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In vitro germination, protocorm formation, and plantlet development of *Orchis coriophora* (Orchidaceae), a naturally growing orchid species in Turkey

Ersan BEKTAŞ*, Mustafa CÜCE, Atalay SÖKMEN
Department of Biology, Faculty of Sciences, Karadeniz Technical University, 61080 Trabzon, Turkey

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Abstract: Some species belonging to the genus *Orchis* Tourn. ex L. (Orchidaceae) are of great economic importance as their tubers or corms are used to produce a hot beverage called salep. Nevertheless, these plants are not cultivated but are rather collected from nature, and due to careless collection many have already been listed as endangered plants. In order to assess the possibility of in vitro propagation, an orchid, *Orchis coriophora* L., was selected as a model plant, and the effects of basal media and plant growth regulators on in vitro seed germination, protocorm development, and plantlet formation were studied. Mature seeds were cultured in 4 different basal media, each supplemented with various concentrations and/or combinations of auxins and cytokinins/cytokinin-like substances. The highest germination rate (44.2%) was observed in Orchimax medium including activated charcoal plus 1 mg/L indole-3-acetic acid. Protocorms developed plantlets in all the tested media. Orchimax medium including activated charcoal and supplemented with 0.25 mg/L 6-benzyladenine was found to be the most suitable medium for the formation of plantlets from protocorms.

Key words: *Orchis coriophora*, protocorm, in vitro germination, Orchimax, mature seed

1. Introduction

The family Orchidaceae is one of the largest flowering plant families, comprising more than 880 genera and around 26,000 species, many of which grow wild throughout the world. Almost 800 species are identified and added to the family list every year. Research on these species continues, and the number of species is estimated to reach around 30,000 (Nicoletti, 2003). The members of this genus are collectively referred to as orchids, and the tropical and subtropical ones are cultivated for their flowers. The underground tubers of terrestrial orchids [mainly *Orchis mascula* (L.) L. (early purple orchid)] are ground into a powder and used for cooking, as in the hot beverage salep or in the Turkish Maraş ice cream. Around 20 million corms are collected from salep species growing naturally in the Turkish flora (Özhatay, 2000).

The number of orchid species is rapidly and steadily declining because of their low rate of propagation in nature and the ongoing collection from nature. Careless collection of these species has led to serious genetic and ecological erosion; many have already been listed as endangered species (Özhatay, 2000; Clemenets, 2003; Machaka-Houri et al., 2012). Therefore, in vitro propagation could be useful for the mass propagation of orchids for commercial purposes. Tissue culture techniques have been widely used for the in vitro mass propagation of several commercially important orchids over the past few decades (Chen & Chang, 2000, 2004). Since Knudson (1922) developed an asymbiotic germination technique, a growing number of orchid species have been produced in vitro. However, in vitro germination of terrestrial orchids is limited to a few genera, e.g., *Cypripedium* L. (Leroux et al., 1995), *Spiranthes* Rich. (Zelmer & Currah, 1997), *Platanthera* (L.) Rich. (Zettler & McInnis, 1994), and *Ophrys* L. (Kitsaki et al., 2004). Various methods including asymbiotic/symbiotic germination, use of mature/immature seeds, light/dark treatments, sonication, sterilisation, and scarification treatments have been tested to overcome the difficulties in germinating orchid seeds (Arditti & Ghani, 2000).

Orchis coriophora L. has been evaluated as a salep source for the Turkish flora, and although it is not listed in the endangered plant category, we have chosen this species as a model plant for in vitro propagation studies. The effects of various basal media preparations, each supplemented with different plant growth regulators (auxins and cytokinins), on seed germination, development of protocorms, and subsequent plantlet formation in *Orchis coriophora* were studied extensively. The results are presented comprehensively in this article.

* Correspondence: ebektas61@yahoo.com

2. Materials and methods

2.1. Seed source and sterilisation

Mature seeds of *Orchis coriophora* were donated by the Ministry of Agriculture's Agricultural Research Centre in Menemen, İzmir, Turkey. After collection, capsules were dried over silica gel desiccant for 2 weeks at 25 ± 2 °C, followed by storage in darkness at 4 °C until use. Before surface sterilisation, seeds were treated with 5% sucrose solution containing several drops of commercial bleach for 12 h. The solution was removed using a Pasteur pipette, and seeds were surface-sterilised with H_2O_2 for 30 min. Viability of the seeds was tested by staining in 2,3,5-triphenyl-tetrazolium chloride solution (1%, pH 7) for 2 days at 32 ± 1 °C (Lauzer et al., 1994). Orange or reddish embryos were considered viable.

2.2. Germination and protocorm formation of seeds

The 4 basal media listed in Table 1 were tested for their effectiveness in promoting the germination and subsequent

Table 1. Components of culture media used in the present study.

		OM	KCM	LM	POMM
Macroelements (mg/L)	$CaCl_2$	166.00	-	-	166.0
	KH_2PO_4	85.00	250.00	135.00	85.00
	KNO_3	950.00	-	-	950.0
	$MgSO_4$	90.35	122.15	58.98	90.35
	NH_4NO_3	825.00	500.00	-	825.00
	$Ca(NO_3)_2$	-	241.30	347.20	-
	KCl	-	250.00	1050.00	-
	$(NH_4)_2SO_4$	-	500.00	1000.00	-
Microelements (mg/L)	$FeSO_4.7H_2O$	-	25.00	-	27.85
	$MnSO_4.H_2O$	8.45	5.68	-	8.45
	$AlCl_3.6H_2O$	-	-	0.56	-
	$CuSO_4.5H_2O$	0.0125	-	0.02	-
	Fe citrate	-	-	4.40	-
	H_3BO_3	3.10	-	1.01	3.10
	KI	0.415	-	0.10	0.415
	$MnSO_4.H_2O$	8.45	-	0.05	-
	$NiCl_2.6H_2O$	-	-	0.03	-
	$ZnSO_4.7H_2O$	5.30	-	0.57	5.30
	$CoCl_2.6H_2O$	0.0125	-	-	0.0125
	FeNaEDTA	36.70	-	-	37.24
	$Na_2Mo_4.2H_2O$	0.125	-	-	0.125
Vitamins (mg/L)	Myo-inositol	100.00	-	-	-
	Picotinic acid	1.00	-	-	0.50
	Pyridoxin HCL	1.00	-	-	0.50
	Thiamine HCL	10.00	-	-	1.00
Organics (g/L)	Sucrose	20.0	-	-	20.0
	Tryptone	2.0	-	-	-
	Peptone	-	-	-	2.0
	Activated charcoal	2.0	-	-	-
	MES (mg/L)	1000.0	-	-	1000.0

Abbreviations: OM = Orchimax medium, KCM = Knudson C orchid medium, LM = Lindeman orchid medium, POMM = Phytamax orchid multiplication medium.

protocorm formation of *Orchis coriophora* seeds. Orchimax (OM), Knudson C (KCM), and Lindeman (LM) basal media were purchased from Duchefa (the Netherlands), while Phytamax orchid multiplication medium (POMM) was purchased from Sigma Chemical Company (USA). All basal media tested for seed germination were supplemented with 2% sucrose without plant growth regulators (PGRs). In order to achieve a high germination rate as well as protocorm formation, Orchimax medium was then supplemented with various PGRs [auxins: indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA); cytokinins: 6-benzyladenine (6-BA), zeatin (ZEA), 6-(γ,γ-dimethylallylamino) purine (2-iP), kinetin (KIN); and the cytokinin-like substance thidiazuron (TDZ)] at fixed concentrations (1 mg/L) individually. In all cases, Orchimax medium was modified by adding 2 g/L activated charcoal. All media were solidified with 0.8% Phyto Agar (Duchefa) and adjusted to pH 5.8 before autoclaving.

Sterile media were dispensed into 9-cm petri plates. Surface-sterilised seeds were evenly spread on the medium, and 10 replicate plates were inoculated with each tested medium. Petri plates were packed with a semitransparent Parafilm and cultured under a white fluorescent lamp (50 μmol m^{-2} s^{-1}) with 16/8 light/dark photoperiod conditions at 25 ± 2 °C for 8 weeks. At the end of 8 weeks, seed germination (protocorm formation) was observed under a dissecting light microscope. Germination percentages were determined by dividing the number of germinated seeds by the total number of viable seeds in the same media. The process of seed germination was divided into the following 5 categories according to the developmental stages of embryos (Yamazaki & Miyoshi, 2006).

Stage 0: 'No germination' stage. No growth of embryo occurs.

Stage 1: 'Pregermination' stage. Embryo swells to fill the seed coat.

Stage 2: 'Germination' stage. Embryo emerges from the seed coat.

Stage 3: 'Protocorm' stage. Embryo is completely discharged from the seed coat.

Stage 4: 'Rhizoid' stage. Rhizoids are formed on the protocorm surface.

Stage 5: 'Shoot' stage. Shoot is differentiated from the protocorm.

2.3. In vitro plantlet development

After 8 weeks, the protocorms were transferred to fresh OM, KCM, and LM media, each supplemented with 2 g/L activated charcoal and various concentrations (0.25, 0.5, 1.0, and 2.0 mg/L) of cytokinins (6-BA, KIN, and 2-iP). Protocorms were planted in Magenta Caps (Sigma) containing 40 mL of solidified media. For each medium 5 Magenta Caps containing 4 protocorms were incubated in the growth chamber under a white fluorescent lamp (50 μmol m^{-2} s^{-1}) at 25 ± 2 °C with a 16/8 light/dark photoperiod for the formation of plantlets. After 8 weeks, the effects of the media and plant growth regulators (cytokinins) on the formation of leaves and roots were recorded (Table 2).

Table 2. Effect of cytokinins on root and shoot formation in Orchimax (OM)[1].

	Concentrations (mg/L)	Root number	Root fresh weight (mg)	Root dry weight	Shoot length (mm)	Shoot fresh weight	Shoot dry weight
6-BA	**0.25**	3.9 ± 0.7	43.1 ± 1.5	5.8 ± 0.9	35.89 ± 1.2	32.2 ± 1.7	4.5 ± 0.5
	0.5	3.1 ± 0.6	33.1 ± 1.8	4.5 ± 0.9	29.65 ± 1.1	26.4 ± 1.2	3.4 ± 0.3
	1	2.9 ± 0.5	30.6 ± 1.3	4.1 ± 0.8	30.13 ± 1.0	27.4 ± 0.9	3.4 ± 0.1
	2	1.8 ± 0.2	21.3 ± 1.2	2.8 ± 0.7	28.59 ± 1.0	25.0 ± 1.8	3.2 ± 0.1
2-iP	**0.25**	2.1 ± 0.5	18.6 ± 1.5	2.4 ± 0.8	23.16 ± 1.0	25.8 ± 1.4	3.2 ± 0.1
	0.5	1.9 ± 0.4	17.2 ± 1.8	2.3 ± 0.5	20.82 ± 0.9	21.1 ± 1.9	2.2 ± 0.3
	1	2.2 ± 0.9	17.7 ± 1.2	2.3 ± 0.5	24.73 ± 1.2	24.1 ± 1.3	2.6 ± 0.2
	2	2.4 ± 0.3	18.9 ± 1.5	2.4 ± 0.6	24.97 ± 1.5	24.6 ± 1.2	2.7 ± 0.1
KIN	**0.25**	1.5 ± 0.7	7.8 ± 1.7	1.0 ± 0.6	14.63 ± 1.3	19.5 ± 1.2	2.2 ± 0.6
	0.5	1.5 ± 0.6	8.1 ± 1.5	1.1 ± 0.5	17.65 ± 1.4	20.7 ± 1.0	2.1 ± 0.3
	1	1.6 ± 0.4	8.3 ± 1.4	1.1 ± 0.5	12.32 ± 1.6	14.2 ± 0.6	1.7 ± 0.1
	2	1.6 ± 0.4	9.0 ± 1.5	1.2 ± 0.4	10.32 ± 1.0	14.1 ± 1.4	1.8 ± 0.4

[1]Each value represents the average of 40 samples, 10 of which were cultured in different Magenta Cups.

2.4. Statistical analysis

Ten petri plates, each containing approximately 100 mature seeds, were used in the seed germination experiments. Each petri plate was considered a replicate. The effects of cytokinins on root and shoot formation were recorded. Each value represents the average of 40 samples. All experiments were carried out in 10 replicates. Seed germination data were analysed using Duncan's test (ANOVA) at $\alpha = 0.05$ (the level of risk), and plantlet formation was calculated using SPSS 11.0.

3. Results and discussion

3.1. Germination and protocorm formation

Beforehand, the mature seeds of *Orchis coriophora* were subjected to the tetrazolium test following methods given elsewhere (Vellupillai et al., 1997); the viability rate of the seeds was 54 ± 2.1%.

Four basal media (OM, POMM, KCM, and LM), each containing 2% sucrose without PGRs, were chosen to assess their effect on seed germination.

Among the basal media tested, Orchimax (OM) was the most effective, with 27.4 ± 2.1% germination, followed by POMM (21.6 ± 2.0%), KCM (13.8 ± 0.6%), and LM (12.4 ± 0.4%). All tested media contain mineral salts that vary not only in their concentrations but also in their available forms. Nitrogen sources vary in all tested media. While KCM and LM contain only inorganic forms of nitrogen (ammonium and nitrate), OM and POMM contain a mixture of both inorganic and organic sources (Table 1). The organic nitrogen source in OM medium (tryptone) may promote seed germination. Inorganic forms of nitrogen may inhibit germination due to low nitrate reductase activity during germination and protocorm development (Raghavan & Torrey, 1964; Van Waes & Debergh, 1986; Malmgren, 1992). According to previous reports, organic nitrogen sources (i.e. amino acids) as opposed to inorganic forms may have a positive effect on seed germination in orchid (Anderson, 1996; Stewart & Kane, 2006). In a previous study, seed germination of *Orchis coriophora* was tested in KCM and Van Waes & Debergh medium with various plant extracts, separately. The highest germination rate (9.24%) was obtained in Van Waes & Debergh medium supported with tomato extract and activated charcoal (Çağlayan et al., 1998).

In this experiment, seeds were cultured using a 16/8 light/dark photoperiod. The negative effects of light application on seed germination have been reported by several researchers (Van Waes & Debergh, 1986; Yamazaki & Kazumitsu, 2006); however, responses may vary (Arditti et al., 1981). In another study, seed germination in terrestrial orchid [*Bletia purpurea* (Lam.) D.C.] was tested under different photoperiod conditions, and 16/8 light/dark was found to be suitable for germination and seedling development (Dutra et al., 2008). According to Valletta et al. (2008), seed germination was not observed under continuous darkness, and the best photoperiod regime for germination was 16/8 light/dark, which is similar to our findings.

Activated charcoal is commonly used in plant tissue culture media. The effects of activated charcoal on plant tissue culture have been reported (Pan & Van Staden, 1998). The addition of activated charcoal to the medium of European orchids resulted in lower germination rates and slower development (Van Waes, 1987). However, Valletta et al. (2008) reported that Orchimax medium supplemented with 6-benzyladenine and active charcoal proved to be best at promoting seed germination in *Orchis mascula*. In the present study, only OM was modified by adding 2 g/L activated charcoal, and the best germination rate was observed using this medium (Figure 1).

Although the investigated basal media supported the in vitro germination of *Orchis coriophora* to varying degrees, none was sufficient to produce protocorms without the addition of PGRs. According to a previous report, some mycorrhizal fungi produce cytokinins, and this phenomenon may aid protocorm formation in orchids in nature (Crafts & Miller, 1974). Based on this report, it appears that the addition of exogenous cytokinins to the medium may increase germination and plantlet development. Accordingly, the most suitable medium for germination (OM with active charcoal in this case) was supplemented with various PGRs, each prepared individually and at a fixed concentration (1 mg/L). Figure 2 shows that the best supportive auxin was IAA with the highest germination rate (46.2%); the other auxins tested showed no positive effect when compared to the control. The effects of cytokinins are also shown in Figure 3. When used alone, germination percentages observed among all tested cytokinins were similar, and no significant difference was found within groups or with the control. Moreover, among all PGRs studied, IAA provided the best germination and thus the best protocorm formation in vitro.

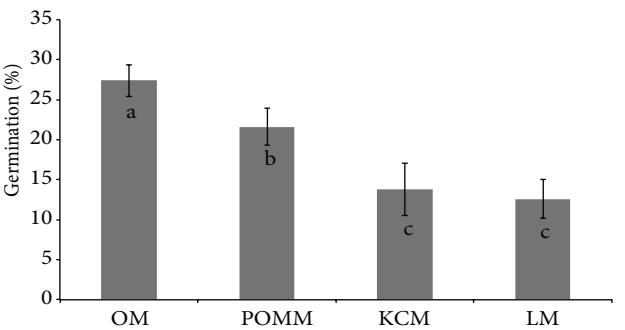

Figure 1. Germination percentages of *Orchis coriophora* seeds in the 4 different basal media.

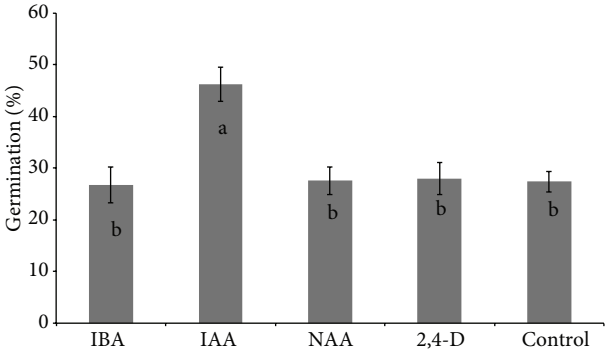

Figure 2. Effects of various auxins on germination of the seeds of *Orchis coriophora*. OM, including activated charcoal and without plant growth regulators, was used as control. Histobars with the same letters are not significantly different (α = 0.05). Auxins were added to the media at a fixed concentration (1 mg/L). IAA has an excellent effect on germination, and the others showed no significant differences compared to the control.

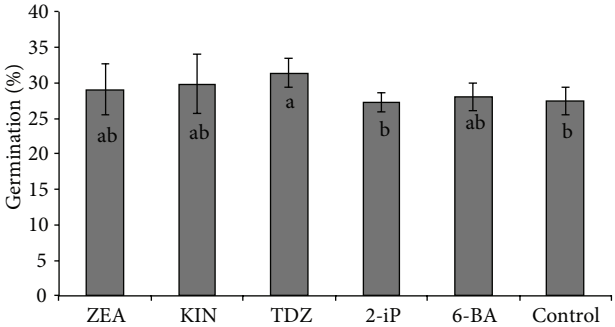

Figure 3. Effects of various cytokinins on germination of the seeds of *Orchis coriophora*. OM, including activated charcoal and without plant growth regulators, was used as control. Histobars with the same letters are not significantly different (α = 0.05). Cytokinins were added to the media at a fixed concentration (1 mg/L). Germination percentages observed from all tested cytokinins were very close to the control.

Stewart and Kane (2006) reported that low concentrations of kinetin and zeatin were more effective in seed germination of *Habenaria macroceratitis* Willd. Miyoshi and Mii (1998) found a similar preferential response for kinetin in the asymbiotic seed germination of *Cypripedium macranthos* Sw. In another study, Orchimax supplemented with BA and active charcoal was the best germination medium for *Orchis mascula* seeds. On the other hand, our results showed that cytokinins had no positive effect on germination (Valetta et al., 2008).

3.2. In vitro plantlet development

After protocorm formation, OM, KCM, and LM were individually evaluated for plantlet formation. Each medium was supplemented with a cytokinin: 6-BA, KIN, and 2-iP at concentrations ranging from 0.25 to 2.0 mg/L. All cultures were incubated at 25 ± 2 °C under a 16/8 light/dark photoperiod in the growth chamber. At the end of 8 weeks, the percentage of shoot and root formation produced by each medium was determined (Table 2). Accordingly, OM including activated charcoal and 0.25 mg/L 6-BA was found to be the best medium for plantlet formation (both root and shoot formation). Shoot lengths and numbers and lengths of roots in media supplemented with 0.25 mg/L 6-BA were longer than those grown in other media. The enhancing effects of cytokinins on morphogenesis were previously reported (Goh & Wong, 1990). TDZ was the most effective plant growth regulator for in vitro shoot formation studies (Nayak et al., 1997a, 1997b; Erişen et al., 2011). However, they also found retarding effects of TDZ on shoot growth and root formation [*Acampe praemorsa* (Roxb.) Blatter and McCann., *Cymbidium aloifolium* (L.) Sw., *Dendrobium aphyllum* (Roxb.) Fisch., and *Dendrobium moschatum* (Buch.-Ham.)]. In order to overcome this problem, a combination of TDZ and an auxin (NAA) at a very low concentration was employed. In the present study, problems in achieving sufficient shoot length and root formation were resolved by using either 6-BA or 2-iP individually instead of using the combinations mentioned above.

The morphological development of *Orchis coriophora* from seed to plantlet is shown in Figure 4. Viable embryos swelled and filled the seed coat in week 2 (Stage 1, Figure 4), and embryos emerged from the seed coat entirely. After 4 weeks, a protocorm was at an early stage of development (Stage 3, Figure 4) showing the basal part covered with simple rhizoids. In week 6, most of the protocorm surface was covered with rhizoids (Stage 4, Figure 4). After 8 weeks of sowing, the vegetative shoot apex was formed (Stage 5, Figure 4).

Orchid seeds are minute and lack an endosperm layer, and embryos of orchid seeds consist of 80–100 undifferentiated cells (Arditti, 1967). Therefore, they must be in a symbiotic relationship with fungi for germination (Ingold and Hudson, 1993). However, they can multiply without a mycorrhiza via in vitro propagation (Rublup et al., 1989).

4. Conclusion

The in vitro propagation of orchids has always been envisaged as problematic since their nutrient requirements have not been fully understood. On the other hand, such plants are economically important since they have long been used as a basic ingredient in the hot beverage salep and in ice creams. As they are collected from nature, not cultivated, their extinction is inevitable; careless collection will cause genetic and ecological erosion. The results presented here show that in vitro seed germination and plantlet formation of *Orchis coriophora* can be achieved at a higher rate by choosing the appropriate basal

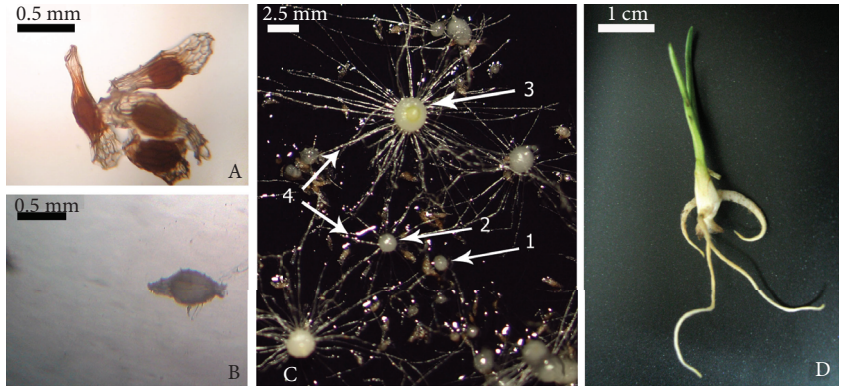

Figure 4. Developmental stages of *Orchis coriophora* from seed germination to protocorm and plantlet formation in asymbiotic culture in vitro. A) Stage 0: viable seeds, 'no germination' stage, no growth of embryo occurs. B) Stage 1: 'pregermination' stage, embryo swells the width of seed coat. C1) Stage 2: 'germination' stage, embryo emerges from the seed coat. C2) Stage 3: 'protocorm' stage, embryo is discharged from the seed coat and rhizoids (arrow 4) are formed in the basal parts of embryo. C3) Stage 4: 'rhizoid' stage, rhizoids (arrow 4) are formed on most of the surface of the protocorm; and Stage 5: 'shoot' stage, shoot is differentiated in protocorm. D) Plantlet formation after 8 weeks of protocorm formation.

medium supplemented with an appropriate PGR (OM supplemented with IAA, in this case). These data may hold the key to the mass propagation of other orchid species via organogenesis or may provide a starting point for the production of synthetic seeds.

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