

## IN VITRO SYMBIOTIC GERMINATION POTENTIALS OF SOME *ANACAMPTIS*, *DACTYLORHIZA*, *ORCHIS* AND *OPHRYS* TERRESTRIAL ORCHID SPECIES

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**Abstract.** Germination and propagation of terrestrial orchids is highly challenging due to their small size seeds and absence of endosperms. Some orchid species are extremely dependent on fungi for germination of their seeds. Although germination occurs symbiotically between the seed and the fungus, it is not coincidental that fungus species are specific to orchid species. Aim of this study is to show the impact of the fungi, isolated from the tubers of ten naturally grown species of orchids, collected from mountainous area of Van province, Turkey, on in vitro symbiotic germination of the seeds. The collected orchids species were *Dactylorhiza romana* (Seb.) Soo subsp. *georgica* (Klinge) Soo ex Renz & Taub., *Orchis pinetorum* Boissier & Kotschy, *Orchis spitzelii* Saut. ex W.D.J. Koch, *Orchis coriophora* L., *Orchis collina* Banks & Solander, *Orchis anatolica* Boiss., *Orchis simia* Lamarck, *Ophrys straussii* H. Fleischmann & Bornmüller, *Dactylorhiza umbrosa* (Kar. & Kir.) Nevski, *Anacamptis pyramidalis* (L.) L. C. Rich. In the study from orchid tubers, *Rhizoctonia* sp., *Aspergillus* sp., *Alternaria* sp., *Penicillium* sp., *Trichoderma* sp. and *Fusarium* sp. fungi species are isolated. Firstly, orchid seeds and fungal isolates placed in oat culture medium were transferred to Van Waes & Debergh culture medium in subculture. The highest germination rates obtained in the seeds are 71.19% in *Dactylorhiza romana* subsp. *georgica* species with *Rhizoctonia* sp.; 78.26% in *Orchis pinetorum* species with *Penicillium* sp.; 27.77% in *Orchis spitzelii* species with *Fusarium* sp.; 83.07% in *Orchis coriophora* species with *Rhizoctonia* sp.; 75.00% in *Orchis collina* species with *Fusarium* sp.; 38.88% in *Orchis anatolica* species with *Alternaria* sp.; 73.91% in *Orchis simia* species with *Fusarium* sp.; 91.60% in *Ophrys straussii* species with *Fusarium* sp.; 93.75% in *Dactylorhiza umbrosa* species with *Fusarium* sp. and 56.00% in *Anacamptis pyramidalis* species with *Rhizoctonia* sp. At the end of the study, it was understood that the fungi isolated from their own or other tubers had different effects on the germination of each orchid species.

**Keywords:** *fungal isolates, in vitro, mycorrhiza effect, symbiotic germination, terrestrial orchid*

### Introduction

The most distinctive features of the orchid seeds are their very small size, absence of endosperm and undeveloped embryo. These seeds have a length of 0.25-1.2 mm, width of 0.09-0.27 mm and weight of 0.3-1.4 mg (Arditti, 1967). The orchids grown at high altitudes occasionally fail to grow and set seed due to low temperatures or the ones grown under dense forests grow slowly and cannot reach to a desired level of development due to lack of light (Sezik, 1984). Thus, in addition to appropriate temperature, light, oxygen, moisture and soil conditions required in the microclimate of the environment in which the seed fail to germinate, an appropriate symbiotic relationship should be established with a mycorrhizal fungus for germination of the

seeds (Sezik, 1984). As the orchid seeds do not contain nutrient reserves, successful germination cannot be achieved unless a carbohydrate source, such as glucose, is provided (Ingold and Hudson, 1993).

The symbiotic capacity of a fungal isolate depends primarily on the nature of the isolate and then the orchid species with which it coexists. The results obtained on the existence of a specificity between orchid species and mycorrhizal fungi indicate that there is no strict specificity at the species-species level, and that, on the other hand, orchid-fungus relationships are not entirely random. According to Batty et al. (2001), terrestrial orchids may have narrow or broad potential specificity (Warcup, 1981; Alexander and Hadley, 1983; Muir, 1989), but the specificity of their associations with endophytes in natural habitats is still poorly understood. The specificity of the orchid–mycorrhiza association is variable between species (Steinfort et al., 2010). An orchid may form mycorrhizal associations with more than one fungal species, and a fungal species might associate with more than one orchid under in situ conditions (Bonnardeaux et al., 2007; Otero et al., 2004).

When these relations existing in the nature are moved to the laboratory conditions; the fungal isolates are isolated from the orchid tubers and roots and inoculated into the medium. In this study, the impacts of the fungal isolates isolated from orchid tubers, collected from the nature and inoculated into nutrient medium, on germination of the seeds are determined.

## Material and methods

The material of the research consists of orchid seeds and fungi obtained from the tubers of the plants. The plants used in the research are naturally grown in Van province, Turkey, collected from Gevaş District Altınsaç neighbourhood in July-2014 when the plants formed capsules, and identified according to Davis (1984).

### *Orchid species*

The orchid species examined in the study are *Dactylorhiza romana* (Seb.) Soo subsp. *georgica* (Klinge) Soo ex Renz & Taub., *Orchis pinetorum* Boissier & Kotschy, *Orchis spitzelii* Saut. ex W.D.J. Koch, *Orchis coriophora* L., *Orchis collina* Banks & Solander, *Orchis anatolica* Boiss., *Orchis simia* Lamarck, *Ophrys straussii* H. Fleischmann & Bornmüller, *Dactylorhiza umbrosa* (Kar. & Kir.) Nevski, *Anacamptis pyramidalis* (L.) L. C. Rich.

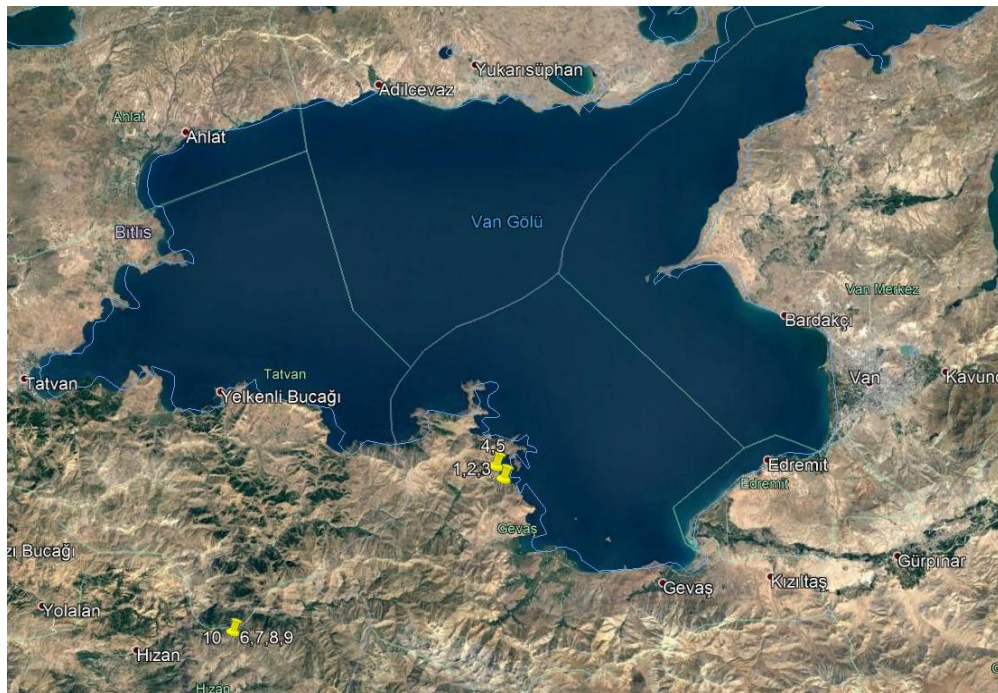
*Dactylorhiza romana* subsp. *georgica*, *Orchis pinetorum* ve *Orchis spitzelii* species were determined in the same location (N 38°23'30.2" and E 42°53'43.8") while *Orchis coriophora* and *Dactylorhiza umbrosa* species were obtained in the same location (N 38°24'16.2" and E 42°53'76.6"). *Anacamptis pyramidalis*, *Orchis collina*, *Orchis simia*, and *Ophrys straussii* species were collected from N 38°23'61.7" and E 42°55'03.2" coordinates while *Orchis anatolica* species was collected from N 38°23'59.3" and E 42°54'98.1" coordinate (Table 1 and Fig. 1).

### *Fungi*

Fungi obtained from isolations in tuber samples, collected during seed collection, are used.

**Table 1.** GPS coordinates where orchid species are collected

No	Orchid species	GPS Coordinates		Altitude (m)
1	<i>Dactylorhiza romana</i> subsp. <i>georgica</i>	N 38°23'30.2"	E 42°53'43.8"	1864
2	<i>Orchis pinetorum</i>	N 38°23'30.2"	E 42°53'43.8"	1864
3	<i>Orchis spitzelii</i>	N 38°23'30.2"	E 42°53'43.8"	1864
4	<i>Orchis coriophora</i>	N 38°24'16.2"	E 42°53'76.6"	1631
5	<i>Dactylorhiza umbrosa</i>	N 38°24'16.2"	E 42°53'76.6"	1631
6	<i>Anacamptis pyramidalis</i>	N 38°23'61.7"	E 42°55'03.2"	1724
7	<i>Orchis collina</i>	N 38°23'61.7"	E 42°55'03.2"	1724
8	<i>Orchis simia</i>	N 38°23'61.7"	E 42°55'03.2"	1724
9	<i>Ophrys straussii</i>	N 38°23'61.7"	E 42°55'03.2"	1724
10	<i>Orchis anatolica</i>	N 38°23'59.3"	E 42°54'98.1"	1724



**Figure 1.** Locations where orchid species are collected (Google Earth, 2018)

### Surface sterilization of the seeds

Orchid seeds are sterilized in small envelopes made from coarse filter papers as they are very small and have a dusty structure. Seeds of each species weighting 100 mg are enveloped and used for experiments. For each experiment, 100 mg of the seeds were planted in total of 9 petri dishes containing Potato Dextrose Agar (PDA) media in 3 replicates, 3 pots in each replicate.

The seeds were shaken for 5 min with 2% sulfuric acid, sterilized with 1-2 drops of Tween-20 and 10% commercial bleach for 12 min and then rinsed 3 times with sterile distilled water before sowing to Çiğ and Yılmaz (2017).

### ***Fungus isolation from tubers***

The orchid tubers, washed from the soil in the tap water, were shaken in the sterilized cabinet for 3 min in 3% bleach solution and then rinsed 3 times with sterile distilled water and wiped with sterilized drying papers. 2-3 pieces of tuber, cut into sections with 0.5-1 cm length with scalpel, were planted in PDA petri dishes and incubated for 3-4 days at 24 °C. The PDA medium was prepared with 39 g/l potato dextrose agar dose (Zettler et al., 2001; Sharma et al., 2003) and sterilized for 20 min in an autoclave operating at 121 °C and 1.2 atmosphere pressure.

Developed fungal hyphae were identified on light microscope after being transferred to water agar (WA, 15 g/l) petri dishes and stored in refrigerator in glass tubes. Fungus isolation procedures and identification were done in Mycology Laboratory of the Department of Plant Protection, Faculty of Agriculture at Yüzüncü Yıl University.

### ***Identification of isolates***

Morphological and microscopic features of the fungi incubated in PDA and WA for 7 days at 25 °C were used in identification of the isolates obtained in the study (Barnett, 1965; Booth, 1971; Burgess et al., 1994; Domsch et al., 1980; Gilman, 1959; Singh et al., 1991; Ogoshi, 1975). Identification of *Trichoderma* species was done by using the interactive key <http://nt.arsgrin.gov/taxadescriptions/keys/TrichodermaIndex.cfm> (Samuel et al., 2006).

### ***Composition of the oat medium (OM)***

In order to create the oat medium described by Clements and Ellyard (1979), 2.5 g milled and powdered oat was boiled in 1000 ml of distilled water for one hour and drained from the gauze. The pH of the cooled medium was adjusted to 5.5 and it was autoclaved by adding 7 g/l agar as in the PDA medium.

### ***Seed sowing in symbiotic culture medium***

The fungi used in the study were planted in petri dishes containing PDA in advance and 0.5 cm discs were taken with cork borer when they were 10 days old. Each disk was placed on one side of the petri dish containing OM, which is a symbiotic medium. Petri dishes were incubated in the dark at 24 °C for a couple of days. Seed sowing was done on the other side of the petri dishes where there is no fungus. The petri dishes were kept at  $23 \pm 1$  °C in the dark during germination. The seeds were taken to Van Waes & Debergh (VWD) (1986) culture medium while sub-culturing.

### ***Statistically analysis***

The data were analyzed using the statistical software package SPSS. The means were grouped using the Duncan multiple comparison test (Düzgüneş et al., 1987).

## **Results and discussions**

### ***Fungus isolation and identification***

Six orchid tuber samples/orchid species were used for fungal isolation. The identification of the fungi was done by the references after isolations from tuber

samples. *Rhizoctonia* sp., *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp., *Trichoderma* sp., and *Alternaria* sp. were isolated from orchid tubers. The identification was based on morphological and microscopic features (Table 2).

**Table 2.** Morphological and microscopic features of the fungi isolated from tuber samples

Fungi	Morphological and microscopic features		References
	Colony color in PDA	Shape of conidia	
<i>Rhizoctonia</i> sp.	White/brown	No conidia	Ogoshi, 1975
<i>Aspergillus</i> sp.	Black/green	Spherical	Singh et al., 1991; Barnett, 1965
<i>Fusarium</i> sp.	Pink/orange/purple	Ovoid to elongated (macroconidia and microconidia)	Booth, 1971; Burgess et al., 1994
<i>Penicillium</i> sp.	Green	Ellipsoidal	Domsch et al., 1980; Gilman, 1959; Singh et al., 1991
<i>Trichoderma</i> sp.	Green	Globose, subglobose/ellipsoidal	Samuels et al., 2006
<i>Alternaria</i> sp.	Brown/black	Ovoid/ ellipsoidal	Domsch et al., 1980; Gilman, 1959

*Rhizoctonia* sp. and *Aspergillus* sp. fungi from *Dactylorhiza romana* subsp. *georgica* (1) species; *Fusarium* sp. and *Penicillium* sp. fungi from *Orchis pinetorum* (2) species; *Rhizoctonia* sp. and *Fusarium* sp. fungi from *Orchis spitzelii* (3) species; *Trichoderma* sp., *Fusarium* sp. and *Penicillium* sp. fungi from *Orchis coriophora* (4) species; *Alternaria* sp. fungus from *Orchis collina* (5) species; *Fusarium* sp. fungus from *Orchis simia* (7) species; *Fusarium* sp., *Aspergillus* sp. and *Penicillium* sp. fungi from *Ophrys straussii* (8) species,; *Rhizoctonia* sp., *Fusarium* sp. and *Alternaria* sp. fungi from *Dactylorhiza umbrosa* (9) species (3 isolates); *Fusarium* sp., *Alternaria* sp., *Penicillium* sp. and *Aspergillus* sp. fungi from *Anacamptis pyramidalis* (10) species were isolated, while no fungus was isolated from *Orchis anatolica* (6) species (Table 3).

Fungi were infected not only in the orchid species they were isolated from, but also into the cultures where the seeds of other species were planted. Photographs of symbiotic germination were taken with OLYMPUS SZ61 binocular microscope dp20 camera, 10X x 1.2 software (Fig. 2).

**Table 3.** Orchid species and isolated fungi

Species	Fungi					
	<i>Rhizoctonia</i> sp.	<i>Aspergillus</i> sp.	<i>Fusarium</i> sp.	<i>Penicillium</i> sp.	<i>Trichoderma</i> sp.	<i>Alternaria</i> sp.
<i>Dactylorhiza romana</i> subsp. <i>georgica</i> (1)*	R	As	-	-	-	-
<i>Orchis pinetorum</i> (2)	-	-	F	P	-	-
<i>Orchis spitzelii</i> (3)	R	-	Fo	-	-	-
<i>Orchis coriophora</i> (4)	-	-	Fe	P	T	-
<i>Orchis collina</i> (5)	-	-	-	-	-	Alt
<i>Orchis anatolica</i> (6)	-	-	-	-	-	-
<i>Orchis simia</i> (7)	-	-	F	-	-	-
<i>Ophrys straussii</i> (8)	-	As	Fs	P	-	-
<i>Dactylorhiza umbrosa</i> (9)	R, R1, R2	-	F	-	-	Alt
<i>Anacamptis pyramidalis</i> (10)	-	As	F	P	-	Alt

\*Each plant species was coded with a number

### Symbiotic germination

Isolated fungal isolates were shown like *Rhizoctonia* (R, R1, R2), *Aspergillus* (As), *Fusarium* (F, Fo, Fs), *Penicillium* (P), *Trichoderma* (T) and *Alternaria* (Alt) codes.

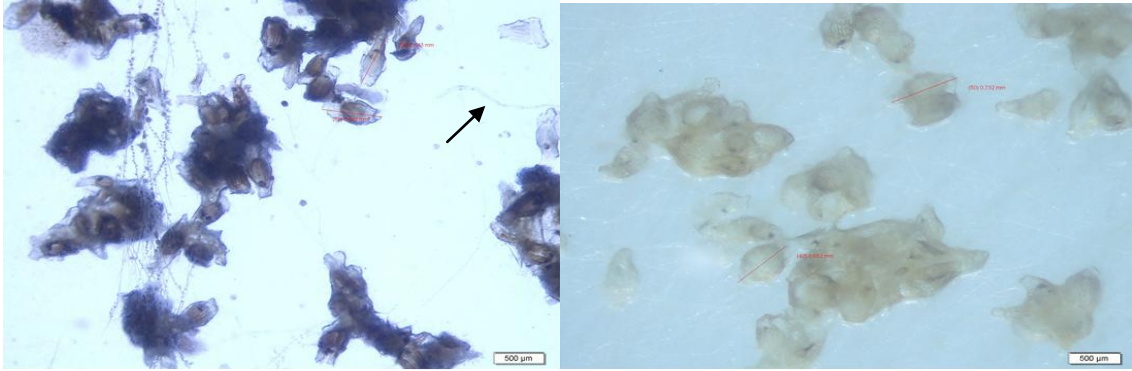
**Table 4.** The effects of isolated fungi on the germination of orchid seeds

Fungi	Germination percentage (%)									
	Species 1	Species 2	Species 3	Species 4	Species 5	Species 6	Species 7	Species 8	Species 9	Species 10
1As							18.18 h		16.66 l	
1R	71.19 a*			45.23 f	20.20 i	6.66 d	34.78 d		53.84 h	18.91 kl
2F				39.47 g	64.00 c		73.91 a	91.60 a	63.79 e	37.23 e
2P				33.33 h				15.38 k	70.86 d	44.44 cd
3Fo	40.00 e		27.77 a	61.53 d	42.64 f			56.25 e	54.85 gh	22.22 hi
3R			16.00 c	48.38 e	41.66 f			42.85 g	54.54 gh	56.00 a
4Fe	26.47 f			41.46 g	15.38 j		35.00 d	83.33 b	60.37 f	
4P		31.57 c			33.33 g		31.25 e		56.49 g	
4T	17.64 g			65.86 c	68.18 b	29.72 b	66.66 b	18.18 j	69.54 d	20.00 jk
5Alt					48.59 e	38.88 a		79.78 c	52.94 h	
7F	55.80 c			27.58 i		14.28 c	25.98 f	57.14 e	79.16 b	28.57 g
8As				36.12 h	27.97 h		37.50 c		37.40 k	42.85 d
8Fs	45.68 d			29.62 i				15.38 k	41.17 j	
8P		78.26 a		60.00 d	62.74 c			20.83 i	50.00 i	
9Alt	37.63 e			66.66 c	66.17 b		37.50 c	15.00 k	68.75 d	53.33 b
9F				80.95 a	27.94 h		25.00 f		17.14 l	44.76 c
9R	45.78 d		20.83 b	83.07 a	18.75 i	38.29 a		30.00 h	61.29 f	17.56 l
9R1				77.77 b	50.00 e			65.38 d	17.64 l	21.05 ij
9R2				66.66 c	58.33 d			11.42 l	76.77 c	
10Alt	63.21 b			59.21 d	15.00 j		22.36 g		74.67 c	7.93 n
10As		48.27 b		13.33 j	10.63 k			9.19 m	41.30 j	23.07 h
10F	57.69 c			67.85 c	75.00 a		12.82 i		93.75 a	34.04 f
10P		34.32 c		40.00 g	16.00 j			50.00 f		12.50 m

\*Means followed by the same letter in the line indicate no statistical difference at 5% level

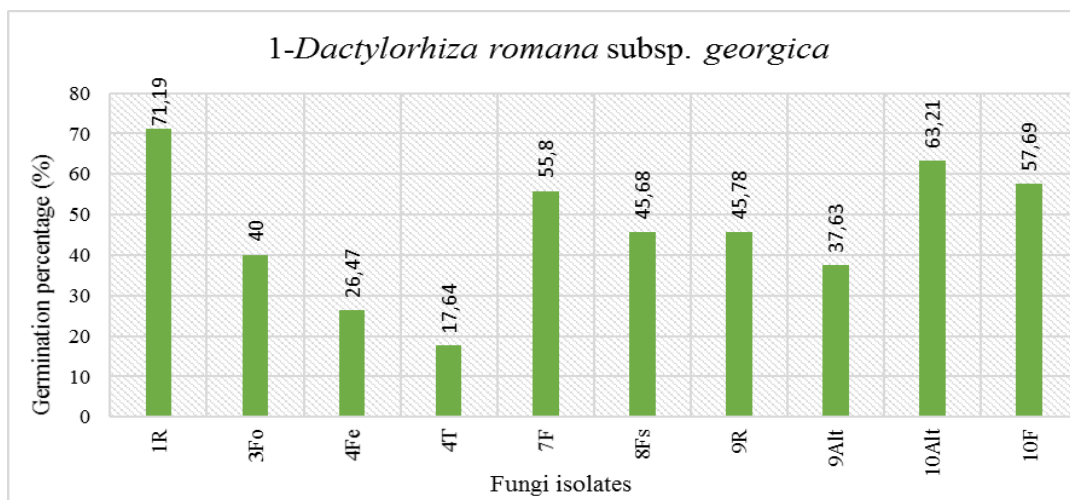
Orchid species 1: *Dactylorhiza romana* subsp. *georgica*, 2: *Orchis pinetorum*, 3: *Orchis spitzelii*, 4: *Orchis coriophora*, 5: *Orchis collina*, 6: *Orchis anatolica*, 7: *Orchis simia*, 8: *Ophrys straussii*, 9: *Dactylorhiza umbrosa*, and 10: *Anacamptis pyramidalis*

Fungal isolates: *Aspergillus* (As), *Alternaria* (Alt), *Fusarium* (F, Fo, Fs), *Penicillium* (P), *Rhizoctonia* (R, R1, R2), and *Trichoderma* (T)

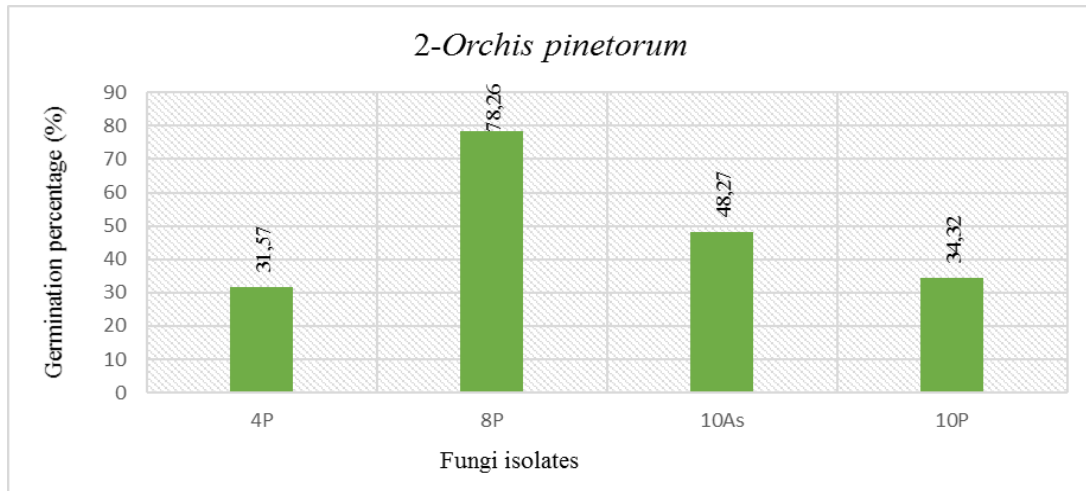


**Figure 2.** Symbiotic germination

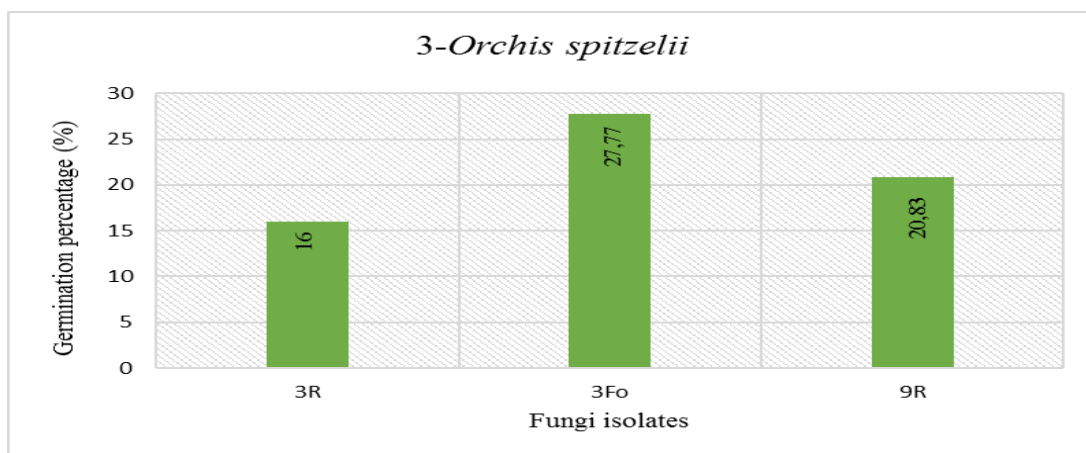
The effects of the isolated fungi on the germination of orchid seeds were found statistically significant ( $p < 0.05$ ) (Table 4). 71.19% germination rate is observed in *Rhizoctonia* (R) fungus obtained from the tuber of *D. romana* subsp. *georgica* (species number 1) (Fig. 3). No germination is observed with the fungi obtained from tubers of species number 2 *O. pinetorum* (Fig. 4), while the impact of R and Fo isolated from the tubers of species number 3 *O. spitzelii* on germination is found as 16% and 27.77%, respectively (Fig. 5). T isolate, isolated from the tubers of species number 4 *O. coriophora*, provided 65.86% germination rate (Fig. 6). From the species number 5 *O. collina*, only sub-culturing is done and the impact of its own seeds on germination was determined as 48.59% (Fig. 7). As no fungus was isolated from the tubers of species number 6 *O. anatolica*, germination is obtained using fungi isolated from tubers of other orchid species (Fig. 8). In the symbiotic germination between the seeds of species number 7 *O. simia* and F fungus isolated from its tubers, a success rate of 25.98% is achieved (Fig. 9). Fs and P fungi isolated from the tubers of species number 8 *O. straussii* had impacts of 15.38% and 20.83% respectively on germination of its own seeds (Fig. 10). R, R1, R2, Alt and F fungi isolated from the tubers of species number 9 *D. umbrosa* had impacts of 61.29%, 17.64%, 76.77%, 68.75% and 17.14% on germination of its own seeds, respectively (Fig. 11). F, Alt, As and P fungi isolated from the tubers of species number 10 *A. pyramidalis* had impacts of 34.04%, 7.93%, 23.07% and 12.5% respectively on germination of its own seeds (Fig. 12).



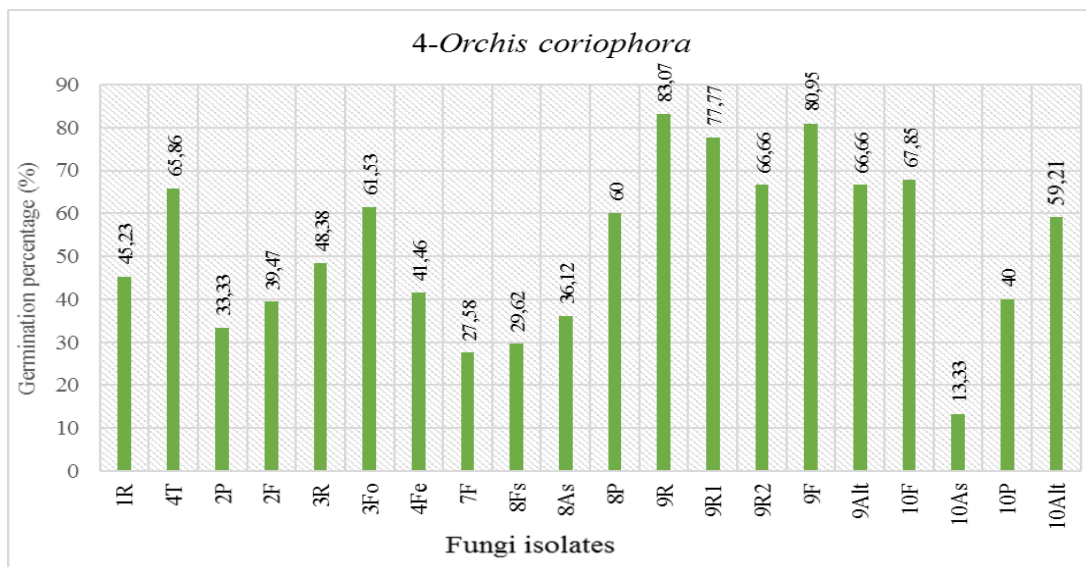
**Figure 3.** Effects on the germination rates of fungi on *D. romana* subsp. *georgica* germination



**Figure 4.** Effects on the germination rates of fungi on *O. pinetorum* germination

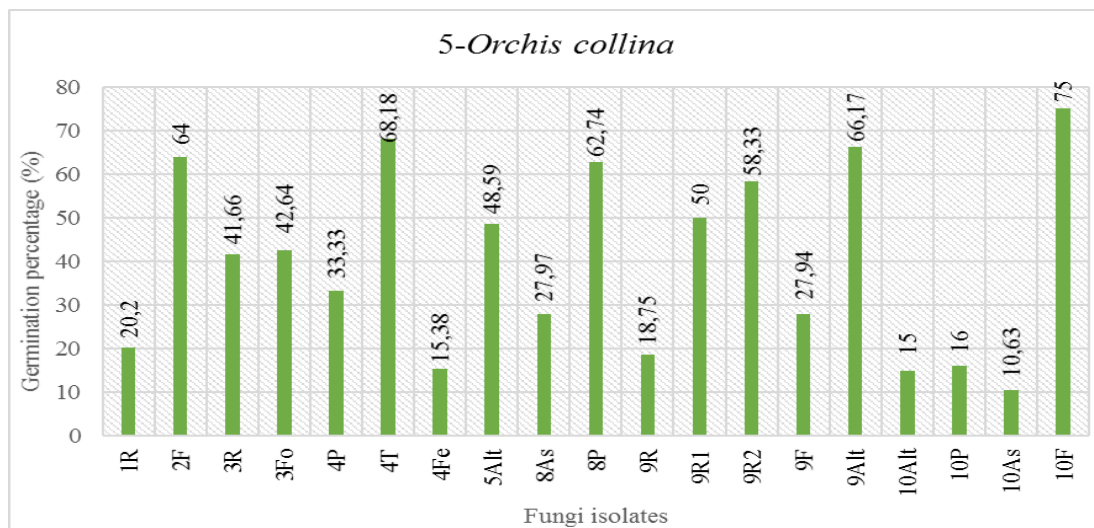


**Figure 5.** Effects on the germination rates of fungi on *O. spitzelii* germination

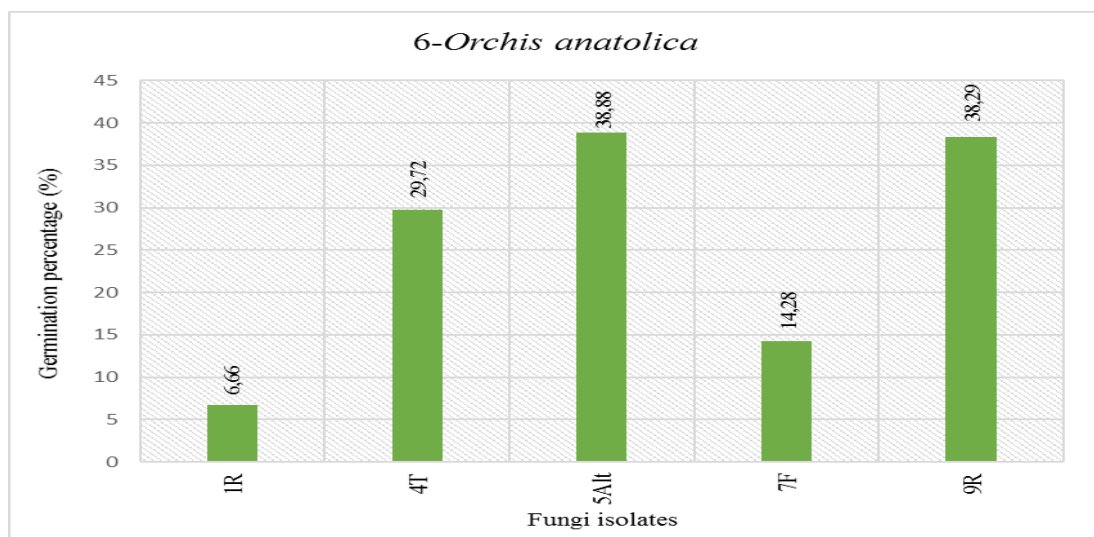


**Figure 6.** Effects on the germination rates of fungi on *O. coriophora* germination

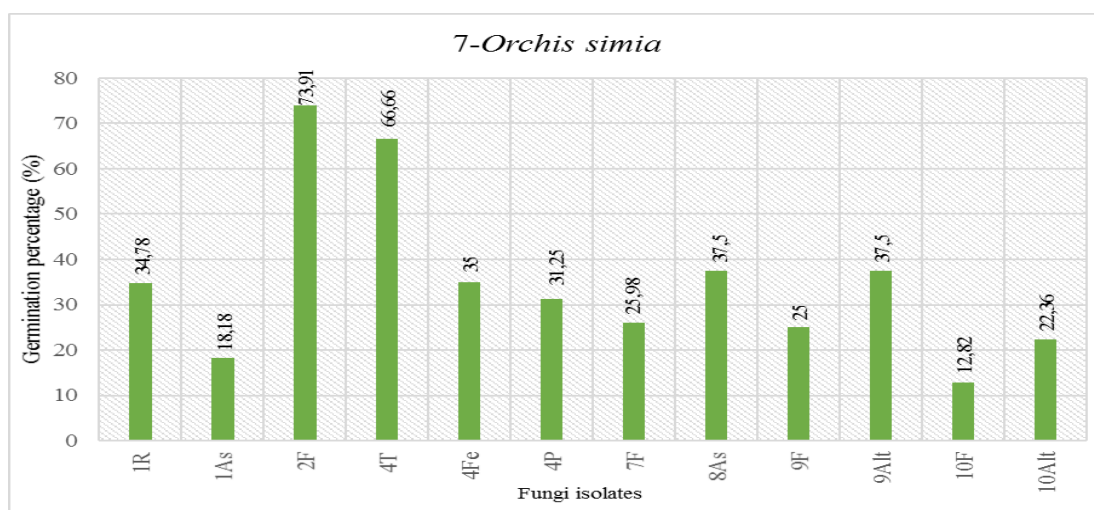




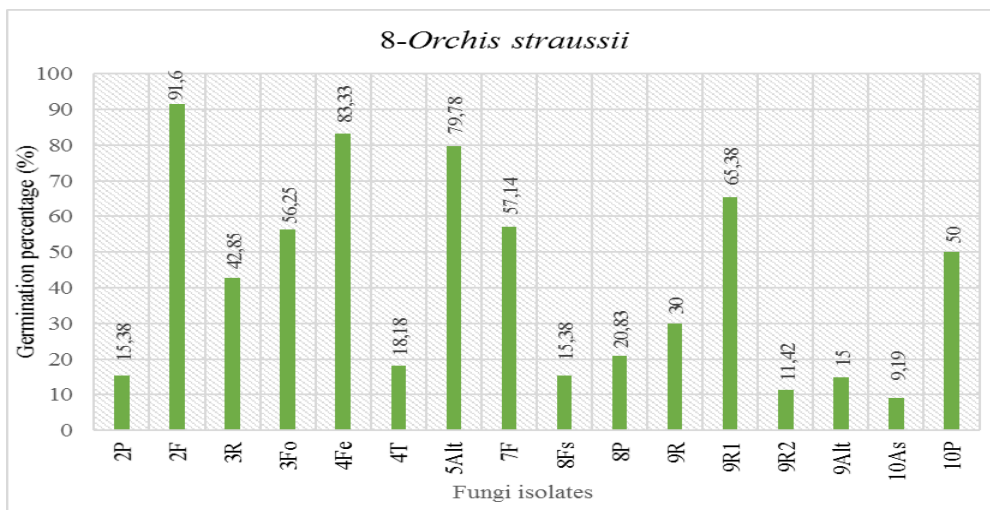
**Figure 7.** Effects on the germination rates of fungi on *O. collina* germination



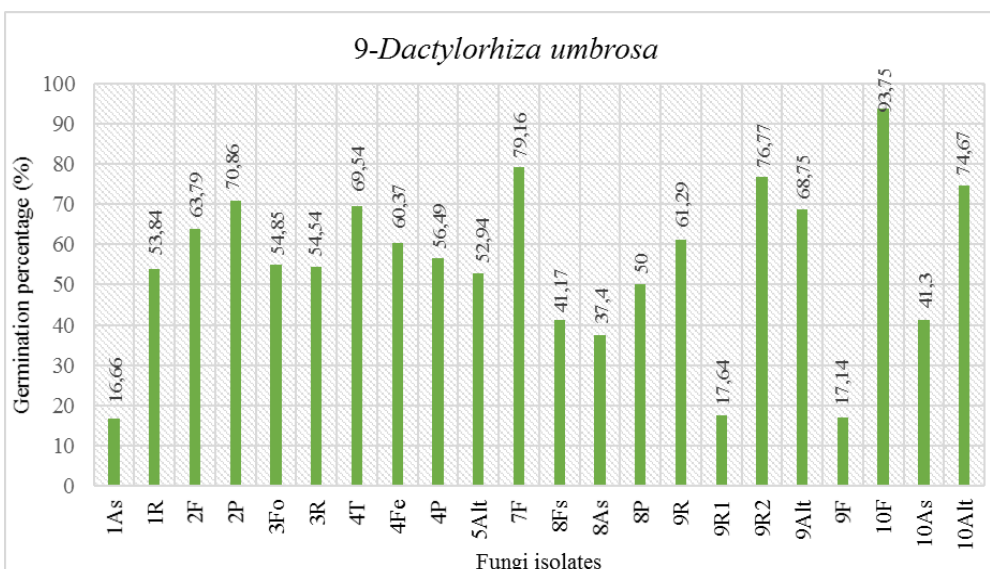
**Figure 8.** Effects on the germination rates of fungi on *O. anatolica* germination



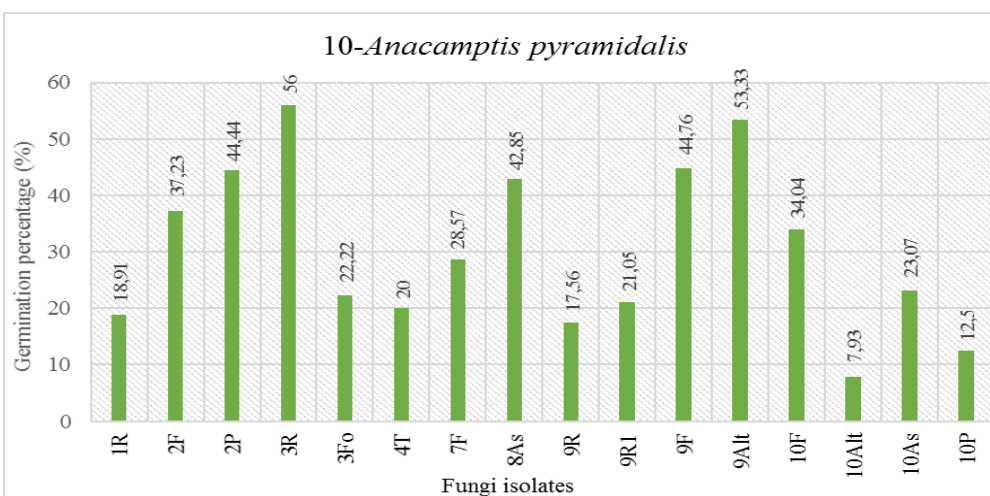
**Figure 9.** Effects on the germination rates of fungi on *O. simia* germination



**Figure 10.** Effects on the germination rates of fungi on *O. straussii* germination



**Figure 11.** Effects on the germination rates of fungi on *D. umbrosa* germination



**Figure 12.** Effects on the germination rates of fungi on *A. pyramidalis* germination

Bernard (1909) stated that the symbiotic relationship between the orchid and the fungus is specific to species, while other researchers reported no such specific relation between a mycorrhizal fungus and an orchid species exists (Burgeff, 1936; Curtis, 1939). In another study, it was determined that fungi isolated from orchid tubers were *Rhizoctonia*, *Corticium*, *Armillaria*, *Fomes*, *Hymenochaeta* species, while *Aspergillus*, *Penicillium*, *Phytophthora* and *Trichoderma* were reported to be initiating and promoting germination (Arditti, 1967).

Clements and Ellyard (1979) reported that the effect of the nutrient medium content on symbiotic culture experiments was important for in vitro germination of orchid seeds and suggested primarily to obtain the most effective *Rhizoctonia* isolate and to prepare the optimal medium for the isolate. In line with this, it has been reported that some fungi have no or little effect on orchid seed germination and the most suitable nutrient medium for controlling parasitic condition is oat medium with additives (Hadley, 1983; Tsutsui and Tomita, 1986). Clements et al. (1986) reported in their study on symbiotic germination of the temperate zone orchids in Europe that *Ceratobasidium*-like fungi are affective on *Dactylorhiza* species and *Tulasnella*-like fungi are affective on *Orchis*, *Ophrys* and *Serapias* orchid species. Researches show that although ground oat added to the symbiotic culture medium used in the studies on *Rhizoctonia* sp. fungus and orchid seed combination has a stimuli effect on germination, amounts above a certain concentration reduces the degree of stimulation. Smreciu and Currah (1989) have shown that the fungal isolates obtained from an orchid species are symbiotic in germination of another orchid species, and even can act in different development stages of the orchid plant. From 19 fungal isolates isolated from different orchid species collected between March and May in the Eastern Mediterranean Region, 8 pieces of *Fusarium* sp., 2 pieces of *R. solani*, 2 pieces of *Macrophomina* sp., 2 pieces of *Trichoderma* sp., 2 pieces of *Pythiaceus* sp. and 2 pieces of *Absidia* sp. fungi are identified (Vakkasoğlu, 1995). 44 out of 47 fungal isolates in a study, in which mycorrhiza forming fungi were isolated from the roots and tubers of various orchid species collected in the Aegean and the Mediterranean Region in April, were identified as *Fusarium*, while 2 were identifies as *Rhizoctonia* and 1 was identified as *Papulaspora* species (Gezgin, 2004). Another study, in which *Fusarium* sp. was isolated in greater proportions than others, was conducted using *Serapias vomeracea* subsp. *laxiflora* and it was determined that they are contaminant (Özkoç, 1991). *Alternaria*, *Aspergillus*, *Fusarium*, *Macrophomina*, *Rhizoctonia*, *Trichoderma* and *Verticillium* fungi species were isolated from eleven orchid species belonging to *Anacamptis*, *Cephalanthera*, *Dactylorhiza* and *Orchis* genus collected in Van province (Çiğ and Yılmaz, 2014). According to the literature, the fungi isolated from orchid tubers in our study stimulate seed germination and plant growth. This is also evident from the results we have obtained. The germination rate varied with species. Not all the orchid species germinated with all fungi. On the contrary, it is observed that one fungal isolate yielding germination success in one species did not achieve the same effect in another one. In some cases, the fungus has not succeeded in germinating the seeds or did not have a high germination rate with the plant it was isolated from. This can be explained by not achieving 100% of the ecological conditions in the aseptic environment and by the special relationship between the orchid species and the fungus. Özkoç and Dalcı (1993) reported that not all the isolates had the same effect on *Orchis laxiflora* seeds cultivated in oat medium (OM) and modified oat medium (MOM) in the presence of 11 fungal isolates brought from different countries and found that two Turkish isolates were

ineffective in germination. In the germination experiment with 11 fungal isolates in *Serapias vomeracea* subsp. *laxiflora* and *Orchis laxiflora* species, it was observed that not all the isolates did have the same effect (Özkoç, 1991). According to the results obtained from our study, the lowest germination rate in species number 1 was 17.64% with T isolate; and the highest germination rate was obtained with R isolate with 71.19%. In species number 2, the lowest and highest germination rates were found as 31.57% and 78.26% respectively with P isolate. In other species, the lowest and highest germination percentages and fungi isolates in which they are symbiotic were obtained as follows: for species number 3 as 16% (R) - 27.77% (Fo); for species number 4 as 27.58% (F) - 83.07% (R); for species number 5 as 10.63% (As) - 75% (F); for species number 6 as 6.66% (R) - 38.88% (Alt); for species number 7 as 18.18% (As) - 73.91% (F); for species number 8 as 9.19% (As) - 91.60% (F); for species number 9 as 16.66% (As) - 93.75% (F) and for species number 10 as 7.93% (Alt) - 56% (R).

Successful germination of *Dactylorhiza iberica* (Bieb. ex Willd.) Soó, *Dactylorhiza umbrosa* (Kar. et. Kir.) Soó and *Orchis palustris* Jacquin species with binucleus *Rhizoctonia* and *Rhizoctonia solani* isolates in the oat medium and the modified oat medium is achieved (Çiğ and Yılmaz, 2017). The overall expected effect of *Rhizoctonia* isolate in orchid germination studies is success, however, as can be seen in our study, *Rhizoctonia* sp. fungus, which plays a role in seed germination of many species, did not have proportionally the highest average germination figure. When we look at the success rates of fungal isolates along with the ones that cannot stimulate germination, the highest average is provided by *Fusarium* isolate by 93.75%. In this case, it is clear that fungal isolates and species give different germination success rates together. According to Salifah et al. (2011), a total of 31 different species of fungus was isolated and inoculated onto *Grammatophyllum speciosum* seed on oat meal agar. The result obtained from the test demonstrated that seed germination rates were best when co-cultured with *Fusarium* sp. number 3. Initial seed germination rates were best when co-cultured with *Fusarium* sp. 3 and *Trichoderma* sp. 2, yielding an increment in 63.3% and 55.7%, respectively when compared to the seeds' original size.

The relationship between orchid species and fungal isolates may vary from symbiosis to parasitism, as some researchers have found that parasitic properties of the fungus can increase and unbalance the relationship between the orchid and fungus by depletion of nutrients in the culture medium and fungus might have a parasitic effect in symbiotic environments with high nutrient content (Tomita and Tsutsui, 1988; Özkoç, 1991). Seeds germinated in oat medium were sub-cultured in VWD culture medium. During this process, fungal isolates have developed to such an extent that encapsulates the whole petri dish due to high nutrient concentration and had parasitic effect on germinating seeds. On the other hand, germinated seeds did not have enough required nutrients to develop in oat medium.

Environmental factors such as relative humidity and temperature were reported to have a profound influence on the infectiveness of a variety of fungi (Ibrahim et al., 2011). According to the researches, growth rate of fungi varies depending on temperature and relative humidity. The optimum growth temperatures for the majority of fungi studied was found to fall from 25 to 30 °C (Sharma and Razak, 2003). For example, the growth of *Fusarium oxysporium* was found to reach its maximum at 30 °C after 7 days of incubation which was drastically reduced below 15 °C and above 35 °C (Farooq et al., 2005). As can be seen, for a successful orchid germination protocol, both fungi and seeds enter into symbiotic life together with the common satisfaction of them.

## Conclusions

As a result of the study, different germination success rates were achieved in each orchid species in the presence of fungal isolates. The idea of using a more diluted media during sub-culturing or even using temperature applications to control fungal growth will be given in the methodology section of subsequent new studies to be undertaken with consideration of culture media in which germination took place. But the most important step that must be taken before anything else is to make a molecular identity in order to reveal the true identity of the isolated fungi.

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