Tarım Bilimleri Dergisi
Tar. Bil. Der.
Dergi web sayfası:
www.agri.ankara.edu.tr/dergi

Journal of Agricultural Sciences
Journal homepage:
www.agri.ankara.edu.tr/journal

In vitro Symbiotic Culture Studies of Some Orchid Species

Arzu ÇIĞ[a], Hüdai YILMAZ[b]

[a]*Siirt University, Agriculture Faculty, Department of Horticulture, Kezer Campus, Siirt, TURKEY*

[b]*Pamukkale University, School of Applied Sciences, Denizli, TURKEY*

ARTICLE INFO
Research Article DOI: 10.15832/ankutbd.385859
Corresponding Author: Arzu ÇIĴ , E-mail: arzucig@yahoo.com, Tel: +90 (531) 623 15 33
Received: 27 August 2014, Received in Revised Form: 19 February 2016, Accepted: 16 September 2017

ABSTRACT

This study investigated the formation of protocorms and shoots from in vitro cultured seeds of *Dactylorhiza iberica* (Bieb. ex Willd.) Soó, *D. umbrosa* (Kar. and Kir.) Nevski, and *Orchis palustris* Jacquin. Culture conditions included binucleate *Rhizoctonia* and *Rhizoctonia solani* isolates, which were symbiotic cultures isolated from the tubers of these plants, and culture media consisting of an oat medium (OM) or a modified oat medium (MOM). The shortest times for protocorm and shoot development of *D. umbrosa* sowed in OM were 42.67 and 66 days, respectively. The highest rate of protocorm development and the lowest percentage of formation of darkened protocorms in *D. umbrosa* were 60% (in OM) and 2.99% (in MOM), respectively. The maximum percentage of shoots obtained from protocorms was 35.04% for *D. iberica* cultured in OM. All data were obtained using binucleate *Rhizoctonia* sp. inoculates in the nutrient media.

Keywords: In vitro; Orchid; Protocorm; *Rhizoctonia* spp.; Shoot

Bazı Orkide Türlerinin *in vitro* Simbiyotik Kültür Çalışmaları

ESER BİLGİSİ
Araştırma Makalesi
Sorumlu Yazar: Arzu ÇIĞ, E-posta: arzucig@yahoo.com, Tel: +90 (531) 623 15 33
Geliş Tarihi: 27 Ağustos 2014, Düzeltmelerin Gelişi: 19 Şubat 2016, Kabul: 16 Eylül 2017

ÖZET

Bu çalışmada *Dactylorhiza iberica* (Bieb. ex Willd.) Soó, *Dactylorhiza umbrosa* (Kar. et. Kir.) Soó ve *Orchis palustris* Jacquin türlerine ait in vitro kültüre alınmış tohumlardan protokorm ve sürgün oluşumları incelenmiştir. Simbiyotik kültürler, bitkilerin yumrularından izole edilen binükleik *Rhizoctonia* ve *Rhizoctonia solani* izolatları ile yulaf ortamı (YO) ve modifiye yulaf ortamından (MYO) oluşmaktadır. Protokorm ve sürgün oluşumunda en erken süre, sırasıyla, 42.67 gün ve 66 gün olarak YO'na ekilmiş olan *D. umbrosa* türünde belirlenmiştir. En yüksek protokorm gelişim ve en az kararan protokorm oluşum oranı sırasıyla % 60 oranında (YO) ve % 2.99 oranında (MYO) *D. umbrosa* türünden elde edilmiştir. Protokormlardan elde edilen sürgünler, en fazla YO'da gelişen *D. iberica* türünde % 35.04 olarak tespit edilmiştir. Tüm veriler, besi ortamlarında, binükleik *Rhizoctonia* izolatının kullanılması ile elde edilmiştir.

Anahtar Kelimeler: In vitro; Orkide; Protokorm; *Rhizoctonia* spp.; Sürgün

1. Introduction

Orchid seeds are among the smallest seeds of all flowering plants because they lack endosperm and the embryo is not developed (Mitchell 1989). Orchid seeds therefore have extremely limited proliferative capacity and require suitable growing conditions (e.g., temperature, light, humidity, and oxygen), as well as the ability to establish appropriate associations with mycorrhizal fungi at the sites where they fall from the parent plant (Sezik 1984). Lacking nutrient reserves means that successful orchid seed germination cannot be realized without an external carbohydrate source such as glucose (Ingold & Hudson 1993). All the orchids are therefore obligate mycorrhizal plants and their associations are mostly with *Rhizoctonia* sp. This association is not species-specific, which means that different orchid species can be infected by the same species of *Rhizoctonia* (Andersen & Rasmussen 1996). A successful attempt to obtain rootlets in the symbiotic culture of *Coelogyne nervosa* seeds, an epiphytic orchid species, and *Epulorhiza* sp. isolate obtained from the *Eulophia epidendrea* root, a terrestrial orchid, indicates that the relationship between orchids and fungus is not species-specific (Sathiyadash et al 2014).

Orchid mycorrhiza can sometimes be described as parasitic due to the attacks of fungi and protocorms (Burges 1939). An infection attempt might be prevented by the plant if the seedling has a strong defense reaction (Garrett 1981; Hudson 1986). Researchers have also reported that only a certain number of the orchids in an appropriate habitat germinate when they encounter *Rhizoctonia* species; a seedling might be infected and might even die if the inoculums potential of the fungus is high. Paradoxically, fungal isolates have different impacts on different species; they can support or hinder plant survival. This emphasizes the importance of studying the survival and propagation capabilities of orchid species under natural or aseptic conditions, as their germination and development is heavily dependent on ambient conditions. Symbiotic in vitro seed germination in the examination of orchid with experimental and protection purpose, is seen as one of the popular tools (Stewart et al 2003; Swarts & Dixon 2009). Related to this, increasing number of symbiotic protocols was prepared for terrestrial orchids around the world (Fracchia et al 2014).

In this study, the protocorm and shoot development stages were examined for some terrestrial orchids started as seedlings using *Rhizoctonia* sp. isolated from their tubers as an in vitro symbiotic culture medium.

2. Material and Methods

2.1. Orchid source

In the study, the seeds and tubers of *Dactylorhiza iberica* (Bieb. ex Willd.) Soó, *Dactylorhiza umbrosa* (Kar. et Kir.) Soó, and *Orchis palustris* (Jacquin) species were used. The plants were collected from the meadows of the Edremit and Erciş Districts of Van Province between July and August when the capsules were brown. The seeds were kept at +4 °C temperature and in the dark until it is used.

2.2. Fungal isolation

The fungus was isolated from tubers washed with tap water and sterilized for 3 minutes in 3% commercial sodium hypochlorite (NaOCl) solution in a sterilized cabin. Tuber explants, 0.5x0.5 cm in size, were sown in three different commercial media [water agar, potato dextrose agar (PDA), and yeast extract agar (YEA)] and incubated for 3-4 days at 24 °C. After mycorrhizal hyphae on the media were detected by light microscopy, Mycelia were purified and kept in glass tubes containing PDA at +4 °C temperature and in the dark.

2.3. Fungal species identification

Test isolates used to determine anastomosis groups are supplied from the collections of the Department of Plant Protection at Faculty of Agriculture, Yüzüncü Yıl University. The origins of test isolate were different plants and they were molecularly identified. After the isolates obtained from the plants and test isolates were developed in PDA at 25±2 °C for 7 days, it was matched in 1.5% of water agar. With this aim, mycelium disks taken with cork

drillers (5 mm) from the isolate obtained from the plant with test isolate were placed at a distance of 2-4 cm reciprocally; the line that hyphae after incubation at 25±2 °C for 48-72 hours was dyed with lactophenol consisting of 0.5% trypan and it was examined directly in optical microscope in order to identify whether there was a unification status of cell wall and cytoplasmic among hyphae (Parmeter et al 1969).

2.4. Symbiotic culture medium

Oat medium (OM) as symbiotic culture (rolled oats 2.5 g L^{-1}, agar 0.7%, pH 5.7) (Clements & Ellyard 1979) and modified oat medium (MOM), ($Ca(NO_3)_2.4H_2O$ 0.2 g L^{-1}, $KH_2PO_4.7H_2O$ 0.2 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.1 g L^{-1}, KCl 0.1 g L^{-1}, $MgSO_4.7H_2O$ 0.1 g L^{-1}, yeast extract 0.1 g L^{-1}, rolled oats 3.5 g L^{-1}, agar 0.6%, pH 5.7) (Clements et al 1986) were used. Medium up to 20 ml was poured into glass petri dishes. Seven-day-fungal isolates were inoculated as disks in the size of 0.5x0.5 cm on one side of culture media.

2.5. Seed sowing

The orchid seeds were sterilized by shaking in 2% sulfuric acid for 5 minutes, followed by a 12-minute treatment with 1-2 drops of Tween-20 and 10% NaOCl, and three rinses for a few minutes in sterilized water. The study was conducted in 3 replicates for each treatment. In each replicate, 6 pieces of petri were used; there were 100 pieces of seeds in each petri dishes. The seeds were sowed on the other side of petri dish where fungal isolate was placed. The culture was kept at 23±1 °C temperature and in the dark until the first protocorm was seen. After the first protocorm was formed, it was kept in climate chamber providing light/dark photoperiod for 16/8 hours. For the protocorm and shoot formation, sub-cultures were made to the jars containing the corresponding cultural medium where the seeds were sowed once a month.

2.6. Statistical analysis

The trials in which R is included were not statistically evaluated. Binucleate *Rhizoctonia* was evaluated by considering the nutrient medium and the species interaction.

In the evaluation of the data "variance analysis in factorial order in the randomized plot design" was made. The measured percentage changes were subjected to angle transformation prior to the analysis of variance. Following the analysis of variance, Duncan's multiple comparison test was used for determination of differences among the species and media. The statistical significance level was set at ($P<0.05$) and all analyses were conducted using the SPSS (ver.13) statistical package software.

3. Results and Discussion

3.1. Fungal isolation

A *Rhizoctonia solani* AG-3 (R) fungal isolate was successfully isolated from a tuber explant of *D. umbrosa* species cultured on water agar (Figure 1); a binucleate *Rhizoctonia* (2R) isolate was isolated from tubers of *O. palustris* species cultured on PDA (Figure 2). The rapid sprouting of the fungus in the nutrient medium in the petri dishes meant that germination could not be precisely defined, but protocorm and shoot formation was observed. None of the media inoculated with *R. solani* fungi resulted in any germination or protocorm formation. That the fungus isolated from the roots and tubers of orchids could not show any symbiotic effect on the seeds is a frequent situation encountered by the researchers. It is not encouraging for the species of *R. solani* isolate, *Orchis italica* and *Serapias vomeracea* (Oğuz et al 2005; Sarı et al 2011) and in *Bipinnula fimbriata* species (Steinfort et al 2010) since fungi have a pathogenic effect on *Cattleya skinneri* seeds, and since they cause the death of all seeds in a few days (Ovando et al 2005), it may be seen as an example. Some fungal isolates isolated from the tubers of *Spiranthes spiralis* (L.) and some fungal isolates brought from abroad did not have any symbiotic effect on the seeds of the species of *D. romana* subsp. *romana* (Sazak 2004). Despite this, in the symbiotic culture carried out with the seeds of *Spiranthes sinensis* var. *amoena*, the isolates of *R. solani* and binucleate *Rhizoctonia* anastomosis groups were used, and it was detected that fungi had positive effects on the germination of

the seeds (Masuhara et al 1993). Each isolate used in the species of *S. vomeracea* subsp. *laxiflora* and *Orchis laxiflora* could not provide the same effect (Özkoç 1991). Although some isolates stimulated the germination, protocorm and plant formation, some isolates had no effect on the symbiotic culture done with *Dendrobium chrysanthum* and pathogenic *Rhizoctonia* isolates, (Hajong et al 2013).

As evidenced in the studies conducted in the expectation that fungi has a symbiotic effect, it causes death due to its pathogenic effect as well as to the fact that it enters the process of plants via seen germination and protocorm.

3.2. Darkened protocorm ratio (%)

Some of the protocorms that developed in both media turned brown and soft shortly after removal, indicating a loss of viability (Figure 3). These protocorms were still counted and evaluated statistically (Table 1). Their percentages were calculated as a proportion of the total number of seeds. Examination of the presence of darkened protocorms among the *D. iberica, D. umbrosa* and *O. palustris* seeds sowed in OM and MOM revealed a statistically significant difference ($P<0.05$). The highest proportion (37.38%) of darkened protocorms was obtained from *O. palustris* in MOM, while the lowest percentage (2.99%) was seen in *D. umbrosa* in MOM, confirming a clear difference among the species. Comparison of the culture media revealed that the lowest and highest darkening ratios for *D. umbrosa* occurred in the MOM and OM, respectively, indicating a correlation between development and culture media. Similarly, the highest and the lowest darkening ratios were obtained in different species grown in the same nutrient medium (MOM), indicating a significant effect of the nutrient media on different species.

Symbiotic germination studies of *Serapias vomeracea* subsp. *laxiflora* and *O. laxiflora* in OM and MOM media reported the darkening of in a 5.1% of the protocorms in MOM medium 1 month

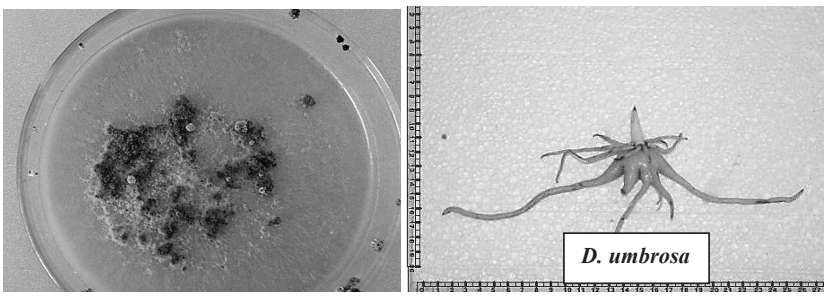

Figure 1- *Rhizoctonia solani* and its source, the tuber of *D. umbrosa* species

Figure 2- Binucleate *Rhizoctonia* (2R) and its source, the tuber of *O. palustris* species

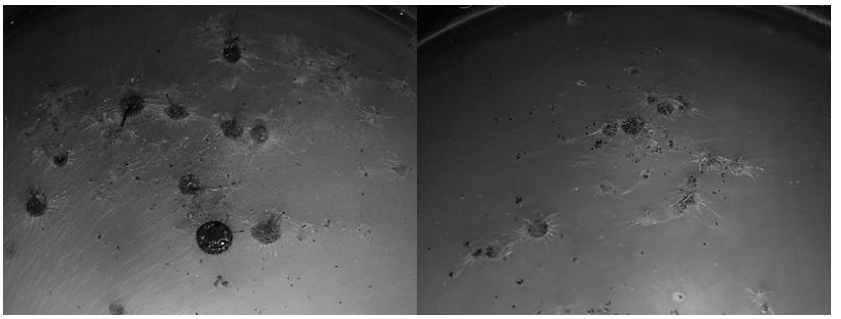

Figure 3- Darkened protocorms developed in the 2R+MOM culture medium

Table 1- The effect of the culture medium on protocorm darkening (%) in *Dactylorhiza iberica, D. umbrosa,* and *Orchis palustris*

Mediums	*Dactylorhiza iberica*	*Dactylorhiza umbrosa*	*Orchis palustris*
Oat medium	9.31±0.27 a* C**	26.43±0.26 a A	15.57±0.35 b B
Modified oat medium	8.71±0.25 a B	2.99±0.11 b C	37.38±0.56 a A

*, in the same column, lower case letters indicate a none significant difference between the averages at the 0.05 level; **, in the same row, upper case letters indicate a none significant difference between the averages at the 0.05 level

after planting (Özkoç 1991). A similar darkening and rotting after germination was observed for the seeds of *D. osmanica* var. *osmanica* sown in OM with *R. solani* (Sazak & Ozdener 2006). Some researchers mentioned that gradual darkening and protocorm putrefaction might be observed in culture studies including fungi and extending more than 16 weeks at 20 °C (Shimura & Koda 2005).

In our study, the 2R isolate stimulated protocorm development, however, it also promoted a substantial protocorm darkening. The darkened protocorms lose their vitality and do not send out shoots, so a darkened protocorm can be interpreted as a loss of the shoot and the plant. The 2R isolate had the strongest influence on *O. palustris* in MOM medium. The fungal isolate obtained from the tuber was thought to possibly have a parasitic effect on the seeds of the same species under appropriate conditions.

3.3. Total protocorm ratio (%)

The total protocorm number was evaluated as the combined total numbers of robust and darkening protocorms and ratios were calculated by dividing the protocorm numbers by the total number of seeds. The differences observed for total protocorm formation among *D. iberica, D. umbrosa,* and *O. palustris* sown in OM and MOM were statistically significant ($P<0.05$) (Table 2). The highest ratio was obtained for *D. umbrosa* in OM, as 60.74%, and the lowest ratio was obtained for *D. iberica* in OM, at 9.45% (Figure 4). In the other nutrient media, the highest ratio was also obtained from *D. umbrosa*; the lowest ratio was obtained from *D. iberica*.

Table 2- The effect of the culture medium on the total number of protocorms (%) developed in *Dactylorhiza iberica, D. umbrosa,* and *Orchis palustris*

Mediums	*Dactylorhiza iberica*	*Dactylorhiza umbrosa*	*Orchis palustris*
Oat medium	12.98±0.38 a* C**	60.74±0.54 a A	25.95±0.29 b B
Modified oat medium	9.45±0.81 b C	56.74±0.30 b A	41.84±0.39 a B

*, in the same column, lower case letters indicate a none significant difference between the averages at the 0.05 level; **, in the same row, upper case letters indicate a none significant difference between the averages at the 0.05 level

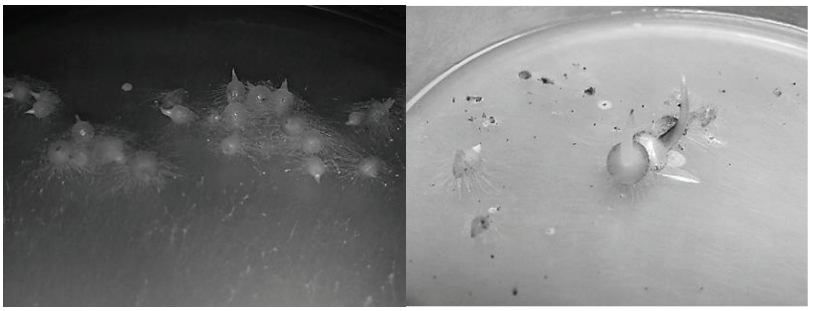

Figure 4- *D. umbrosa* protocorms in 2R+MOM culture medium

The highest protocorm development was 2.35% for *D. iberica* seeds cultured in OM with mycorrhizal fungi isolates and 4.72% when cultured in MOM (Özdener 1994). For *D. aristata* the highest protocorm development was 45.10% when cultured in OM (Hayakawa et al 1999). In a study the highest protocorm development rate was 22.1% in OM and 3.3% in MOM for *S. vomeracea*, whereas the rate was 4.8% for *O. laxiflora* cultured in OM and 5.2% when cultured in MOM (Özkoç 1991). In agreement with the present study, the highest and the most positive results in these studies were generally obtained with OM. In a study inoculated with binucleate *Rhizoctonia* and *R. solani* isolate, in *O. palustris* and *S. vomeracea* subsp. *vomeracea* species, protocorm formation was seen in a previous study (Esitken et al 2005). In symbiotic study conducted in OM with *Spiranthes brevilabris* Lindley seeds, *Epulorhiza repens* become successful among 4 fungal isolates. The first protocorms were emerged around 7-8% after 10-12 weeks of the culture (Stewart & Kane 2007). In OM conducted between fungal isolates isolated from the species of *Disa bracteata* and 6 orchid species; 14-90% protocorms were obtained in the species of *Caladenia falcata* and *Pterostylis sanguinea*. In this study, it is understood that entophytic orchids can be germinated with fungus but the protocorm formation is due to the physiological imbalance of the fungi (Bonnardeaux et al 2007). The seeds of *Pecteilis susannae* increased the protocorm formation with *Epulorhiza* isolate over 60% (Chutima et al 2011).

As it is mentioned above, the highest and positive results, consisted with the previous studies, were obtained from OM to a great extent. These results can be interpreted that nutrient mediums have important effects on protocorm formation. Similarly, fungi cause protocorm formation in media that may be regarded as low in some species where they may behave different from orchid species while higher level of formation was obtained in some species.

3.4. Ratio of shoots obtained from protocorms (%)

The shoot ratio was expressed as the percentage of developed shoots to the total number of protocorms, regardless of whether the protocorms were darkened. The differences between the average numbers of protocorms and shoot formation were statistically significant ($P<0.05$) (Table 3).

Table 3- The effect of the culture medium on shoot formation (%) from the protocorms of *Dactylorhiza iberica, D. umbrosa,* and *Orchis palustris*

Mediums	*Dactylorhiza iberica*	*Dactylorhiza umbrosa*	*Orchis palustris*
Oat medium	35.04±1.12 a* A**	18.00±0.51 b B	15.56±0.17 a B
Modified oat medium	14.28±0.58 b B	32.59±1.00 a A	7.77±0.59 b C

*, in the same column, lower case letters indicate a none significant difference between the averages at the 0.05 level; **, in the same row, upper case letters indicate a none significant difference between the averages at the 0.05 level

The highest mean values belonging to shoot formation were obtained in the ratio of 35.04% in the species of *D. iberica* in OM; however these shoots were not developed as healthy as in other species; they continued their lives as short and weak (Figure 5). The lowest shoot formation was found to be 7.77% from the species of *O. palustris* in MOM. The shoot was obtained from the protocorms formed approximately at the rates of 7-35% in our study.

Shoot formation failure is frequently observed in the protocorms developing from symbiotically germinated seeds (Hayakawa et al 1999). A study carried with nine fungal isolates reported the highest shoot formation in *D. urvilleana* as 55.80% in OM and 60.60% in MOM; while the highest rate for *D. iberica* was 3.10% in OM and 4.80% in MOM (Özdener 1994). Further experiments using six different nutrient media, prepared by modifying MOM, gave results that varied from no shoot formation to as much as 1.20% shoot formation. Symbiotic germination studies with *S. vomeracea* and *O. laxiflora* reported the highest shoot formation of 14.3% and 2.8% in OM and MOM, respectively, in *S. vomeracea* and 6.9% and 4.1%, respectively, in *O. laxiflora* (Özkoç 1991). In this study, inoculations of BNR 8-3 mycorrhizal isolate isolated in the species of *D. urvilleana* and in the species of *O. palustris*, to which polyethylene glycol was applied in OM, the first output of the leaf has a 21% chance of success (Esitken et al 2004).

Symbiotic germination studies of *D. hatagirea* (with YEA addition) and *Ceratobasidium* sp. in OM resulted in shoot initiation in 61% of all the germinated seeds (Aggarwal & Zettler 2010). In that study, the germinated seeds developed protocorms in 40 days. In a symbiotic study conducted in OM with binucleate *Rhizoctonia* and *Rhizoctonia solani* AG-6 isolated from the plant of *Anoectochilus formosanus* Hayata, both isolates increase the growth of the plants of *A. formosanus* to a great extend (Chang & Chou 2007). In OM, the seed of *Spiranthes brevilabris* Lindley and *Epulorhiza repens* isolate indicated successful results. The first shoots were observed around 10% and 6% in 10th and 12th weeks of the culture, respectively, and extention of the shoots were detected around 50% and 60% in 10th and 12th weeks, respectively (Stewart & Kane 2007).

3.5. Protocorm formation time (days)

The period between the planting of the orchid seeds in the petri dish containing the culture medium with mycorrhizal fungus and the appearance of the first protocorm was designated as the protocorm development time. The nutrient media had statistically significant effects on it ($P<0.05$) (Table 4). The shortest development time (42.67 days) was observed in *D. umbrosa* seeds planted in OM, while the longest time (55.33 days) was observed in *O. palustris* seeds planted in OM. The seeds of different species showed clearly different growth in the nutrient media and therefore had different protocorm development times. All the plant species examined had protocorm development times of approximately 2 months.

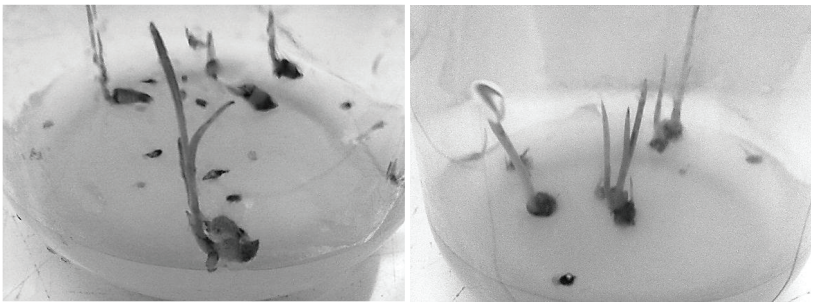

Figure 5- *D. iberica* shoots in 2R+OM culture medium

Table 4- The effect of the culture medium on the protocorm formation time (days) for *Dactylorhiza iberica, D. umbrosa,* and *Orchis palustris*

Mediums	*Dactylorhiza iberica*	*Dactylorhiza umbrosa*	*Orchis palustris*
Oat medium	48.67±0.88 b* B**	42.67±0.88 b C	55.33±0.67 a A
Modified oat medium	53.00±1.15 a A	51.67±0.88 a AB	49.67±0.33 b C

*, in the same column, lower case letters indicate a none significant difference between the averages at the 0.05 level; **, in the same row, upper case letters indicate a none significant difference between the averages at the 0.05 level

In a study, the protocorms of the species of *D. hatagirea* sowed in OM with fungal isolates belonging to the species of *Ceratobasidium* developed in 40 days (Aggarwal & Zettler 2010). Seeds of *D. maculata* germinated with an *R. stahlii* isolate developed protocorms in two weeks (Beyrle et al 1985); however, the authors reported that this rapid protocorm development might have been a result of stimulation by cold treatment. Protocorm development occurred 1.5-2 months in *O. coriophora* seeds cultured in OM with *R. solani* (Vakkasoğlu 1995). The *Dactylorhiza* and *Orchis* species showed protocorm development in 40-60 days when cultured with a symbiotic fungus on either OM or MOM.

3.6. Shoot formation time (days)

The period between the planting of the orchid seeds in the petri dishes (containing culture medium and the mycorrhizal fungus) and the appearance of the first shoot was designated the shoot formation time. The different species showed statistically significant differences in the shoot formation time ($P<0.05$) (Table 5). The earliest and the latest shoot formation times in MOM were 63.67 days for *O. palustris* and 78.67 days for *D. iberica,* respectively. The timing of the initiation of shoot formation after protocorm development varied depending on the species and the medium; however, green shoot formation was rapid in all three species. This means that healthy protocorms can develop and form shoots more rapidly in the presence of symbiotic fungi. All three species formed shoots in 14-27 days after protocorm development (Table 4).

Species of the *Dactylorhiza* genus are reported as a difficult group of orchids to cultivate (Mitchell 1989). The results of the present study confirmed that species of the *Dactylorhiza* genus formed shoots later than a member of the *Orchis* genus did. A previous study reported that the time required for the development of green leaves in *Spiranthes sinensis* var. *amoena,* when grown in symbiotic culture in OM and various *Rhizoctonia* isolates, was 90 days (Masuhara & Katsuya 1994). In a symbiotic culture between the seeds of *Rhizoctonia cerealis* isolate and *D. maculata* spp. *ericetorum*, green shoot growth from the protocorms began 90 days after the seed sowing (Weber & Webster 2001). With being infected of *Spathoglottis plicata* seeds with *Epulorhiza repens* and *Rhizoctonia globularis* isolates, the time passed between the seed sowing and the beginning of leafing was 60 days (Athipunyakom et al 2004). In the symbiotic culture of fungal isolates obtained from the species of orchids and the seeds of *Oncidium flexuosum*, some isolates started to germinate but it did not contribute

Table 5- The effect of the culture medium on the shoot formation time (days) of *Dactylorhiza iberica, D. umbrosa* and *Orchis palustris*

Mediums	*Dactylorhiza iberica*	*Dactylorhiza umbrosa*	*Orchis palustris*
Oat medium	73.33±0.33 b* A**	66.00±1.00 b B	75.67±0.88 a A
Modified oat medium	78.67±0.88 a A	78.33±0.88 a A	63.67±0.88 b B

*, in the same column, lower case letters indicate a none significant difference between the averages at the 0.05 level; **, in the same row, upper case letters indicate a none significant difference between the averages at the 0.05 level

to the protocorm and plant formation at a desired level and some of them promoted leaf formation 50 days after protocorms (Pereira et al 2005). With 5 fungal isolates isolated from it, the species of *Aa achalensis* promoted 2-4 leaf formation from 30% of the protocorms in 16 weeks after the growth (Sebastián et al 2014). The similarity between the nutrient media and fungal isolates used in these studies suggests the possibility that the observed shoot formation differences might be species specific.

4. Conclusions

The choice of the nutrient medium and the fungal isolate for in vitro symbiotic culture of orchid seeds has extremely important effects on germination, protocorm formation, and shoot initiation. Some fungi never promote the germination of orchid seeds, or have a very insignificant impact on orchid development, and sometimes they even have a parasitic effect. The differences observed in the present study with respect to protocorm formation from the germinated seeds sown in oat medium and modified oat medium are likely to reflect the combination of agents added to the culture medium. The data obtained from our study were detected in the cultures to which 2R is infected. The observation that orchid species developed more protocorms in oat medium culture indicated that this nutrient medium favored optimum growth of the binucleate *Rhizoctonia* symbiont enabling a better symbiosis. The differences due to the nutrient media can be explained by the different effects of a given fungal isolate on different orchid species. The more successful germination and protocorm development seen in *Dactylorhiza umbrosa* seeds with the binucleate *Rhizoctonia* isolate, obtained from an *Orchis palustris* tuber, supports the view of some researchers that the degree of effectiveness of fungal isolates might vary with species. The formation of darkened protocorms, which was evaluated separately in the present study, could be viewed as a loss, as these protocorms could not form shoots. The nutrient medium had a significant effect on the the total number of protocorms developed, with the highest number of protocorms seen with *Dactylorhiza umbrosa* and the lowest with *Dactylorhiza iberica*. The lowest success rate was obtained with *Orchis palustris* in both nutrient media. The other species showed their best performances in oat medium and modified oat medium at different levels. Protocorm development took approximately 1.5-2 months after planting in all the species and each species had a different timing in different nutrient media. Accordingly, the shoot formation times also varied. The shortest shoot and protocorm development occurred in oat medium culture. The use of oat medium and modified oat medium infected with binucleate *Rhizoctonia* sp. gave the least amount of protocorm darkening, the highest protocorm number, and the fastest development of protocorms in *Dactylorhiza umbrosa*, it gave the highest number of shoots in *Dactylorhiza iberica,* and it resulted in the fastest shoot formation in *Orchis palustris*.

Acknowledgements

This study is a part of a Doctorate Dissertation Project (2009, FBE-D036) financed by Yüzüncü Yıl University, Van, Turkey Scientific Research Projects Department.

References

Aggarwal S & Zettler L W (2010). Reintroduction of an endangered terrestrial orchid, *Dactylorhiza hatagirea* (D. Don) Soo, assisted by symbiotic seed germination: First report from the Indian subcontinent. *Nature and Science* **8**(10): 139-145

Andersen F T & Rasmussen H N (1996). *The Mycorrhizal Species Of Rhizoctonia, Rhizoctonia Species, Taxonomy, Molecular Biology, Ecology, Patology, And Disease Control* (B. Sneh, S. Jabaji-Hare, S. Neate & G. Dijst). Kluwer Academic Publishers, Dordrecht. pp. 379-390

Athipunyakom P, Manoch L, Piluek C, Artjariyasripong S & Tragulrung S (2004). Mycorrhizal fungi from *Spathoglottis plicata* and the use of these fungi to germinate seeds of *S. plicata* in vitro. *Kasetsart Journal (Natural Sciences)* **38**(1): 83-93

Beyrle H, Pennigsfeld F & Hock B (1985). Orchid mycorrhiza: Symbiotic propagation of the some *Dactylorhiza* species. *Zeitscrift-fur-Mykologie* **51**(2): 185-198

Bonnardeaux Y, Brundrett M, Batty A, Dixon K, Koch J & Sivasithamparam K (2007). Diversity of mycorrhizal fungi of terrestrial orchids: compatibility webs, brief encounters, lasting relationships and alien invasions. *Mycological Research* **111**: 51-61

Burges A (1939). The defensive mechanism in orchid mycorrhizas. *New Phytologist* **38**(3): 273-283

Chang D C-N & Chou L-C (2007). Growth responses, enzyme activities, and component changes as influenced by *Rhizoctonia* orchid mycorrhiza on *Anoectochilus formosanus* Hayata. *Botanical Studies* **48**: 445-451

Chutima R, Dell B, Vessabutr S, Bussaban B & Lumyong S (2011). Endophytic fungi from *Pecteilis susannae* (L.) Rafin (*Orchidaceae*), a threatened terrestrial orchid in Thailand. *Mycorrhiza* **21**(3): 221-229

Clements M A & Ellyard R K (1979). The symbiotic germination of Australian terrestrial orchids. *American Orchid Societly Bulletin* **48**: 810-816

Clements M A, Muir H & Cribb P J (1986). A preliminary report on the symbiotic germination of European terrestrial orchids. *Kew. Bulletin* **41**(2): 437-445

Esitken A, Ercisli S, Eken C & Tay D (2004). Seed priming effect on symbiotic germination and seedling development of *Orchis palustris* Jacq. *HortScience* **39**(7): 1700-1701

Esitken A, Ercisli S & Eken C (2005). Effects of mycorrhiza isolates on symbiotic germination of terrestrial orchids (*Orchis palustris* Jacq. and *Serapias vomeracea* subsp. *vomeracea* (Burm. f.) Briq.) in Turkey. *Symbiosis* **38**(1): 59-68

Garrett S D (1981). *Soil Fungi and Soil Fertility.* 2nd. Edition, Perganon. Oxford, pp. 150

Hajong S, Kumaria S & Tandon P (2013). Compatible fungi, suitable medium, and appropriate developmental stage essential for stable association of *Dendrobium chrysanthum*. *Journal of Basic Microbiology* **53**(12): 1025-1033

Hayakawa S, Uetake Y & Ogoshi A (1999). Identification of symbiotic *Rhizoctonia*s from naturally occurring protocorms and roots of *Dactylorhiza aristata (Orchidaceae)*. *Journal of the Faculty of Agriculture Hokkaido University* **69**(2): 129-141

Hudson H J (1986). Fungal Biology. Edward Arnold, London, pp. 298

Ingold C T & Hudson H J (1993). The Biology of Fungi, Sixth Edition (Chapman & Hall). London, pp. 224

Masuhara G & Katsuya K (1994). In situ and in vitro specificity between *Rhizoctonia* spp. and *Spiranthes sinensis* (Persoon) Ames. var. *amoena* (M. Bieberstein) Hara *(Orchidaceae)*. *New Phytologist* **127**(4): 711-728

Masuhara G, Katsuya K & Yamaguchi K (1993). Potential for symbiosis of *Rhizoctonia solani* and binucleate *Rhizoctonia* with seed of *Spiranthes sinensis* var. *amoena in vitro*. *Mycological Research* **97**(6): 746-752

Mitchell R B (1989). Growing hardy orchids from seeds at Kew. *The Plantsman* **1**: 152-169

Oğuz B, Sari A O & Bilgiç A (2005). Ege Bölgesi'nde Yayılış Gösteren Bazı Salep Orkidelerinin Üretim Olanaklarının Araştırılması. Tagem Proje Sonuç Raporu, Proje No: Tagem/Ta/02/02/03/010

Ovando I, Damon A, Bello R, Ambrosio D, Albores V, Adriano L & Salvador M (2005). Isolation of endophytic fungi and their mycorrhizal potential for the tropical epiphytic orchids *Cattleya skinneri*, *C. aurantiaca* and *Brassavola nodosa*. *Asian Journal of Plant Sciences* **4**(3): 309-315

Özdener Y (1994). *Dactylorhiza urvilleana* (Steudel) Bauman Künkele ve *Dacytlorhiza iberica* (Bieb. Ex Willd) Soo *(Orchidaceae)* türlerinin köklerinden fungusların izole edilmesi, bu türlere ait tohumların simbiyotik ve asimbiyotik kültür ortamlarında çimlenme ve gelişmesi üzerinde bir araştırma. Doktora tezi, Ondokuz Mayıs Üniversitesi Fen Bilimleri Enstitüsü (Basılmamış), Samsun

Özkoç İ (1991). *Serapias vomeracea* (Burmfil.) Briq. Sub sp. *laxiflora* (Soo) Gölz et. Reinhard ve *Orchis laxiflora* Lam. *(Orchidacea)* tohumlarının simbiyotik ve asimbiyotik kültürlerde çimlenme ve gelişmesi üzerinde araştırılması. Doktora tezi, Ondokuz Mayıs Üniversitesi Fen Bilimleri Enstitüsü (Basılmamış), Samsun

Parmeter J R, Sherwood R T & Platt W D (1969). Anastomosis grouping among isolates of *Thanatephorus cucumeris*. *Phytopathology* **59**(9): 1270-1278

Pereira O L, Kasuya M C M, Rollemberg C & Borges A C (2005). In vitro symbiotic seed germination of *Oncidium flexuosum (Orchidaceae)* by *Rhizoctonia-*

like mycorrhizal fungi. *Revista Brasileira de Ciência do Solo* **29**(2): 199-206

Sari A O, Tutar M & Çiçek F (2011). Ege Tarımsal Araştırma Enstitüsü salep üretim çalışmaları. *I. Salep Orkidesi Çalıştayı,* 24-25 Mayıs, Kahramanmaraş, s. 65-86

Sathiyadash K, Muthukumar T, Murugan S B, Sathishkumar R & Pandey R R (2014). In vitro symbiotic seed germination of South Indian endemic orchid *Coelogyne nervosa*. *Mycoscience* **55**(3): 183-189

Sazak A (2004). Bazı orkide türlerine ait tohumların simbiyotik ve asimbiyotik olarak çimlendirilmesi ve fide gelişimi. Yüksek lisans tezi, Ondokuz Mayıs Üniversitesi Fen Bilimleri Enstitüsü (Basılmamış), Samsun

Sazak A & Ozdener Y (2006). Symbiotic and asymbiotic germination of endangered *Spiranthes spiralis* (L.) Chevall. and *Dactylorhiza osmanica* (Kl.) Soó var. *osmanica* (endemic). *Pakistan Journal of Biological Sciences* **9**(12): 2222-2228

Sebastián F, Vanesa S, Eduardo F, Graciela, T & Silvana S (2014). Symbiotic seed germination and protocorm development of *Aa achalensis* Schltr., a terrestrial orchid endemic from Argentina. *Mycorrhiza* **24**(1): 35-43

Sezik E (1984). Orkidelerimiz, Türkiye'nin Orkideleri. Sandoz Kültür Yayınları. No: 6, s. 166

Shimura H & Koda Y (2005). Enhanced symbiotic seed germination of *Cypripedium macranthos* var. *rebunese* following inoculation after cold treatment. *Physiologia Plantarum* **123**(3): 281-287

Steinfort U, Verdugo G, Besoain X & Cisternas M A (2010). Mycorrhizal association and symbiotic germination of the terrestrial orchid *Bipinnula fimbriata* (Poepp.) Johnst *(Orchidaceae*). *Flora-Morphology, Distribution, Functional Ecology of Plants* **205**(12): 811-817

Stewart S L & Kane M E (2007). Symbiotic seed germination and evidence for *in vitro* mycobiont specificity in *Spiranthes brevilabris* (*Orchidaceae*) and its implications for species-level conservation. *In Vitro Cellular & Developmental Biology-Plant* **43**(3): 178-186

Stewart S L, Zettler L W, Minso J & Brown P M (2003). Symbiotic germination and reintroduction of *Spiranthes brevilabris* Lindley, an endangered orchid native to Florida. *Selbyana* **24**(1): 64-70

Swarts N D & Dixon K W (2009). Terrestrial orchid conservation in the age of extinction. *Annals of Botany* **104**(3): 543-556

Vakkasoğlu F (1995). Orkidelerde mikorizal fungusların orkide tohumlarının çimlenmesi ve büyümeleri üzerine etkisi. Yüksek lisans tezi, Çukurova Üniversitesi Fen Bilimleri Enstitüsü (Basılmamış), Adana

Weber R W S & Webster J (2001). Teaching techniques for mycology: 14. Mycorrhizal infection of orchid seedlings in the laboratory. *Mycologist* **15**(2): 55-59