shown).

Multiplication or shoot proliferation

Based on tables 1-3, simulatanious presence of NAA and BA is more suitable than the presence of each one of them for shoot proliferation. The highest leaf length (4.3 cm per explant) was achieved on medium enriched with 1.5 mg l^{-1} NAA together with 1.5 mg l^{-1} BA along with 1.0 mg l^{-1} AC. The content of 3.0 mg l^{-1} NAA together with 3.0 mg l^{-1} BA along with 1.0 mg l^{-1} AC in medium was suitable for inducing the leaf length. The lowest leaf length (1.13 cm per explant) was obtained on medium without PGRs (control). The highest mean values

of leaf width was 2.66 cm per explant, followed by 2.53 cm per explant, observed on media supplemented with 1.0 mg l^{-1} NAA together with 1.5 mg l^{-1} BA without AC, and 1.5 mg l^{-1} NAA together with 1.0 mg l^{-1} BA along with 1.0 mg l^{-1} AC, respectively. The lowest leaf length (0.4 cm per explant) was observed on medium without PGRs. Our results demonstrated that leaf number on medium enriched with 1.0 mg l^{-1} NAA in combination with 1.5 mg l^{-1} BA was significantly different compared to other treatments, as they produced the maximum number of leaf per explant (5.76 with 1.0 mg l^{-1} AC and 5.4 with 0.5 mg l^{-1} AC, respectively) (Tables 1-3, Fig. 2A, B). The medium without BA and AC, but containing 0.5 mg l^{-1} NAA induced the minimum number of leaf per explant (1.66). Present research showed that the 10 treatments containing different concentrations of NAA and BA induced the production of leaf more than 4 per explant.

Table 1. Mean comparison of the effect of different concentrations of NAA and BA without activated chaircol on the measured characteristics of *Phalaenopsis schilleriana* 'Karen Rockwell'.

NAA × BA (mg l^{-1})	Leaf length (cm)	Leaf width (cm)	Leaf number	Root length (cm)	Root number	Callus number	Viability (%)
0.0 × 0.0	1.33^{h}	0.76^{fg}	2.26^{d-g}	2.50^{i}	3.63^{bc}	4.36^{cde}	70.0^{d}
0.0 × 0.5	1.63^{e-h}	0.53^{g}	3.36^{bcd}	2.70^{i}	3.93^{abc}	4.63^{cde}	83.0^{bcd}
0.0 × 1.0	1.73^{d-h}	0.80^{efg}	2.53^{c-g}	3.13^{f-i}	3.60^{bc}	4.70^{cde}	73.0^{cd}
0.0 × 1.5	2.46^{b-e}	1.16^{d-g}	1.8^{fg}	4.33^{b-f}	4.46^{abc}	4.13^{cde}	83.0^{bcd}
0.0 × 3.0	2.00^{c-h}	1.60^{cde}	2.43^{d-g}	2.90^{hi}	3.36^{c}	7.56^{ab}	96.0^{ab}
0.5 × 0.0	1.43^{f-h}	1.26^{c-g}	1.66^{g}	3.80^{b-i}	4.40^{abc}	3.23^{cde}	83.0^{bcd}
0.5 × 0.5	1.90^{c-h}	1.16^{d-g}	2.8^{c-g}	3.56^{c-i}	4.16^{abc}	2.76^{e}	90.0^{ab}
0.5 × 1.0	2.03^{c-h}	2.03^{abc}	2.7^{c-g}	4.73^{bcd}	4.76^{abc}	5.20^{cd}	100.0^{a}
0.5 × 1.5	1.86^{c-h}	2.46^{ab}	3.7^{abc}	4.03^{b-h}	4.13^{abc}	3.36^{cde}	93.0^{ab}
0.5 × 3.0	1.40^{gh}	0.86^{d-g}	2.6^{c-g}	3.80^{b-i}	3.66^{bc}	4.20^{cde}	100.0^{a}
1.0 × 0.0	2.00^{c-h}	0.76^{fg}	2.16^{efg}	4.90^{b}	4.56^{abc}	3.16^{cde}	100.0^{a}
1.0 × 0.5	2.03^{c-h}	1.40^{c-f}	3.36^{bcd}	3.80^{b-i}	5.00^{ab}	8.70^{a}	90.0^{ab}
1.0 × 1.0	2.70^{a-d}	1.63^{cd}	3.33^{b-e}	6.20^{a}	5.00^{ab}	4.36^{cde}	90.0^{b}
1.0 × 1.5	2.36^{b-g}	2.66^{a}	4.7^{a}	4.70^{bcd}	5.23^{a}	3.00^{de}	96.0^{ab}
1.0 × 3.0	3.26^{ab}	1.56^{c-f}	3.33^{b-e}	4.56^{bcde}	4.50^{abc}	4.20^{cde}	86.0^{abc}
1.5 × 0.0	1.76^{d-h}	0.93^{d-g}	2.43^{d-g}	4.83^{bc}	4.50^{abc}	4.33^{cde}	86.0^{abc}
1.5 × 0.5	1.93^{c-h}	1.30^{c-g}	3.06^{b-e}	4.00^{b-h}	4.80^{abc}	3.53^{cde}	96.0^{ab}
1.5 × 1.0	2.50^{a-e}	1.66^{bcd}	3.26^{b-e}	4.80^{bc}	4.10^{abc}	4.46^{cde}	96.0^{ab}
1.5 × 1.5	3.23^{ab}	0.93^{d-g}	4.2^{ab}	4.26^{b-g}	4.16^{abc}	3.00^{de}	96.0^{ab}
1.5 × 3.0	1.80^{c-h}	0.90^{d-g}	2.8^{c-g}	3.00^{ghi}	4.66^{abc}	4.33^{cde}	86.0^{abc}
3.0 × 0.0	1.86^{c-h}	1.16^{d-g}	2.46^{d-g}	3.33^{e-i}	4.16^{abc}	4.03^{cde}	96.0^{ab}
3.0 × 0.5	1.73^{d-h}	1.26^{c-g}	2.73^{c-g}	3.80^{b-i}	4.23^{abc}	5.33^{bc}	86.0^{abc}
3.0 × 1.0	2.43^{b-f}	1.56^{c-f}	2.63^{c-g}	3.46^{d-i}	3.93^{abc}	4.53^{cde}	90.0^{ab}
3.0 × 1.5	2.80^{abc}	1.23^{c-g}	4.16^{ab}	3.46^{d-i}	4.30^{abc}	3.63^{cde}	93.0^{ab}
3.0 × 3.0	3.50^{a}	1.00^{d-g}	2.86^{c-e}	3.66^{b-i}	4.16^{abc}	3.80^{cde}	86.0^{abc}

Means with different letters on the same column are significantly different ($P<0.05$) based on LSD test.

Table 2. Mean comparison of the effect of different concentrations of NAA and BA along with 0.5 mg l^{-1} activated chaircol on the measured characteristics of *Phalaenopsis schilleriana* 'Karen Rockwell'.

NAA × BA (mg l^{-1})	Leaf length (cm)	Leaf width (cm)	Leaf number	Root length (cm)	Root number	Callus number	Viability (%)
0.0 × 0.0	1.13^{g}	0.40^{f}	3.00^{cd}	2.8^{g}	3.46^{gh}	2.60^{g}	86.0^{abc}
0.0 × 0.5	1.36^{efg}	1.33^{b-e}	2.26^{d}	2.93^{efg}	2.83^{h}	5.20^{bcd}	83.0^{bc}
0.0 × 1.0	2.20^{b-e}	0.90^{c-f}	3.23^{bcd}	2.76^{g}	3.70^{fgh}	3.03^{fg}	90.0^{abc}
0.0 × 1.5	2.36^{a-d}	1.20^{b-e}	2.50^{d}	2.83^{fg}	4.73^{b-g}	2.83^{g}	100.0^{a}
0.0 × 3.0	1.93^{b-g}	0.93^{c-f}	2.50^{d}	2.93^{efg}	4.40^{b-g}	3.33^{d-g}	86.0^{abc}
0.5 × 0.0	1.93^{b-g}	0.73^{ef}	2.80^{d}	4.66^{a-d}	5.40^{a-e}	2.86^{fg}	96.0^{ab}
0.5 × 0.5	1.70^{d-g}	1.20^{b-e}	2.26^{d}	3.36^{d-g}	5.76^{abc}	5.10^{bcd}	93.0^{abc}
0.5 × 1.0	2.03^{b-g}	1.66^{abc}	2.46^{d}	3.83^{c-g}	5.43^{a-e}	6.16^{bc}	93.0^{abc}
0.5 × 1.5	1.26^{fg}	2.26^{a}	4.73^{a}	3.46^{c-g}	5.56^{a-d}	3.00^{fg}	96.0^{ab}
0.5 × 3.0	2.13^{b-f}	1.16^{b-f}	2.93^{d}	4.30^{a-e}	4.50^{b-g}	3.13^{efg}	96.0^{ab}
1.0 × 0.0	2.43^{a-d}	0.80^{def}	2.56^{d}	5.56^{ab}	3.33^{gh}	3.50^{d-g}	80.0^{c}
1.0 × 0.5	2.23^{b-e}	1.53^{a-d}	2.86^{d}	4.23^{b-f}	5.70^{abc}	4.96^{cde}	96.0^{ab}
1.0 × 1.0	1.83^{c-g}	1.86^{ab}	3.40^{bcd}	5.66^{a}	6.66^{a}	5.03^{b-e}	90.0^{abc}
1.0 × 1.5	1.76^{c-g}	1.43^{b-e}	5.40^{a}	4.86^{abc}	5.16^{a-f}	8.16^{a}	96.0^{ab}
1.0 × 3.0	2.83^{ab}	1.33^{b-e}	3.43^{bcd}	3.6^{c-g}	4.10^{d-h}	2.76^{g}	90.0^{abc}
1.5 × 0.0	1.70^{d-g}	0.96^{c-f}	2.70^{d}	4.00^{c-g}	5.00^{b-f}	6.90^{ab}	90.0^{abc}
1.5 × 0.5	2.30^{a-d}	1.23^{b-e}	4.23^{abc}	3.40^{d-g}	5.83^{ab}	3.93^{d-g}	96.0^{ab}
1.5 × 1.0	2.33^{a-d}	1.50^{a-e}	2.46^{d}	4.13^{c-g}	4.53^{b-g}	4.76^{c-f}	93.0^{abc}
1.5 × 1.5	3.16^{a}	1.06^{c-f}	4.36^{ab}	3.60^{c-g}	4.26^{c-h}	4.36^{c-g}	83.0^{bc}
1.5 × 3.0	2.53^{a-d}	1.30^{b-e}	3.13^{bcd}	4.16^{b-g}	3.73^{fgh}	4.50^{c-g}	100.0^{a}
3.0 × 0.0	2.63^{abc}	1.16^{b-f}	2.46^{d}	3.26^{d-g}	4.33^{b-h}	2.80^{g}	90.0^{abc}
3.0 × 0.5	1.96^{b-g}	1.26^{b-e}	2.96^{cd}	4.16^{b-g}	4.13^{d-h}	5.20^{bcd}	80.0^{c}
3.0 × 1.0	2.53^{a-d}	1.53^{a-d}	3.00^{cd}	4.13^{c-g}	4.03^{d-h}	4.23^{d-g}	90.0^{abc}
3.0 × 1.5	2.50^{a-d}	1.06^{c-f}	4.33^{ab}	3.86^{c-g}	3.73^{fgh}	3.53^{d-g}	83.0^{bc}
3.0 × 3.0	2.33^{a-d}	1.23^{b-e}	3.36^{bcd}	3.93^{c-g}	3.80^{fgh}	3.53^{d-g}	96.0^{ab}

Means with different letters on the same column are significantly different (P<0.05) based on LSD test.

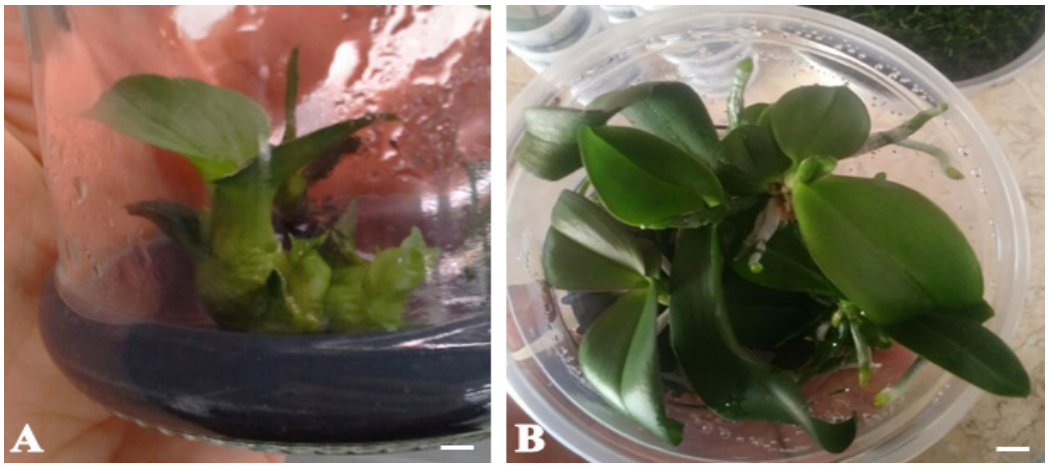

Fig. 2. Effect of NAA and BA along with activated chaicol on PLBs growth of *Phalaenopsis schilleriana* 'Karen Rockwell'. A) On medium enriched with 1.0 mg l^{-1} NAA together with 1.5 mg l^{-1} BA along with 0.5 mg l^{-1} AC; B) On medium enriched with 1.0 mg l^{-1} NAA together with 1.5 mg l^{-1} BA along with 1.0 mg l^{-1} AC (scale bar = 5 mm).

Table 3. Mean comparison of the effect of different concentrations of NAA and BA along with 1.0 mg l^{-1} activated chaircol on the measured characteristics of *Phalaenopsis schilleriana* 'Karen Rockwell'.

NAA × BA (mg l^{-1})	Leaf length (cm)	Leaf width (cm)	Leaf number	Root length (cm)	Root number	Callus number	Viability (%)
0.0 × 0.0	1.9^{de}	1.01^{d}	2.90^{e-h}	3.06^{ef}	4.10^{c-f}	5.40^{abc}	90.0^{a-d}
0.0 × 0.5	2.16^{cde}	1.20^{d}	2.70^{e-h}	2.80^{f}	3.50^{f}	4.16^{bc}	73.0^{e}
0.0 × 1.0	2.33^{cde}	1.80^{a-d}	3.00^{d-h}	3.76^{c-f}	4.43^{b-f}	5.26^{abc}	76.0^{de}
0.0 × 1.5	1.93^{de}	1.50^{bcd}	3.03^{d-h}	3.33^{def}	4.50^{b-f}	4.03^{bc}	76.0^{de}
0.0 × 3.0	1.80^{de}	1.90^{a-d}	2.66^{e-h}	4.13^{b-f}	3.93^{def}	3.46^{c}	90.0^{a-d}
0.5 × 0.0	1.90^{de}	1.10^{d}	2.60^{e-h}	3.70^{c-f}	4.96^{b-e}	3.43^{c}	96.0^{ab}
0.5 × 0.5	2.76^{cd}	1.60^{a-d}	2.43^{gh}	4.13^{b-f}	4.93^{b-f}	6.50^{ab}	90.0^{a-d}
0.5 × 1.0	1.83^{de}	1.70^{a-d}	2.86^{e-h}	5.73^{ab}	5.46^{abc}	5.40^{abc}	83.0^{b-e}
0.5 × 1.5	3.06^{bc}	2.23^{ab}	4.63^{ab}	4.73^{a-d}	5.36^{a-d}	4.80^{abc}	93.0^{abc}
0.5 × 3.0	2.66^{cd}	1.66^{a-d}	2.50^{fgh}	4.10^{b-f}	4.33^{b-f}	3.86^{bc}	96.0^{ab}
1.0 × 0.0	2.50^{cde}	1.03^{d}	1.86^{h}	4.30^{b-f}	5.70^{ab}	3.40^{c}	80.0^{cde}
1.0 × 0.5	2.80^{cd}	1.50^{bcd}	3.40^{b-g}	3.66^{c-f}	4.00^{def}	6.50^{ab}	100.0^{a}
1.0 × 1.0	2.20^{cde}	2.16^{abc}	2.83^{e-h}	6.26^{a}	5.70^{ab}	4.63^{bc}	86.0^{a-e}
1.0 × 1.5	2.33^{cde}	2.46^{a}	5.76^{a}	5.00^{abc}	4.03^{cdef}	3.96^{bc}	83.0^{b-e}
1.0 × 3.0	3.03^{bc}	1.60^{a-d}	3.80^{b-f}	4.80^{a-d}	4.46^{b-f}	3.40^{c}	90.0^{a-d}
1.5 × 0.0	1.80^{de}	1.40^{bcd}	2.60^{e-h}	4.26^{b-f}	4.96^{b-e}	5.76^{abc}	83.0^{b-e}
1.5 × 0.5	2.30^{cde}	1.16^{d}	4.23^{bcd}	4.00^{c-f}	6.73^{a}	4.86^{abc}	93.0^{abc}
1.5 × 1.0	2.40^{cde}	2.53^{a}	3.33^{b-g}	3.46^{c-f}	3.70^{ef}	4.06^{bc}	96.0^{ab}
1.5 × 1.5	4.30^{a}	1.03^{d}	4.53^{abc}	3.96^{c-f}	4.36^{b-f}	3.63^{c}	96.0^{ab}
1.5 × 3.0	2.50^{cde}	1.36^{bcd}	3.26^{c-g}	4.00^{c-f}	4.10^{c-f}	4.70^{abc}	96.0^{ab}
3.0 × 0.0	2.6^{cde}	1.66^{a-d}	2.66^{e-h}	3.56^{c-f}	4.6^{b-f}	3.73^{c}	80.0^{cde}
3.0 × 0.5	2.13^{cde}	1.26^{cd}	2.93^{d-h}	4.66^{a-e}	4.46^{b-f}	7.33^{a}	93.0^{abc}
3.0 × 1.0	1.56^{e}	1.66^{a-d}	3.46^{b-g}	4.46^{b-e}	4.56^{b-f}	4.50^{bc}	90.0^{a-d}
3.0 × 1.5	2.46^{cde}	1.46^{bcd}	3.90^{b-e}	3.90^{c-f}	4.36^{b-f}	4.16^{bc}	83.0^{b-e}
3.0 × 3.0	4.06^{ab}	1.30^{bcd}	3.40^{b-g}	3.40^{c-f}	4.43^{b-f}	3.40^{c}	80.0^{cde}

Means with different letters on the same column are significantly different (P<0.05) based on LSD test.

Root induction and growth

Based on tables 1-3, simulatanious presence of NAA and BA is more suitable than the presence of each one of them for root induction and growth. We found that a combination of 1.00 mg l^{-1} of both NAA BA, with and without AC, provoked the highest length of root (6.26, 6.2 and 5.66 cm per explant, respectively). The lowed root length (2.5 cm per explant) was achieved on medium without NAA, BA and AC. The media containing 0.5, 1.0 and 1.5 mg l^{-1}

NAA combined with 0.5 and 1.0 mg l^{-1} BA were effective for root production. The medium fortified with 1.5 mg l^{-1} NAA together with 0.5 mg l^{-1} BA along with 1.0 mg l^{-1} AC resulted in the highest increase in root number (6.73) (Fig. 3A). High root number was also observed using 1.0 mg l^{-1} NAA together with 1.0 mg l^{-1} BA along with 0.5 mg l^{-1} AC. The difference between these two media was not significant. The highest reduction in root production was found in medium containing 0.5 mg l^{-1} BA without NAA.

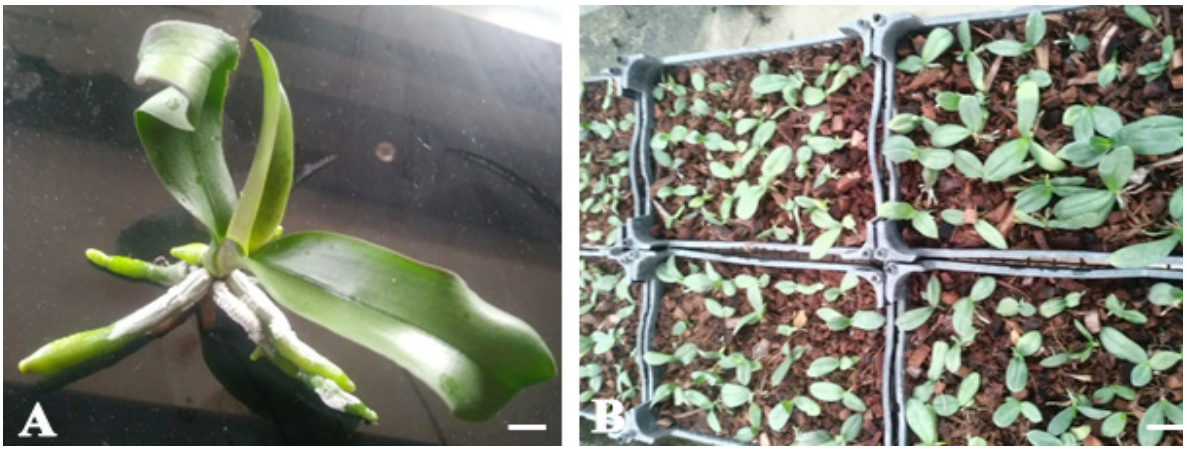

Fig. 3. Rooting and transplanting of *Phalaenopsis schilleriana* 'Karen Rockwell'. A) Rooting plantlet on medium enriched with 1.5 mg l^{-1} NAA together with 0.5 mg l^{-1} BA along with 1.0 mg l^{-1} AC; B) Plantlets transplanted to trays filled out with a mixture of LECA (Light Expanded Clay Aggregate), peat moss, coco peat, charcoal soil, coco chips and perlite (A: scale bar = 5 mm; B: scale bar = 20 mm).

Callus induction and plantlets viability

The treatments containing 1.0 mg l^{-1} NAA together with 0.5 and 1.5 mg l^{-1} caused an increase in the number of callus per explant (more than 8), compared with the control (2.6). Explants cultured on medium supplemented with 3.0 mg l^{-1} NAA together with 0.5 mg l^{-1} BA along with 1.0 mg l^{-1} AC showed high callus number. Plantlets obtained from six treatments resulted in the 100% viability. Plantlets produced in medium without NAA, BA and AC showed least viability (70%) (Tables 1-3).

***Ex vitro* establishment of plantlets**

Well-developed plantlets were transferred to plastic pots for *ex vitro* establishment and acclimatization (Fig. 3B). A 100% establishment rate was obtained and plantlets were morphologically identical to the mother plants.

DISCUSSION

Our findings demonstrated that the spontaneous use of an auxin and a cytokinin is important in the shoot and root production in *Phalaenopsis schilleriana* 'Karen Rockwell'. The PGR-free culture medium resulted in low survival of plantlets and low shoot multiplication and root induction rates. Reports of many researchers showed better results when they were used an auxin and a cytokinin, in combination (Panwar *et al.,* 2012; Zakizadeh *et al.,* 2019; Mohammadi *et al.,* 2019; Asa and Kaviani, 2020). Bhattacharyya *et al.* (2016) revealed that when the explants were grown in medium containing cytokinin and auxin, a higher rate of response frequency of shoot buds and PLBs was observed in all PGRs combinations. A combination of 1.0 mg l^{-1} KIN and 1.0 mg l^{-1} IBA was found to be suitable for regeneration of most measured characteristics especially leaf and root number in *Phalaenopsis amabilis* (L.) Blume var. Jawa. Also, the

maximum number of plantlet was obtained on medium supplemented with 1.0 mg l^{-1} KIN and 0.5 mg l^{-1} IBA (Asa and Kaviani, 2020). In our study, the percentage of the explant response to leaf, root and callus formation was generally enhanced through the use of 0.5-1.5 mg l^{-1} of both NAA and BA. BA treatment was significantly better for shoot induction compared with NAA. On the other hand, NAA treatment was significantly better for root induction compared with BA. Although, the differences between NAA and BA were insignificant. Contrary to our finding, BA individually was better than in combination with NAA for shoot production of orchid *Oncidium* (Kalimuthu *et al.,* 2007). BA is known to promote seedling leaf formation in some *Paphiopedilum* species (Chen *et al.,* 2015). BA is the cytokinin most commonly used in plant tissue culture, and it is also efficient in promoting shoot development (Zanello *et al.,* 2022). The ratio of auxin and cytokinin for PLB, shoot and root formation depends upon the species studied. Similar findings were reported on *Phalaenopsis* and other orchid's species (Baker *et al.,* 2014; Zakizadeh *et al.,* 2019; Lo *et al.,* 2022; Zargar *et al.,* 2023; Kiaheirati *et al.,* 2024). Study on orchid *Dendrobium nobile* demonstrated that when explants were cultured in medium enriched with BAP solely, PLBs was formed but direct shoot formation was not observed (Bhattacharyya *et al.,* 2016). The presence of cytokinins alone promoted optimal shoot proliferation from protocorm explants in some orchids like *Dendrobium nobile* and *C. aloifolium* (Nayak *et al.,* 1997b), *C. ensifolium* (Chang and Chang, 1998), *Rhynchostylis gigantea* (van Le *et al.,* 1999), *D. nobile* and *C. aloifolium* (Nayak *et al.,* 2002), and *Dendrobium* (Ferreira *et al.,* 2006). The effect of NAA and BA at different concentrations on the induction of PLBs, leaf, root and callus was assessed on *Phalaenopsis* (Bali Lashaki *et al.,* 2014).

The most commonly used auxins in orchid culture media are indole-3-acetic acid (IAA), NAA, IBA, and 2,4-D. On the other hand, Kin, BA, BAP, TDZ, and zeatin (Zt) are the most commonly used cytokinins in orchid culture media (Yam and Arditi, 2018). Some orchid tissue culture studies found that auxins alone or in combination with cytokinins increased overall shoot growth (Parvathy, 2022). Some researchers have recognized that the effect of a single PGR alone on shoot multiplication is better than the effect of that in combination with another PGRs in orchids (Martin and Madassery, 2006; Zhao *et al.,* 2007; Mahendran and Narmatha Bai, 2009; Luo *et al.,* 2009; Panwar *et al.,* 2012; Parthibhan *et al.,* 2015). These findings are in contrast with our findings, because maximum shoot number was produced in media fortified with NAA in combination with BA. Study of Hossain *et al.* (2010) on *Cymbidium giganteum* and Bali Lashaki *et al.* (2014) on *Phalaenopsis amabilis* var. 'Manila' revealed that multiple shoot formation were induced on medium supplemented with different concentrations and combinations of BAP together with NAA, and BA together with NAA, respectively.

The present study showed that BA in combination with NAA induced better rooting. Some reports showed that cytokinins would be associated with a subsequent inhibition of *in vitro* rooting (Podwyszynska, 2003). However, many researches has shown that BA-derived shoots resulted in better rooting compared with shoots derived from BA-free culture medium (Iiyama and Cardoso, 2021; Zanello *et al.,* 2022). Our study also showed that NAA was suitable for root length and number. Similar to our finding, in *Vanda coerulea* Griff ex. Lindl. (Blue Vanda), NAA was found to be the most effective for production of maximum numbers of PLBs, shoots and roots which simultaneously differentiated in the same medium (Roy *et al.,* 2011). NAA was found more effective than IBA for micropropagation of *Orchis catasetum* (Baker *et al.,* 2014). Kiaheirati *et al.* (2024) demonstrated that the longest roots in *Phalaenopsis circus* were induced using both NAA and Kin in combination. The best root induction in *Phalaenopsis amabilis* cv. Cool 'Breeze' was achieved with 1.0 mg l^{-1} IAA (Bali Lashaki *et al.,* 2014). Baker *et al.* (2014) showed that a combination of 0.5 mg l^{-1} BA and 0.5 mg l^{-1} NAA induced the

largest number of root and the highest length of root in orchid *Catasetum*. These researchers also showed that a combination of 1.0 mg l^{-1} BA and 0.5 mg l^{-1} NAA was a suitable treatment for induction of root number and root length.

Callus was produced at the base of shoots of *Eulophia nuda* cultured on medium supplemented with higher concentration of BA, while lesser number of shoots were produced on medium with lower BA concentration (Panwar *et al.,* 2012). In the present study, induction of callus was occured in some treatments. This is in contradict with the findings for *Paphiopedilum* spp. (Guo *et al.,* 2024; Kiaheirati *et al.,* 2024). Similarity, calluses have been successfully induced from the seeds or protocorms of some species of *Paphiopedilum* (Zeng *et al.,* 2013; Guo *et al.,* 2024).

The better development and proliferation of shoots and induction of roots in culture media containing AC may be related to the effect of AC as an anti-browning agent. Browning is one of the major problems affecting *in vitro* cultivation of *Phalaenopsis* and some other orchids. This problem is frequently associated with high content of phenolics and increases in polyphenol oxidase activity (Xu and Li, 2006; Zanello *et al.,* 2022). The high rate of explants browning, and its association with phenolic oxidation, has been previously reported in the genus *Phalaenopsis* and is caused by physical damage to tissues, with phenolic oxidation being toxic to plant tissues and in some cases leading to plant death (Minamiguchi and Machado Neto, 2007; Zanello *et al.,* 2022).

The *in vitro* rooted plantlets were successfully acclimatized in the greenhouse through their cultivation in pots containing a mixture of LECA (Light Expanded Clay Aggregate), peat moss, coco peat, charcoal soil, coco chips and perlite in the proportion of 15:10:20:5:30:20%. Similar results were reported on *Phalaenopsis circus* (Kiaheirati *et al.,* 2024). Coconut powder, sphagnum, and vermiculite, also cocochips and sphagnum moss were also applied for acclimatization of *Phalaenopsis* plantlets (Venturieri and Arbieto, 2011; Asa and Kaviani, 2020; Zanello *et al.,* 2022). The successful use of clay, sand, vermicompost (1:1:1), moss and charcoal (1:1), charcoal and brick pieces (1:1), sand, vermiculite and chopped dry leaves (1:1:1), moss, peat and perlite (3:1:1); vermiculite, bark, soil (1:2:2), sphagnum and coconut fibres (1:1), and peat and perlite (1:1) was reported in some other orchid species (Panwar *et al.,* 2012; Teixeira da Silva *et al.,* 2017; Lo *et al.,* 2022; Zargar *et al.,* 2023). The highest water-holding capacity was pointed out as the main difference between various substrates (Venturieri and Arbieto, 2011).

CONCLUSION

In conclusion, our investigation into the tissue culture of *Phalaenopsis schilleriana* 'Karen Rockwell' under varying exogenous NAA and BA treatments has presented an efficient and reliable procedure. The treatments containing 1.0 mg l^{-1} NAA together with 1.5 mg l^{-1} BA, and 1.5 mg l^{-1} NAA together with 0.5 mg l^{-1} BA, both along with 1.0 mg l^{-1} AC induced the highest number of leaves and roots, respectively. These findings have significant implications for optimizing *P. schilleriana* 'Karen Rockwell' micropropagation protocols.

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