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In Vitro Propagation of *Cephalanthera rubra* (L.) Rich., an Endangered Orchid, Using 2,4-D, NAA and BA

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Abstract

Orchids are universally popular due to the wide range of colors, sizes, shapes, and scents in their flowers. The demand for orchids as cut flowers and pot plants is increasing in the flower market. Seed germination of orchids is comparatively low in the wild as they typically require fungal symbionts during their germination. Micropropagation of orchids is a major approach to orchid conservation and commercial production. *Cephalanthera rubra* (L.) Rich., one of Iran's endangered orchid species, has been cloned using leaf as explant, Murashige and Skoog (MS) as culture medium, and α-naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) (both at the concentrations of 0, 0.1, 0.2, 0.3 and 0.4 mg L^{-1}) as well 6-benzyladenine (BA) (at 0, 1, 2, 3 and 4 mg L^{-1}) as plant growth regulators (PGRs). In order to initiate an axenic culture, the disinfection of leaf explants was performed with sodium hypochlorite and mercury chloride. The highest number of shoots (4.33) was obtained in medium enriched with 4 mg L^{-1} BA. Maximum stem length (4.73 cm), leaf number (5.33) and node number (2.86) was obtained in medium supplemented with 3 mg L^{-1} BA. The largest number of root (5) and the highest length of root (4.83 cm) was produced on medium augmented with 0.3 mg L^{-1} NAA. Rooted plantlets were transferred to pots filled with peat and perlite in 1:1 proportion and acclimatized to ambient greenhouse conditions with an average of 90% survival rate. This is the first report on the micropropagation of *C. rubra* (L.) Rich.

Keywords: Micropropagation, Orchidaceae, Plant growth regulators, Threatened ornamental plants, Tissue culture.

INTRODUCTION

Orchids are commercially important plants because of their exotic values, such as variety in colors, sizes, shapes, fragrances, and long vase life of their flowers. A number of orchids have medicinal importance (Park *et al*., 2018). These plants are cultivated as cut flowers and pot plants around the world (Park *et al*., 2018). Red Helleborine *Cephalanthera rubra* (Orchidaceae), one of the rarest orchids, is an broad-leaf herb orchid found in Europe, North Africa, southwest Asia and in Iran. Geographical distribution of this species is Europa-Siberia, Mediterranean and Iranian-Turani. The main place of distribution of *C. rubra* in Iran is the jungle of Dodangeh, Sari city, Mazandaran province. Phenology of this plant is geophyte containing rhizome. The shoots grow from a creeping rhizome and have between 2 and 8 lanceolate leaves. Each shoot may carry up to 20 flowers, which may be pink to red or rarely white. The importance of this species is that it is ornamental. Its other importance, including its medical and pharmaceutical is not fully known.

Orchids produce numerous minute seeds, which have a low propagation rate in nature, thus propagation of these group of flowering plants in nature is a slow process. Conventional propagation of orchids utilizes vegetative propagules. Asymbiotic seed germination is another method for large-scale propagation of orchids; however, the plants produced by this method are highly heterozygous (Murthy *et al*., 2018). Therefore, to meet the demand of the horticultural industry and preservation of orchids, especially those in danger of extinction, alternative techniques such as the *in vitro* techniques are needed. *In vitro* propagation is important and a suitable alternative technique for propagation of orchids. This technique can be used also for conservation of rare and endangered plant species (Engelmann, 2011). Plant tissue culture technology is one of the main sections of biotechnology, especially applied for the multiplication, conservation, and improvement of crops. A number of orchids have medicinal importance (Park *et al*., 2018).

Success in orchid micropropagation depends on some factors, such as the choice of explants, media used, handling techniques, equipment, and culture conditions, particularly plant growth regulators (PGRs) (Yeung *et al*., 2018). PGRs are the most important factors affected on success of plant tissue culture. Different PGRs such as α-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), TDZ, BA and kinetin (Kn) have been used for tissue culture of some threatened and endangered orchids (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). Various explants such as seed, leaf, node, root, flower bud, flower, inflorescence, meristem, protocorm, protocorm-like body (PLB), tuber, rhizome and shoot tip have been used for *in vitro* proliferation of endangered orchids (Yam and Arditti, 2018; Zakizadeh *et al*., 2019; Mohammadi *et al*., 2019). The present study, carried out for the first time, aimed to evaluate the effect of different concentrations of BA, NAA and 2,4-D, added individually into the Murashige and Skoog (MS) medium, on the *in vitro* propagation of *C. rubra* (L.) Rich.

MATERIALS AND METHODS

Plant material and initiation of *in vitro* culture

Plant material (*Cephalanthera rubra* (L.) Rich.) was collected from jungle of Dodangeh, Sari city, Mazandaran province. Plant material was transferred to a Plant Tissue Culture Lab. in Amol city, Mazandaran province, the northern part of 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). Leaf segments were used as primary explants. To eliminate surface contamination, the leaves were washed carefully with dishwashing liquid together with a drop of Tween-20 for 20 min. Next, the explants were placed under running tap water for half an hour. After preliminary washing, all the explants were treated with 15% (v/v) sodium hypochlorite (NaOCl) for 10 min. Then, the samples were disinfected with 0.1 mg L^{-1} (w/v) mercury chloride ($HgCl_2$) for 10 min followed by washed in distilled water for 15 min. Finally, ethanol 70% for one min. was used. All disinfection steps were performed under a laminar flow cabinet. The disinfected explants were washed three times in sterile distilled water, each time for 5 min. To remove the parts of the explants damaged in the disinfection treatments, the margins of the explants were cut off.

Organogenesis induction

Disinfected explants were placed inside sterile Petri dishes with 9 cm size (three explants per dish) filled with 50 mL of basal MS culture medium containing 3% (w/v) sucrose and 0.7% (w/v) agar (SIGMA Aldrich, USA). The media were augmented with 0, 0.1, 0.2, 0.3 and 0.4 mg L^{-1} of NAA (Merck, Germany) and 2,4-D (Merck, Germany), and 0, 1, 2, 3 and 4 mg L^{-1} BA (Merck, Germany) for the induction of direct or indirect organogenesis. The pH of the medium was adjusted to 5.6–5.8 with 0.1 N NaOH or HCl after adding all components and prior to autoclaving at 105 kPa and 121°C for 20 min. Cultures were kept in a growth room at 24 ± 2°C with a 16/8 h light/dark regime with the light intensity of 50–60 µmol m^{-2} s^{-1} provided by cool-white fluorescent tubes. Stem length, shoot number, node number, leaf number, root length, and root number were measured.

Acclimatization process

The rooted microshoots were removed from the Petri dishes and the remains of the culture medium were separated from the roots. Plantlets were placed in the perlite and peat substrate (in a ratio of 1:1) in 18-cm plastic pots (with capacity of 4 kg) and watered with sterile water. In each pot, 30 plantlets were planted. The pots were placed in a greenhouse with high humidity (70-80%), controlled photoperiod (16/8 light/dark with intensity of 4000-5000 lux), and temperature (24 ± 2°C) for acclimatization. All surviving plantlets in the culture media were transferred to pots for acclimatization.

Experimental design and data analysis

The *in vitro* experiment was conducted in a completely randomized block design with three replications. Each experimental unit consisted of 5 Petri dishes and 3 explants were cultured in each dish. Data analysis was done with SAS software and averages were compared using LSD test.

RESULTS

Results presented in tables 1-4 revealed that different concentrations of BA, NAA and 2,4-D had significant effect on changing the measured characteristics. The highest number of shoots (4.33/explant) was obtained in medium containing 4 mg L^{-1} BA (Table 4, Fig. 1). Totally, BA was better than NAA and 2,4-D for production of more shoots. The lowest number of shoots (1/explant) was produced in medium containing 0.1 and 0.4 mg L^{-1} 2,4-D (Table 2, Fig. 2). Maximum stem length (4.73 cm/explant), leaf number (5.33/explant) and node number (2.86/explant) was obtained in medium augmented with 3 mg L^{-1} BA (Table 4). Minimum stem

length (2.13 cm/explant) was observed in medium containing 0.4 mg L^{-1} 2,4-D (Table 2). This length was even less than that induced in control medium. Totally, BA was better than NAA and 2,4-D for production of more leaves. Minimum leaf number (1.33/explant) was produced in explants grown on medium containing 0.4 mg L^{-1} 2,4-D and control (Table 2). Least node (1/explant) was produced in control medium.

The largest number of root (5/explant) and the highest length of root (4.83 cm/explant) was produced on medium augmented with 0.3 mg L^{-1} NAA (Table 3, Fig. 3). NAA was more effective than BA and 2,4-D, in terms of root number and length. The number of roots produced in media enriched with 2,4-D was less than other treatments (Table 2). Root length in media containing BA was short. The lowest root length (0.93 cm/explant) was produced in control medium.

Table 1. Analysis of variance of the effect of different concentrations of 2,4-D on measured characteristics of *Cephalanthera rubra* (L.) Rich.

S.o.V	df	MS					
		Shoot number	Stem length	Leaf number	Node number	Root number	Root length
Replication	2	0.018^{ns}	0.032^{ns}	0.831^{*}	0.056^{ns}	0.0024^{ns}	0.144^{ns}
2,4-D	4	0.105^{*}	0.179^{**}	0.597^{*}	0.359^{*}	0.568^{*}	1.136^{**}
Error	8	0.023	0.0122	0.118	0.07933	0.167	0.072
CV (%)	-	12.65	4.27	19.7	17.3	25.5	14.65
Replication	2	0.215^{ns}	0.424^{ns}	0.082^{ns}	0.200^{ns}	0.61^{ns}	0.402^{ns}
NAA	4	0.735^{*}	3.215^{**}	0.239^{*}	0.782^{*}	7.29^{**}	4.782^{**}
Error	8	0.135	0.294	0.064	0.138	0.773	0.353
CV (%)	-	17.12	14.5	8.27	19.91	31.2713	21.39
Replication	2	0.253^{ns}	1.854^{*}	0.804^{ns}	0.209^{ns}	0.465^{ns}	0.312^{ns}
BA	4	2.70^{*}	2.403^{**}	5.27^{**}	1.19^{*}	2.68^{**}	1.086^{*}
Error	8	0.51667	0.289	0.538	0.237	0.314	0.254
CV (%)	-	25.07	14.55	23.81	22.98	25.64	24.25

*, ** and ^{ns}: Significant at $P < 0.05$, $P < 0.01$ and insignificant based on the LSD test, respectively. CV: coefficient of variation; df: degrees of freedom.

Table 2. Mean comparison of the effect of different concentrations of 2,4-D on measured characteristics of *Cephalanthera rubra* (L.) Rich.

2,4-D (mg L^{-1})	Shoot number	Stem length (cm)	Leaf number	Node number	Root number	Root length (cm)
Control	$1.33^{a} \pm 0.049$	$2.63^{a} \pm 0.321$	$1.33^{c} \pm 0.37$	$1.33^{b} \pm 0.000$	$1.66^{ab} \pm 0.533$	$0.933^{c} \pm 0.252$
0.1	$1.00^{b} \pm 0.000$	$2.60^{a} \pm 0.165$	$1.66^{bc} \pm 0.65$	$1.66^{ab} \pm 0.577$	$1.33^{b} \pm 0.058$	$1.66^{b} \pm 0.153$
0.2	$1.33^{a} \pm 0.289$	$2.70^{a} \pm 0.173$	$2.33^{a} \pm 0.57$	$1.66^{ab} \pm 0.115$	$2.33^{a} \pm 0.061$	$2.63^{a} \pm 0.503$
0.3	$1.33^{a} \pm 0.153$	$2.73^{a} \pm 0.061$	$2.00^{ab} \pm 0.000$	$2.00^{a} \pm 0.000$	$1.33^{b} \pm 0.000$	$2.00^{b} \pm 0.265$
0.4	$1.00^{b} \pm 0.000$	$2.13^{b} \pm 0.115$	$1.33^{c} \pm 0.463$	$1.33^{b} \pm 0.044$	$1.33^{b} \pm 0.366$	$1.93^{b} \pm 0.153$

*In each column, means with similar letter(s) are not significantly different ($P < 0.05$) using the LSD test.

Table 3. Mean comparison of the effect of different concentrations of NAA on measured characteristics of *Cephalanthera rubra* (L.) Rich.

NAA (mg L^{-1})	Shoot number	Stem length (cm)	Leaf number	Node number	Root number	Root length (cm)
Control	$1.67^{b} \pm 0.049$	$2.96^{b} \pm 0.321$	$1.66^{b} \pm 0.37$	$1.34^{b} \pm 0.000$	$1.66^{b} \pm 0.533$	$1.13^{c} \pm 0.252$
0.1	$2.00^{ab} \pm 0.000$	$3.36^{b} \pm 0.651$	$3.00^{ab} \pm 0.023$	$1.66^{b} \pm 0.058$	$2.00^{b} \pm 0.941$	$2.23^{bc} \pm 0.751$
0.2	$2.35^{ab} \pm 0.467$	$3.73^{ab} \pm 0.740$	$3.00^{ab} \pm 0.00$	$2.02^{ab} \pm 0.000$	$3.00^{b} \pm 0.873$	$2.56^{b} \pm 0.603$
0.3	$2.67^{a} \pm 0.289$	$4.66^{a} \pm 0.153$	$3.33^{a} \pm 0.048$	$2.66^{a} \pm 0.307$	$5.00^{a} \pm 1.000$	$4.83^{a} \pm 0.651$
0.4	$1.71^{b} \pm 0.613$	$3.90^{ab} \pm 0.436$	$3.33^{a} \pm 0.066$	$1.67^{b} \pm 0.418$	$2.33^{b} \pm 0.577$	$2.83^{b} \pm 0.351$

*In each column, means with similar letter(s) are not significantly different ($P < 0.05$) using the LSD test.

Table 4. Mean comparison of the effect of different concentrations of BA on measured characteristics of *Cephalanthera rubra* (L.) Rich.

BA (mg L^{-1})	Shoot number	Stem length (cm)	Leaf number	Node number	Root number	Root length (cm)
Control	$1.66^{b} \pm 0.049$	$2.46^{c} \pm 0.321$	$2.00^{b} \pm 0.37$	$1.00^{b} \pm 0.000$	$1.33^{b} \pm 0.533$	$1.33^{c} \pm 0.252$
1	$2.58^{b} \pm 0.463$	$4.40^{ab} \pm 0.700$	$2.66^{b} \pm 0.577$	$1.67^{b} \pm 0.548$	$1.66^{b} \pm 0.091$	$1.71^{bc} \pm 0.100$
2	$3.00^{ab} \pm 0.923$	$3.46^{bc} \pm 0.666$	$3.00^{b} \pm 1.000$	$1.66^{b} \pm 0.086$	$3.66^{a} \pm 0.540$	$2.83^{a} \pm 0.777$
3	$2.66^{b} \pm 0.533$	$4.73^{a} \pm 0.886$	$5.33^{a} \pm 0.893$	$2.86^{a} \pm 0.581$	$2.33^{b} \pm 0.429$	$2.50^{ab} \pm 0.529$
4	$4.33^{a} \pm 0.570$	$3.41^{bc} \pm 0.569$	$2.33^{b} \pm 0.466$	$1.33^{b} \pm 0.228$	$1.66^{b} \pm 0.310$	$2.03^{a-c} \pm 0.611$

*In each column, means with similar letter(s) are not significantly different ($P < 0.05$) using the LSD test.

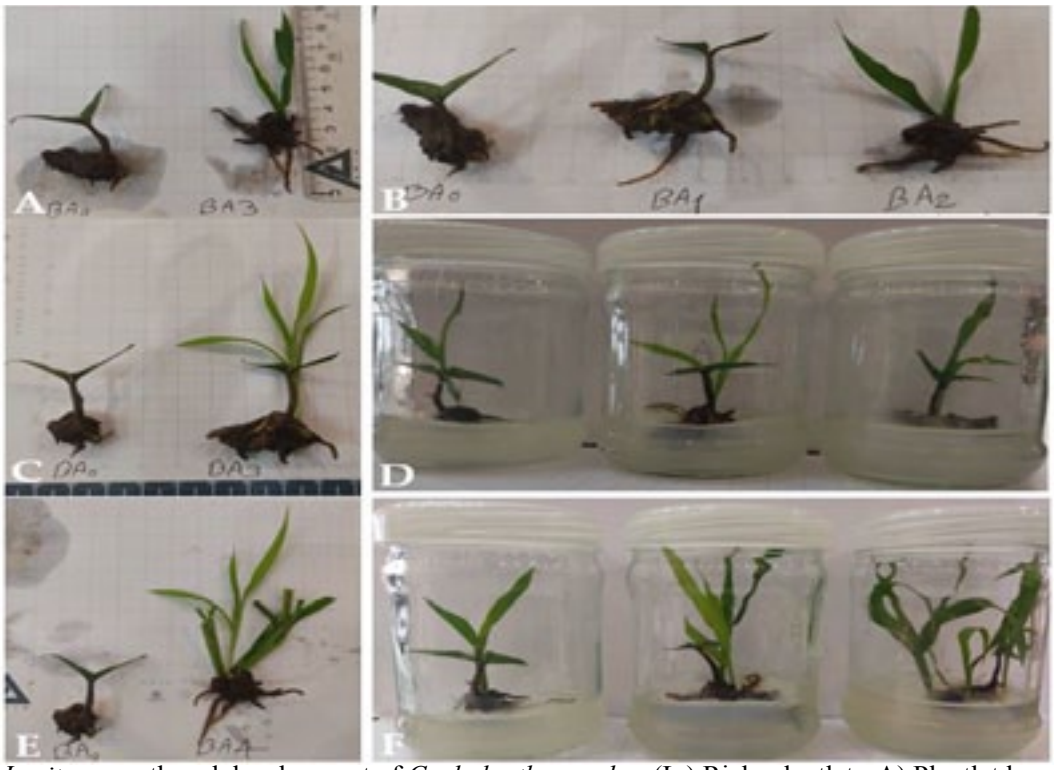

Fig. 1. *In vitro* growth and development of *Cephalanthera rubra* (L.) Rich. plantlets. A) Plantlet length after 4 weeks of culturing on basal MS medium containing 3 mg L^{-1} BA (on the right) and control (on the left); B) plantlet regeneration on the medium containing 2 mg L^{-1} BA (on the right), 1 mg L^{-1} BA (on the medial) and control (on the left); C) Like A, but after 6 weeks; D) growing plantlets on medium supplemented with 1 mg L^{-1} BA (on the right), 3 mg L^{-1} BA (on the medial), and 2 mg L^{-1} BA (on the left); E) shoot and root regeneration after 6 weeks of culturing on the medium containing 4 mg L^{-1} BA (on the right) and control (on the left); F) growing plantlets on medium supplemented with 4 mg L^{-1} BA (on the right), 2 mg L^{-1} BA (on the medial), and 3 mg L^{-1} BA (on the left).

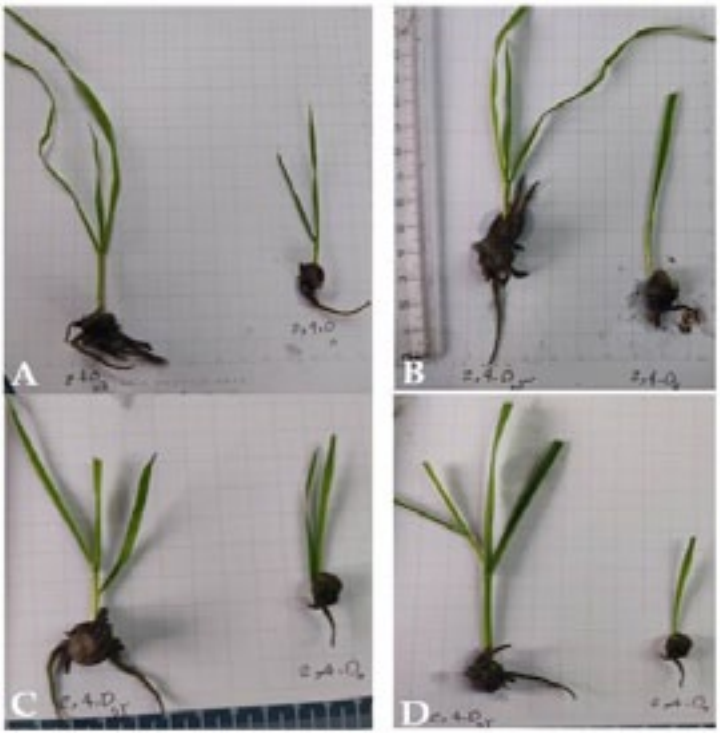

Fig. 2. *In vitro* growth and development of *Cephalanthera rubra* (L.) Rich. plantlets. A) Plantlet regeneration on the medium enriched with 0.3 mg L^{-1} 2,4-D (on the left) and control (on the right); B) plantlet length after 4 weeks of culturing on basal MS medium enriched with 0.3 mg L^{-1} 2,4-D (on the left) and control (on the right); C and D) shoot and root growth on medium enriched with 0.2 mg L^{-1} 2,4-D (on the left) and control (on the right).

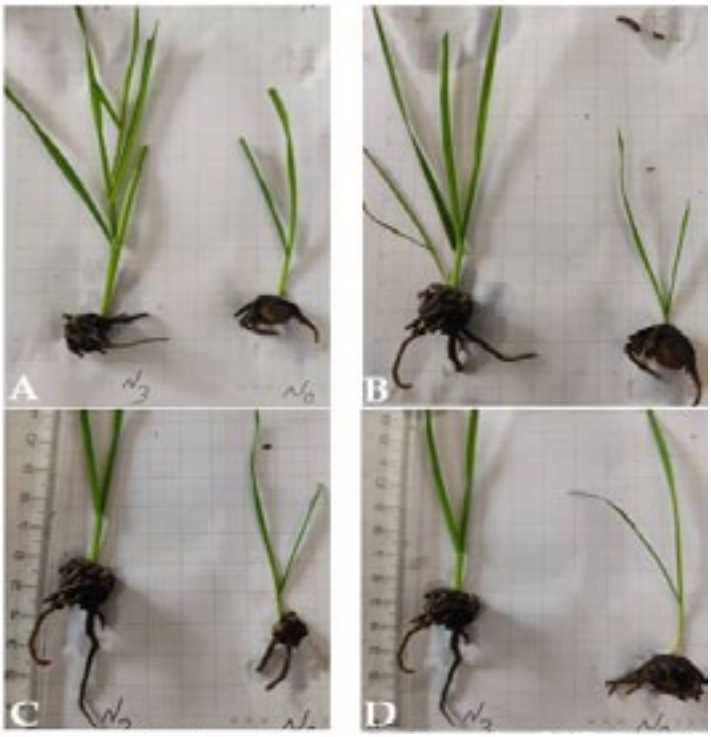

Fig. 3. *In vitro* growth and development of *Cephalanthera rubra* (L.) Rich. plantlets. A) Plantlet regeneration on the medium augmented with 0.3 mg L^{-1} NAA (on the left) and control (on the right); B) shoot and root growth on medium augmented with 0.3 mg L^{-1} 2,4-D (on the left) and control (on the right); C) plantlet length after 4 weeks of culturing on basal MS medium augmented with 0.2 mg L^{-1} NAA (on the left) and control (on the right); D) shoot and root growth on medium augmented with 0.3 mg L^{-1} 2,4-D (on the left) and control (on the right).

DISCUSSION

Some orchid species are threatened. In the present study, we tried to evaluate the influence of BA, NAA and 2,4-D, on *in vitro* propagation of *C. rubra* (L.) Rich., a threatened orchid species. The present study demonstrated that the addition of external PGRs in appropriate concentrations induced shoot and root formation from the leaves explants cultured on the MS medium. The ideal type, concentration and combination of PGRs and type of culture medium for the shoot and root production from different orchids species is known to be species-specific (Chen *et al*., 2015). The type, concentrations and different combinations of PGRs plays an important role during *in vitro* propagation of many orchid species (Baker *et al*., 2014; Panwar *et al*., 2012; Bhattacharyya *et al*., 2016; Kaviani *et al*., 2017; Zakizadeh *et al*., 2019).

Cytokinins are the most suitable PGRs to induce shoots. The ideal type, concentration and combination of cytokinins for the shoot production is species-specific (Chen *et al*., 2015). In our studies, BA was found to be suitable for increasing the number of shoot, leaf and node, as well the length of stem. Similar to our finding, BA individually was better than in combination with NAA for shoot production of orchid *Oncidium* (Kalimuthu *et al*., 2007). BA in varying concentrations is known to promote seedling leaf formation in some *Paphiopedilum* species (Huang *et al*., 2001; Chen *et al*., 2015). Production of multiple shoots is closely related with the type and concentration of cytokinins utilized (Amoo *et al*., 2014). In *Eulophia nuda* Lindl., maximum shoot multiplication and elongation were obtained on MS medium containing 2.00 mg L^{-1} BA and 1.00 mg L^{-1} Kn (Panwar *et al*., 2012). In *Dendrobium huoshanense*, Kn was reported to be more effective for plantlet regeneration from PLBs than BAP, TDZ, isopentenyl adenine (2-iP) and Zeatin (Zt) (Luo *et al*., 2009). Study on *Dendrobium nobile* revealed that when explants were cultured in medium enriched with BAP alone, formation of PLBs was done but direct shoot formation was not observed (Bhattacharyya *et al*., 2016). Study of Mahendran and Narmatha Bai (2009) demonstrated that among the cytokinins used for multiple shoot induction of *Satyrium nepalense,* TDZ was found to be superior. In most of the orchids the presence of cytokinins singly promoted optimal shoot proliferation (Mahendran and Narmatha Bai, 2009). In the present study, addition of TDZ and BA in suitable concentrations induced plantlets growth and leaf formation. The main advantage of direct organogenesis without an intervening callus phase is that somaclonal variation is reduced (Roy *et al*., 2011). The regulatory role of cytokinins on different physiological and developmental processes in plants is well known. TDZ was found to be the effective PGR affecting the development of *D. nobile* (Bhattacharyya *et al*., 2016). Panwar *et al*. (2012) showed that shoot multiplication was influenced by cytokinin type, concentration and combinations. The highest shoot multiplication and elongation in *Eulophia nuda* Lindl., were obtained in medium containing 8.88 µM BA and 4.64 µM Kn after 4 weeks of cultures (Panwar *et al*., 2012). Callus was produced at the base of shoots of *Eulophia nuda* cultured on medium containing higher concentration of BA, while lesser number of shoots were produced on medium with lower BA concentration (Panwar *et al*., 2012).

Orchids require auxins and/or cytokinins for formation of PLBs and plantlet development (Roy *et al*., 2011). In orchids, PLB regeneration from explants such as shoot, root, leaf and stem segments is a suitable technique for micropropagation (Seeni and Latha, 2000; Dohling *et al*., 2012). Production of PLB are closely related with the type and concentration of cytokinins used. Among all cytokinins used for *in vitro* PLBs production of orchids, BA, BAP, TDZ and Kn have the most application (Kaviani *et al*., 2017; Zakizadeh *et al*., 2019).

Current investigation revealed that NAA was more suitable in terms of root number and root length than 2,4-D. In *Vanda coerulea* Griff ex. Lindl. (Blue Vanda), NAA (2.68 µM) 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).

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). 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.

In conclusion, based on our findings that present for the first time, the largest number of shoots and roots was obtained in media augmented with 4 mg L^{-1} BA and 0.3 mg L^{-1} NAA, respectively. *Cephalanthera rubra* (L.) Rich. is one of Iran's endangered orchid species. *In vitro* propagation of plant species, particularly those in danger of extinction is a way to propagate and conserve of these valuable species.

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