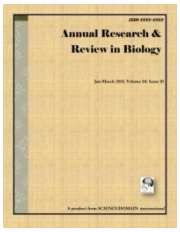

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Effect of Plant Growth Regulators on Seed Germination and Development of Protocorm and Seedling of *Phalaenopsis amabilis (L.)* Blume (*Orchidaceae*)

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Phalaenopsis amabilis (L.) Blume is economically ornamental species of epiphytic orchids, considered for its evergreen and clustered foliage and pendulous racemes containing several yellow-green flowers. Conventional propagation of *Phalaenopsis amabilis (L.)* Blume restricted by unsatisfactory rhizogenesis of shoot cutting or germination problems of lack of endosperm and low seed viability. In order to investigate the effects of some plant growth regulators on seed germination and development of protocorm and seedling of *Phalaenopsis amabilis (L.)* Blume disinfected capsules (21 wap) was longitudinally cutted by scalpel and its content were immediately transferred to test tube containing about 30ml MS medium supplemented by different concentration and combinations of NAA, 2,4-D and BAP. The highest value of germination percentage (83.75%) was occurred in NAA (1.5mgl^{-1}) or combination of BAP (0.5mgl^{-1}) + NAA (0.5mgl^{-1}) on after/during25thday. Plant growth regulators highly affected the initiation time

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of protocorm formation and shortest time (53.50 days) was recorded in MS medium supplemented by NAA (1mgl^{-1}) + BAP (1mgl^{-1}). The best time (157.50 days) for seedling development was observed in NAA (1mgl^{-1}) + BAP (1mgl^{-1}) and 2, 4-D (0.5mgl^{-1}) + NAA (1mgl^{-1}) combination treatments.

Keywords: Phalaenopsis amabilis (L.) Blume 2, 4-D; protocorm; NAA; seedling development; In vitro.

ABBREVIATIONS

BAP, 6-Benzylaminopurine; NAA, Alpha-naphthalene acetic acid; 2, 4-D, 2, 4-Dichlorophenoxyacetic acid; IAA, Indole-3-acetic acid; MS, Murashige and Skoog medium (1962).

1. INTRODUCTION

The *Orchidaceae* family has 850 genera, 35,000 species and considered as the largest family of plants and including 7-11 % of flowering plants [1,2]. Orchid flowers produce large amounts of dusty and conical- shaped seed. Each orchid capsule contains a millions of microscopic pollen- liked seeds [3,4], which Contain undifferentiated and endosperm free embryos enclosed by transparent seed coats [4,5]. A symbiotic association with a mycorrhizal fungus is required because the orchid seeds contain poor quantity of reserve material for the development of embryos[6] and lack of glyoxysomes for conversion of their lipidaceous reserve food material into more utilizable forms [7,8]. Alternatively, *In vitro* supplying of required nutrition and plant growth regulators can be substituted in orchid seed germination and seedling growth. Seed germination and seedling development were prominently affected by the choice of medium. NAA, coconut water (CW), and activated charcoal (AC) were added to media to improve orchid seed germination [9]. Plant growth regulators such as NAA have been used for enhancement of seed germination of *Nothodoritis zhejiangensis* Chinese Orchid [10]. Seed germination in orchids is correlated only with symbiosis to mycorrhizal fungus. This limit can be removed by supplying of sugars and other required mineral nutrients in vitro condition. A little species of orchids have responded to asymbiotic germination [11,12], but the requirements of commercially important and endangered orchid species under *In vitro* culture, is still unknown [4,11,13]. *In vitro* asymbiotic seed germination of five endangered European orchid species– *Cypripedium calceolus* L., *Dactylorhiza majalis* (Rchb.) Hunt et Summerh., *Epipactis Atrorubens* (Hoffm. ex Bernh.) Besser, *Epipactis palustris* (Will.) Cr. and *Orchis morio* L., was performed [14]. Asymbiotic germination of immature embryos of a medicinally important epiphytic orchid *Acampe papillosa* (Lindl.) was also reported (4). *Phalaenopsis amabilis (L.)* Blume as an economically ornamental species of epiphytic orchids, considered for its evergreen and clustered foliage and pendulous racemes containing several flowers. Conventional propagation of *Phalaenopsis amabilis (L.)* Blume is restricted by unsatisfactory rhizogenesis of shoot cutting or germination problems of lack of endosperm and low seed viability. So, its natural habitats faced to destruction and commercial production cannot be scaled up. In this study we report an efficient procedure for *In vitro* asymbiotic germination of immature embryos of *Phalaenopsis amabilis (L.)* Blume which is prerequisite for its mass propagation.

2. MATERIALS AND METHODS

2.1 Preparation of Plant Material

Green capsules of *Phalaenopsis amabilis (L.)* Blume were collected after 3 month of self-pollination from potted plants. For surface sterilization, the capsules were rinsed in distilled water containing 5 ml Teepol 1% for 15 minutes to remove the primary infection and were transferred to ethanol 70% (V/V%) for 30-60 seconds under laminar air flow cabinet and were moved over flame for cutting out remaining ethanol. Bavistin solution 0.03% (W/V %) and streptomycin 0.03% (W/V %) for 5 min were respectively used to cutout the fungi and bacterial contamination and then were washed with sterile distilled water 3 times. Next, the capsules were disinfected in 0.3% $HgCl_2$ solution + 1 to 2 drops of Tween 20 and finally were washed 3-4 times with sterile distilled water to remove the effect of any agents used in the disinfection process.

2.2 Culture Media and Procedure

Each disinfected capsule was longitudinally cutted by scalpel and its content (about 200 seeds) were immediately transferred to petri dishes containing about 30ml MS medium (1962) containing 3% w/v sucrose and gelled with 0.8% w/v agar which supplemented by different concentration and combination of plant growth regulators (PGRs) as mentioned in Table 2. Cultures were kept at 25ºC with illumination provided by cool white fluorescent tube at a fluency rate of 2700 Lux at medium level for $16hd^{-1}$. The percentage of seed germination and days to protocorm initiation and seedling development were recorded after 2 weeks. The seed germination percentage was calculated as the number of germinated seeds out of the total number of cultured seeds in Petri dishes.

2.3 Statistical Analysis

The data were statistically analyzed, using ANOVA by randomized complete design (RCD) with 8 replications (culture bottle) in SAS Statistical Software (Ver. 9.1). Duncan's multiple range mean comparisons were performed for significant treatment effects at a probability level of 1% ($P \leq 0.01$).

3. RESULTS AND DISCUSSION

The result of ANOVA (Table 1) showed significant differences in the effect of different concentrations of plant growth regulators on germination percentage (GP), protocorm initation and seedling development of *Phalaenopsis amabilis (L.)* Blume ($P \leq 0.01$). Based on Duncan's multiple range test different types and concentrations of plant growth regulators caused various responses in all measured parameters (Table 2).

3.1 Percentage of Seed Germination

Plant growth regulators are important factors for *In vitro* germination of orchid seeds. In our experiment, the symptoms of germination observed on 16–24 days after seeds were cultured and the highest value of germination percentage (83.75%) was occurred in MS media containing NAA ($1.5mgl^{-1}$) or combination of BAP ($0.5mgl^{-1}$) + NAA ($0.5mgl^{-1}$) on 25th day. These findings are in agreement with previous study reported that media supplemented by $1mgl^{-1}$NAA (alone), 10% CW, 1% AC caused the germination percentage (64.7%) of

Nothodoritis zhejiangensis [10]. 6-Benzylamine purine (BAP) in combination with α-Napthalene Acetic Acid (NAA) in MS medium enhanced seed germination in comparison to control in two orchid plant species *Epipactis royleana* Lindl. and *Dactylorhiza hatagirea*(D. Don) Soo [15]. Auxin IAA suggested as the best supportive PGRs with the highest germination rate (46.2%) on *In vitro* germination of *Orchis coriophora* (*Orchidaceae*), a naturally growing orchid species in Turkey [16]. Presence of BAP (especially at $1mgl^{-1}$alone or in combination) was also favorite to seed germination which confirmed with previous reports stated that the addition of exogenous cytokinins to the medium may raise germination and plantlet development [16]. Low concentrations of kinetin and zeatin were reported as more effective in seed germination of *Habenaria macroceratitis* Willd [17]. Found a similar preferential response for kinetin in asymbiotic seed germinating *Cypripedium macranthos* [18]. Orchimax medium containing BA and actived charcoal was the best germination medium for *Orchis mascula* seeds [19]. Among the treatment tested, control medium (without PGRs) was the least effective, with 40.50% germination (Table 2) after 45 days.

3.2 Days to Protocorm Initiation and Seedling Development

The investigated basal media supported *In vitro* germinating *Orchis coriophora* to varying, but none was enough to produce protocorms without the addition of PGRs [16]. Some mycorrhizal fungi produce cytokinins, and this phenomenon may aid protocorm formation in orchids in nature [20] and can be bypassed with addition of exogenous cytokinins to the asymbiotic seed germination. Based on Table 1, plant growth regulators highly affected the initiation time of protocorm formation and shortest time (53.50 days; Fig. 1) was recorded in MS medium supplemented by NAA ($1mgl^{-1}$) + BAP ($1mgl^{-1}$) and the 2,4-D ($0.5mgl^{-1}$) + NAA ($1mgl^{-1}$). Similar to germination percentage, IAA (in combination or alone) also needed for best responses of protocorm formation that consistent with among all PGRs studied, IAA provided the best germination and thus the best *In vitro* protocorm formation of *Orchis coriophora* (*Orchidaceae*). Time of starting vegetative apex on protocorm of *Cymbidium devonianum* was significantly rapid in MS medium (11.4 days) as compared to B5 and Mitra medium. 2, 4-D ($2mgl^{-1}$) caused callusing of protocorms and NAA ($2mgl^{-1}$) proved effective for protocorm multiplication [21]. In case *of Dactylorhiza hatagirea*, BAP ($2mgl^{-1}$) and NAA ($1.5mgl^{-1}$) was the optimum hormonal combination on which maximum quantitative increase in protocorm like bodies (PLBs) was achieved [15]. Other treatment such as BAP ($0.5mgl^{-1}$), 2, 4-D ($0.5mgl^{-1}$) and control caused to rise the required time (>70 days) for protocorm formation (Table 2). Reported that the seeds of *Hygrochilus parishii* (*Orchidaceae*) cultured in eight basal nutrient medium germinated and developed into protocorm within 100 days of inoculation [22].

The best time (157.50 days) for seedling development (Fig. 2) was observed in NAA ($1mgl^{-1}$) + BAP ($1mgl^{-1}$) and 2, 4-D ($0.5mgl^{-1}$) + NAA ($1mgl^{-1}$) treatments followed by NAA ($1.5mgl^{-1}$). The application of 2, 4-D ($0.5mgl^{-1}$) resulted in retardation of this condition to 190 days. Combination of BAP ($1mgl^{-1}$), 2, 4-D ($2mgl^{-1}$) and IAA ($1mgl^{-1}$) favored formation of shoot buds. Development of seedlings was also recorded after 172.50 days in control treatment (Table 2). Incorporation of auxin (IAA) and (BAP) had important effects on seedling development. Maximum number of shoots (4.4) per plantlet was recorded in MS medium containing $1mgl^{-1}$ BAP and addition of 1 $mgl^{-1}$of IAA singly in the medium showed significant response only on root number of *Cymbidium devonianum.* The raise in fresh weight (0.33g) and root number (3.9) per plantlet recorded in MS medium supplemented with $1mgl^{-1}$ BAP + $1mgl^{-1}$ IAA. But, Seedling length and shoot number were significantly influenced by different combination of BAP and NAA in the medium [21].

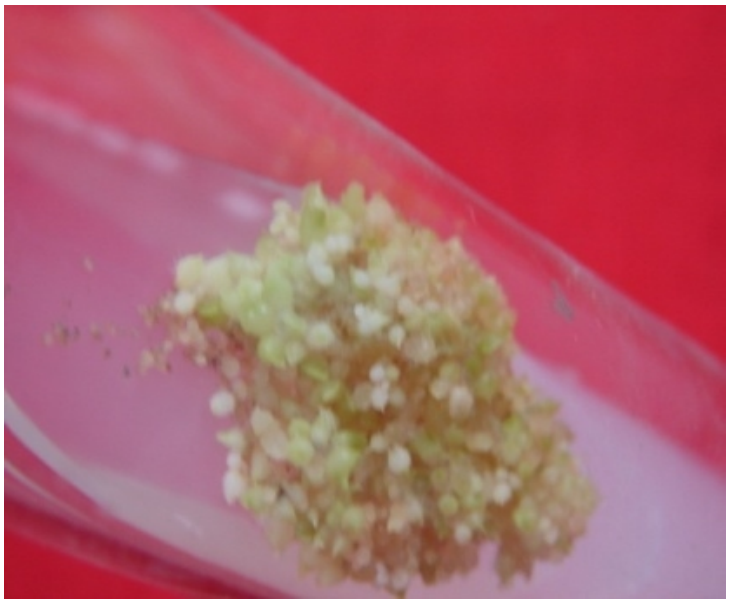

Fig. 1. Development of protocorm after 53.50 days in MS medium supplemented by NAA (1 mgl^{-1}) + BAP (1$mg\ l^{-1}$)

Fig. 2. Seedling development (10 ×) after 157.50 days in MS media containing NAA (1 mgl^{-1}) + BAP (1mgl^{-1})

Table 1. Mean squares (MS) of ANOVA on the basis of CRD for germination percentage and days to protocorm initiation and seedling development of *Phalaenopsis amabilis (L.)* blumein MS medium by SAS v 9.1

Sources changes	df	Germination percentage	Days to	
			Protocorm initiation	Seedling development
Treatment	24	445.97**	140.66**	288.35**
Error	75	40.69	3.91	4.47

***: significant at P≤0.01, ns: not statistically significant*

Table 2. Comparison of the effect of plant growth regulators on germination percentage and days to protocorm initiation and seedling development of *Phalaenopsis amabilis (L.)* blume by using duncan's multiple range test

PGR(S)	Germination percentage	Day to	
		Protocorm initiation	Seedling development
Control treatment	40.50^{I}	71.50^{a}	172.50^{fg}
NAA(0.5mgl^{-1})	25.67^{edf}	65.50^{bcd}	179.75^{cd}
NAA(1mgl^{-1})	71.50^{cde}	72.00^{a}	186.50^{b}
NAA (1.5mgl^{-1})	83.75^{a}	57.50^{g}	161.25^{h}
NAA (2mgl^{-1})	71.25^{cde}	72.50^{a}	185.00^{b}
2,4-D (0.5mgl^{-1})	53.75^{h}	73.75^{a}	190.00^{a}
2,4-D (1mgl^{-1})	62.50^{efgh}	65.00^{bcd}	180.00^{cd}
2,4-D (1.5mgl^{-1})	72.50^{bcde}	66.50^{b}	172.25^{fg}
2,4-D (2mgl^{-1})	66.25^{detg}	68.00^{b}	177.00^{de}
BAP (0.5mgl^{-1})	60.00^{gfh}	74.00^{a}	170.00^{cd}
BAP (1mgl^{-1})	82.50^{cde}	61.00^{ef}	171.25^{g}
BAP (1.5mgl^{-1})	71.50^{ab}	63.00^{cdef}	172.00^{fg}
BAP (2mgl^{-1})	80.00^{abc}	73.00^{a}	181.00^{c}
NAA+BAP(0.5+0.5mgl^{-1})	83.75^{a}	57.50^{g}	161.25^{h}
NAA+BAP(0.5+1mgl^{-1})	82.50^{ab}	61.00^{ef}	171.25^{g}
NAA+BAP(1+0.5mgl^{-1})	72.50^{bcde}	66.50^{b}	172.25^{fg}
NAA+BAP(1+1mgl^{-1})	78.75^{abc}	53.50^{h}	157.50^{I}
2,4-D+NAA(0.5+0.5mgl^{-1})	78.75^{abc}	68.00^{b}	175.00^{ef}
2,4-D+NAA(0.5+1mgl^{-1})	56.75^{gh}	53.50^{h}	157.50^{i}
2,4-D+NAA(1+0.5mgl^{-1})	78.75^{abc}	63.00^{cdef}	176.00^{e}
2,4-D+NAA(1+1mgl^{-1})	66.25^{defg}	65.00^{bcd}	178.00^{cde}
2,4-D+BAP(0.5+0.5mgl^{-1})	71.25^{abc}	71.25^{fg}	178.00^{cde}
2,4-D+BAP(1+0.5mgl^{-1})	63.00^{efgh}	63.25^{cde}	177.00^{de}
2,4-D+BAP(0.5+1mgl^{-1})	75.00^{abcd}	66.00^{bc}	184.00^{b}
2,4-D+BAP(1+1mgl^{-1})	62.50^{efgh}	62.50^{def}	172.00^{gf}

Values followed by a similar letter are not statistically significantly different (P≤0.01)

4. CONCLUSION

In summary, we have presented an effective technique for the seed germination and development of protocorm and seedling of *Phalaenopsis amabilis (L.)* Blume (*Orchidaceae*) through *In vitro* culture system. It is concluded that MS media containing NAA (alone or with combination) had a key role on seed germination, the initiation time of protocorm formation and time for seedling development. Similarly, IAA also needed for best responses of protocorm formation. Among tested PGRs, 2, 4-D caused callus initiation of protocorms and retardation of seedling development. This simple and efficient system is recommended to *In vitro* asymbiotic germination of immature embryos of *Phalaenopsis amabilis (L.)* Blume which is prerequisite for its mass propagation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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