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Original Article

Micropropagation of *Phalaenopsis amabilis* var. 'Manila' by Leaves Obtained from *in Vitro* Culturing the Nodes of Flower Stalks

Khosro BALILASHAKI[1], Masood GHASEMI GHEHSAREH[2]*

[1]*Guilan University, Faculty of Agriculture, Department of Horticultural Sciences, Rasht, Iran; khosrobali@alumni.ut.ac.ir*
[2]*Shahrekord University, Faculty of Agriculture, Department of Horticultural Sciences, Shahrekord, Iran; mghasemi1352@gmail.com (*corresponding author)*

Abstract

Orchids are one of the most popular plants in the world and among them the species of *Phalaenopsis* have the most sales on the global market. Because of its difficult propagation, micropropagation has been suggested recently. In the current study, the leaves obtained from *in vitro* culture of flower stalk nodes were used as explants and were cultured on MS medium with different concentrations of NAA, BA and TDZ. Protocorm-like bodies (PLBs) were produced and transferred to medium without growth regulators. Finally, the adaptation of plants was evaluated in a medium of cocopeat + coal (3:1 v/v) and another medium of cocopeat + charcoal + LECA (2:1:2 v/v). Results showed that the highest percentage of active samples was 100% which could regenerate the PLBs by being treated with 4 mg/l TDZ. The lowest active samples (60%) were those treated in the medium with 4 mg/l BAP + 0.5 mg/l NAA. The highest PLBs per explant (50.65) were obtained in the medium supplemented with 15 mg/l BAP + 3 mg/l NAA. Best acclimation (90%) of plants was obtained when medium of cocopeat + charcoal + LECA (2:1:2 v/v) was used. According to the results of the current experiment, the MS culture medium containing 15 mg/l BAP + 3 mg/l NAA was thereby considered as the best medium for *Phalaenopsis* micropropagation.

Keywords: cytokinin, micropropagation, naphthalene-acetic acid, *Phalaenopsis*, protocorm

Abbreviations: BA (BAP) - benzyladenine; NAA - naphthaleneacetic acid; KIN - kinetin; TDZ - thidiazuron

Introduction

Orchids are comprised of 800 genera and approximatively 25,000 species. They are one of the oldest and most highly developed families of plants. These flowers contribute to 8% of the global flower trade (Chugh *et al.*, 2009). Orchids are important pot plants and they are also used as cut flower plants because of their unique beauty, their diverse colours and shapes and their durable vase-life (Debarg and Zimmerman, 1991). Orchids are one of the first ten popular cut flowers in the world (Chugh *et al.*, 2009). Their cultivation and production is known to be a large business in the realm of horticulture (Goh and Kavalijan, 1989; Hew, 1989; Alam *et al.*, 2002).

The cultivation of orchids in Iran started officially in 1949, concurrent with the establishment of the tropical and semi-tropical complex of agriculture in the Iranian city of Noshahr, the biggest center for the production of orchids in Iran. Nonetheless, recent years have had establishments of more centers for orchid production.

Phalaenopsis are commonly known as moth orchids and are characterized by long, arcuate branches. Most of them are epiphytic plants, meaning that they grow on other plants (trees for instance) and only very few of them are lithophytes (living on rocks) (Rittirat *et al.*, 2014). *Phalaenopsis* are commonly used as pot plants and cut-flower plants in the world flower trade. They owe their popularity and prevalence to their magnificent flowers, to their convenient cultivation under controlled conditions and their particularly long decorative effect (Guo *et al.*, 2012; Liu *et al.*, 2013; Lin and Hsu, 2004). The *Phalaenopsis* have a monopodial pattern of growth; it rarely exhibits branching and it seldom initiates secondary new shoots. Normally, a flower branch is produced from the axial bud of each leaf (Christenson, 2001). The vegetative propagation (or asexual propagation) of this type of orchid is difficult and it naturally grows slowly (Košir *et al.*, 2004). This orchid is naturally propagated via seeds and kieiz; the latter are newborn orchid individuals that naturally form on the flower stalk (Chugh *et al.*, 2009). Orchid seeds do not contain endosperm and therefore the seed will only germinate under special nutritional and environmental conditions (Arditti *et al.*, 1990). Furthermore, the life cycle of *Phalaenopsis* is considerably lengthy and takes 1-3 years. Genetically, some species of this orchid are self-incompatible (Tang and Chen, 2007). Therefore, an optimal approach would be to consider *in vitro* clonal propagation so as to produce *Phalaenopsis* on a large scale of commercial level.

Frequent researches on the production of *Phalaenopsis* have indicated that it can be propagated by cultivating the seeds, by culturing the nodes that occur on the inflorescence, by *in vitro* culturing of the leaves that grow

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from those nodes, by culturing protocorm-like bodies (PLB) and by plantlets obtained from seed (Reuter, 1983; Tanaka and Sakanishi, 1977; 1980; 1985; Tanaka, 1987; 1992; Tokuhara and Mii, 1993; Arditti and Ernst, 1993; Tokuhara and Mii, 2001; Park *et al*., 2002; Chugh *et al*., 2009). And yet, further research is necessary to optimize the methods of micropropagation for *Phalaenopsis*, in light of its great commercial value. The current research is therefore in line with the optimization of relevant micropropagation protocol.

Protocorms are immature organs in orchids that can differentiate into new shoots. The cells of protocorms are meristematic and have strong potential for totipotency; therefore, protocorms can be used for the proliferation of orchids and the production of plantlets (Teixeira da Silva *et al*., 2005). Gow *et al*. (2008) and Penggow *et al*. (2010) examined embryogenesis in orchids by using leaf explants of two orchid species, *P. nebula* and *P. amabilis*. Accordingly, the authors evaluated the effects of explant size, the location whereof the explant was taken from the plant and the effects of various growth regulators such as auxins, cytokinins, polyamines, gibberellic acid and ACC. They discovered that 2ip and BA had the greatest encouraging effect on embryogenesis, whereas auxins, GA_3 and polyamines exhibited preventive effects. Furthermore, they discovered that the best size of explants is 1 cm and that the basal segment of the leaf has more success in producing somatic embryos, compared to the terminal segment of the leaf. Feng and Chen (2014) and Rittirat *et al*. (2014) studied the effects of TDZ and BA on the production of protocorms in two orchid species, *P. cornu-cervi* and *P. aphrodite* spp. Formosana. They observed that TDZ is more effective than BA.

Kośir *et al*. (2004), David and Bala (2012) found that the combined application of BAP and NAA in *Phalaenopsis* has more positive effects on shoot regeneration than that occured from nodes of flower stalks. Tokuhara and Mii (1993) observed that the best combination of plant growth regulators which can be used for *in vitro* media culture was 20 mg l^{-1} BAP and 0.1 mg l^{-1} NAA, so as to yield the best result of producing the most protocorms growing from the buds of flower stalks.

Chen and Chang (2004) examined the effects of TDZ and NAA on the embryogenesis of protocorms resulted from culturing the seeds of *P. amabilis* 'Formosa'. They found that the best condition for embryogenesis occurred in half-strength MS medium with 3 mg l^{-1} TDZ. Niknejad *et al*. (2011) conducted a research by tissue culturing from leaf segments of young *P. gigantea* plants (*in vitro*). The media for their tissue culture contained either of BAP, TDZ or KIN, either with or without NAA. They observed that the best combination of growth regulators that can be used for the optimum production of callus and protocorms was the combination of 0.1 mg l^{-1} TDZ + 1 mg l^{-1} NAA.

With respect to the history of research on the tissue culture of orchids, the current study was designed to optimize and improve the micropropagation of *P. amabilis* 'Manila'. Accordingly, the effects of BAP, NAA and TDZ were examined on the direct embryogenesis and proliferation of clonal plants of the studied orchid species. The explants used in the research were leaves that were obtained by tissue culturing the nodes located on flower stalks.

Materials and Methods

Plant materials

Two-year-old orchid plants of the species *P. amabilis* var. 'Manila' were purchased from a specialized company. On the structure of plants, the branches with at least three blooms and the ones with the biggest flower stalks were cut from the mother plants and their basal ends were placed in special containers having distilled water. The containers were packed and taken to the lab. The terminal ends of the branches, bearing the blooms and flowers, were dissected and the flower stalks were used for the experiment. The flower stalks contained several nodes for the purpose of the experiment. The flower stalks were cleaned three times with cotton that was soaked in ethanol (70%). Each node-bearing explant measured 5-6 cm in length. The explants were pre-treated in a litre of water solution comprised of benomyl (1%), a drop of Tween 20 and a drop of liquid soap, for 10 minutes. Then, the extra moisture on the explants was dried and the explants were exposed to the flow of air under the hood used for tissue culture. Thereafter they were disinfected in ethanol (70%) for 20 seconds and in sodium hypochlorite (10%) for 10 minutes. The explants were washed with sterilized water and then both ends of each explant were cut thus shortened by 1.5 cm. The resultant explants measured 1 cm in length. The bracts adjacent to the flower buds were dissected so as to better expose the buds to the culture media. The explants were cultured horizontally on culture media containing 4.4 mg l^{-1} BA and 1 mg l^{-1} NAA in order to produce leaves. The culture media was MS, containing 8 g/l agar and 30 g/l sucrose. Prior to autoclaving, the pH of media was adjusted to 5.7 by HCl or NaOH (1 Normal solution). The explants were maintained under a photoperiodic condition of 16 (h) of daylight and 8 (h) of darkness through the experiment. The temperature was controlled at 25 ± 1 °C. The explants were sub-cultured every 14 days.

The production of plantlets from sterile leaves

After 150 days, following the first culture of explants, each node had yielded 10 shoots on average. From those shoots, leaf explants were taken in sizes of 1 cm × 0.5 cm and were cultured on MS media containing various concentrations of NAA, BA and TDZ (Table 1). These were sub-cultured every 14 days. Protocorms gradually emerged from the edges of the cultured leaves after six weeks

Table 1. Treatments in embryogenesis from leaf explants

Treatments	Treatments No.
MS (control)	1
MS + 1 mg l^{-1} TDZ	2
MS + 2 mg l^{-1} TDZ	3
MS + 3 mg l^{-1} TDZ	4
MS + 4 mg l^{-1} TDZ	5
MS + 4 mg l^{-1} BAP + 0.5 mg l^{-1} NAA	6
MS + 6 mg l^{-1} BAP + 1 mg l^{-1} NAA	7
MS + 10 mg l^{-1} BAP + 2 mg l^{-1} NAA	8
MS + 15 mg l^{-1} BAP + 3 mg l^{-1} NAA	9

following the first culture. Some explants died under several of the growth regulator treatments and failed to produce protocorms.

In order to produce shoots, the leaves (or explants) were removed from the culture media that contained growth regulators. This was done in the twelfth week following the first culture of the explants. The leaves were subsequently cultured in half-strength MS media, without growth regulators, containing 8 g/l agar, 30 g/l sucrose and 2 g/l activated charcoal. The samples were maintained in that culture for 8 weeks. Thereafter they had grown into plantlets and were separated from each other after growing reasonably enough and after they had produced shoots and roots. Then, they were sub-cultured in similar MS media. Finally, after 190 days, they were ready to be tested for acclimation and thus were cultured in two different media (soil). They were moved into a greenhouse.

Evaluating the soil for the acclimation of plantlets

In order to determine acclimation based on the two soil media in the greenhouse, the plantlets were firstly decontaminated by carboxitiram (1%) and were then moved into the soil in groups of 50, in 10 plastic containers measuring 45 × 30 cm with a height of 10 cm. The first medium was comprised of cocopeat and coal (volume ratio 3:1). The second medium was cocopeat + charcoal + LECA (lightweight expanded clay aggregate) (volume ratio 2:1:2). The plantlets had 3-6 roots on average, which measured 5-12 cm in length. The daytime temperature in the greenhouse was 25 °C and was 20 °C for night time. During the first week of the acclimation process, the plantlets were irrigated daily, twice a day. Thereafter they were irrigated every 3 days with the Kristalon fertilizer (20-20-20) at a concentration of 2,000 mg l^{-1}. The percentage of plantlets survival was recorded after a month following the initiation of the acclimation process.

Statistical analysis

The experiment was conducted on a completely randomized design with 20 replications. Each replicate included one explant in each container. In the end, indices for the number and percentage of viable protocorms and also the indices for protocorms that died during the experiment were recorded in addition to the percentage of survival. Analyses of data were conducted by the SAS software and mean values were compared against each other through the Duncan's multiple-range test ($p \leq 0.05$).

Results and Discussion

Production of plantlets from leaf explants

The data of statistical variance shows that there were significant differences with regard to the number of successful protocorms and the number of unsuccessful ones, as a result of the different treatments. Protocorms began to emerge from the edges of the cultured leaves after six weeks of the first culture. Results showed that the presence of growth regulators in the media of tissue culture can positively affect the formation of protocorms.

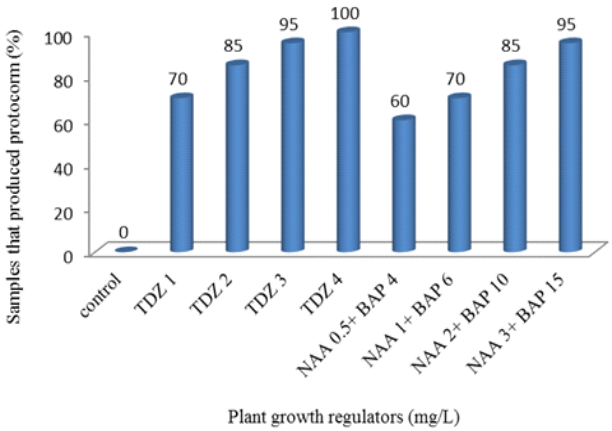

Fig. 1. The effect of growth regulators on the percentage of leaf samples that produced protocorms

Protocorms first emerged in samples that were treated with 4 mg l^{-1} TDZ. Protocorms eventually appeared on the samples of all treatments after 7 weeks. The control group, however, produced no protocorm. Explants of the control group turned yellow in color and died after twelve weeks (by the end of the experiment). All samples treated with 4 mg l^{-1} TDZ were observed to produce protocorms. On the other hand, only 60% of samples that were treated with 4 mg l^{-1} BAP and 0.5 mg l^{-1} NAA succeeded in producing protocorms (Fig. 1). Protocorms were mostly formed at the base of leaves, where they were attached to the branches. This result is in agreement with the findings of Penggow *et al*. (2010), Arditti (1977) and Pierik (1989). The researchers reported that the best size of leaf explants for tissue culture is 1 cm and that the basal segment of the leaf is used best.

According to the hereby results, the highest production of protocorms pertained to the treatment containing both cytokinins and NAA (Table 2) which confirms the results reported by Tokuhara and Mii (1993), Kim and Kim (2003), Puchooa (2004), David and Bala (2012), Niknejad *et al*. (2011), Chen and

Table 2. The effects of growth regulators (TDZ, BAP and NAA) on the production and survival of protocorms obtained from leaf explants of *P. amabilis* var. 'Manila'

Treatment	No. of protocorms	No. of degenerated protocorms	Viability (%)
Control	0.0 e	0.0 f	-
TDZ 1	14.25 d	1.9 e	60.66 b
TDZ 2	27.15 c	3.2 d	74.98 ab
TDZ 3	40.55 b	5.95 b	81.08 ab
TDZ 4	49.6 a	7.1 a	85.63 a
NAA 0.5 + BAP 4	2.6 e	0.0 f	60.00 b
NAA 1 + BAP 6	10.8 d	0.0 f	70.00 ab
NAA 2 + BAP 10	31.9 c	2.55 de	78.17 ab
NAA 3 + BAP 15	50.65 a	4.95 c	85.75

*Mean values with similar letters in each column are not significantly different at the $p \leq 0.05$ level

Chang (2006), Balilashaki *et al*. (2014), Feng and Chen (2014) and Rittirat *et al*. (2014).

The production of protocorms increased parallel to the increase in the concentration of growth regulators. The least number of protocorms was 2.6 per explant which was observed in samples treated with 4 mg l^{-1} BAP + 0.5 mg l^{-1} NAA. The highest number of protocorms was 50.65 per explant which was observed in samples treated with 15 mg l^{-1} BAP + 3 mg l^{-1} NAA. This was not significantly different compared to samples treated with 4 mg l^{-1} TDZ (Table 2). The highest number of protocorms per explant produced in the current study was higher than that of the study of Rittirat *et al*. (2014) who obtained a maximum number of 30 protocorms per explant and an average number of 5.3 by treating the samples with 9 mM TDZ.

Contrary to the results of Feng and Chen (2014) and Rittirat (2014), it can be concluded based on the current results that BA was more effective than TDZ. Results showed that even though increasing the concentration of growth regulators caused an increase in the number of protocorms and the percentage of active samples, nevertheless the numbers of protocorms that died as a result of chlorosis also increased. According to the obtained results, most instances of chlorosis and death of protocorms was observed in 4 mg l^{-1} TDZ. The lowest percentage of survival at the lowest concentration of growth regulators pertained to the concentration of 1 mg l^{-1} TDZ and the concentration of 0.5 mg l^{-1} NAA + 4 mg l^{-1} BAP. The best concentration of growth regulators for the production and survival of protocorms was determined to be 15 mg l^{-1} BAP + 3 mg l^{-1} NAA which was not significantly different compared to the treatment with 4 mg l^{-1} TDZ (Table 2).

Evaluating the acclimation of plantlets

The best acclimation success was 90% which pertained to plants acclimated in the medium of cocopeat + charcoal + LECA (volume ratio 2:1:2). In the medium of cocopeat + charcoal (volume ratio 3:1), 84.6% of the acclimated plants survived. This medium was characterized by a high water-maintaining capacity, which means that it could hold more water in its microscopic pores. This led to a higher incidence of root rot due to the excess presence of water. The medium that contained LECA and charcoal was able to supply the roots with more oxygen due to the nature of its microscopic pores. Therefore the plantlets therein had

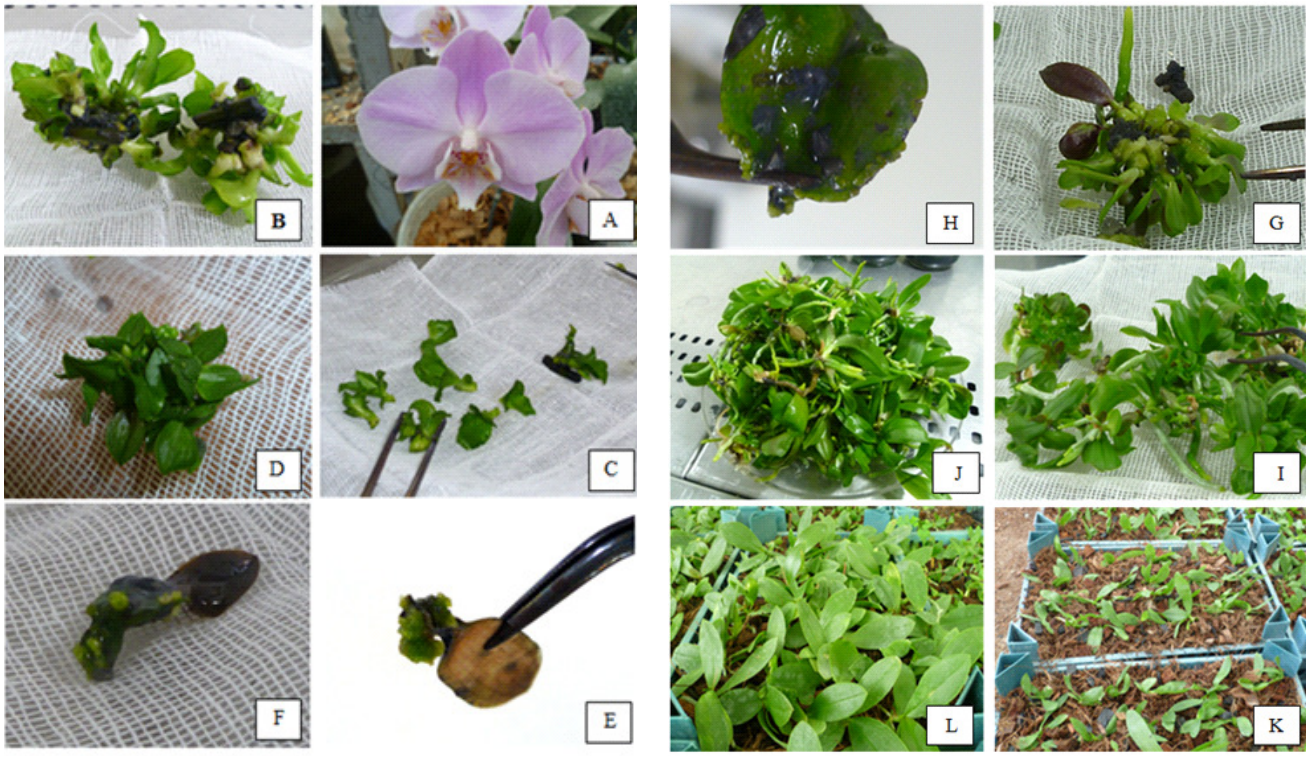

Fig. 2. The main stages of producing plantlets from leaves that had grown *in vitro* from the initial tissue culture of nodes of flower stalks. A: the *Phalaenopsis* orchid var. 'Manila'. B: shoots that grew by tissue culturing the nodes of flower stalks on MS medium containing 4.4 mg l^{-1} BA and 1 mg l^{-1} NAA. C: separating the leaves from the shoots resulted from tissue culturing the nodes of flower stalks. D: protocorms that grew on the edges of leaves in MS medium containing 4 mg l^{-1} TDZ. E: demonstrating the capability of the base of leaves in producing protocorms. F: protocorms that are produced in culture medium with 1 mg l^{-1} TDZ

Fig. 2 (continued): G: protocorms produced in medium with 15 mg l^{-1} BAP and 3 mg l^{-1} NAA. H: protocorms produced in medium with 6 mg l^{-1} BAP and 1 mg l^{-1} NAA. I: separating the explants from each other and moving them to half-strength MS medium containing 2 g^{-l} activated charcoal, with no growth regulators. J: removing the plantlets from jars of the tissue culture and preparation for acclimation. K: plantlets moved to medium of cocopeat + coal (volume ratio 3:1) for acclimation to greenhouse conditions. L: healthy plantlets after 2 months following the acclimation, positioned in cocopeat + coal (volume ratio 3:1)

more success in acclimation and exhibited more growth. The *Phalaenopsis* orchids are epiphytic plants and prefer moist, porous establishments (Arditti and Ernst, 1993). Fig. 2 shows several steps of the experiment.

Conclusions

With respect to the cross-pollinated nature of orchids, sexual propagation will result in heterozygote progeny with lower quality. Furthermore, the long duration of juvenility in sexually propagated plantlets is another issue of difficulty and is therefore accompanied by higher costs of production. The tissue culture of leaf explants is a suitable candidate for the commercial propagation of the *Phalaenopsis* orchid because it requires convenient methods of decontamination, the use of leaf explants in tissue culture is convenient, tissue culturing the orchid provides an acceptable level of efficiency in production and can yield clones of a given plantlet because of the direct production of protocorms from leaves. The fact that plantlets can skip their stage of juvenility is yet another benefit of tissue culturing the orchid. According to the results of the hereby experiment, the MS culture medium containing 15 mg l^{-1} BAP + 3 mg l^{-1} NAA was able to yield 50.65 protocorms per explant and was thereby considered as the best medium. It is cheaper and also more efficient compared to media containing TDZ. Therefore it serves as an optimum formula for the proliferation of *Phalaenopsis* orchid var. 'Manila'. Further research can test similar experiments on the micropropagation of other species of orchid.

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