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Effects of Mycorrhiza Isolates on Symbiotic Germination of Terrestrial Orchids (*Orchis palustris* Jacq. and *Serapias vomeracea* subsp. *vomeracea* (Burm.f.) Briq.) in Turkey

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Abstract

The objective of this study was to determine the effects of a number of *Rhizoctonias*, isolated from wild orchids from different parts of the Northeast Anatolia Region of Turkey, on seed germination and protocorm development of *Orchis palustris* Jacq. and *Serapias vomeracea* subsp. *vomeracea* (Burm.f.) Briq. Fifteen binucleate *Rhizoctonia* and one *Rhizoctonia solani* isolates were obtained from *Dactylorhiza*, *Orchis* and *Serapias* in Erzurum, Erzincan and Artvin regions. When *O. palustris* and *S. vomeracea* seeds were inoculated with these isolates, they germinated within 21 days. Germination was slightly higher in *O. palustris* (27%) than in *S. vomeracea* (23%). Among the mycorrhizal fungus isolates tested, two strains (BNR 8-3 and BNR 15-2) gave the highest germination percentage in both species (42%), while uninoculated control seeds of both species showed the lowest germination rate (<1%). Although all of the isolates promoted seed germination in both species, protocorm development was only observed in *O. palustris* seeds inoculated with BNR 8-3 (5.92%), BNR 10-8 (2.34%) and RS 22-2 (2.14%). All of the isolates did not promote protocorm development in *S. vomeracea*.

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1. Introduction

Turkey is one of the most important countries in the world in terms of plant diversity. Studies showed that of 8,745 species of vascular plants found in Turkey, 2,763 are endemic (Davis, 1992). For many botanists, Turkey is a favorite destination for studying wild orchids (Kreutz, 1998). The Anatolia Region in the Northeast part of the country is especially rich in terms of orchid biodiversity. However, most of the orchid species of that region have become extinct or are close to extinction in the wild. Many orchid growing areas of that region have been destroyed by human activities such as agriculture, pasture, mining, logging, roads, industry and settlements, and many species have been reduced to dangerously low levels. Therefore, careful restorative conservation is increasingly needed for this region.

Orchids have dust-like seeds lacking endosperm and are difficult to germinate relative to other plants (Arditti, 1992). Orchids are also unusual in that they consume mycorrhizal fungi as an energy source in a parasitic symbiosis (Clements, 1988; Rasmussen, 1995) to initiate seed germination and seedling development (Arditti et al., 1984). The establishment of orchids in restored habitats requires the presence of mycorrhizal fungi to recruit seedlings (Zettler, 1997). While it is possible to germinate orchid seeds *in vitro* on artificial media containing carbohydrates (Arditti, 1992), symbiotic germination using appropriate fungi is generally more efficient (Rasmussen, 1995).

For examples, growth and development of protocorms after symbiotic germination is significantly accelerated compared to those that germinate asymbiotically (Hadley, 1983; Smreciu and Currah, 1989; Anderson, 1991). Once established on soil, a mycorrhizal relationship also increases plant access to soil resources, thereby increasing its tolerance to environmental stresses (Rasmussen, 1995). The presence of mycorrhizal fungi in the roots of young seedlings may protect seedlings against pathogens (Clements, 1985). Thus, mycorrhizal fungi are often considered essential to achieving success in re-planting of the host plants (Jackson and Mason, 1984).

Seed germination in natural habitats requires the presence of suitable fungi for seedling development and establishment (Zelmer et al., 1996). Until now, symbiotic techniques have not been applied to the terrestrial orchids in Northeast Anatolia Region of Turkey.

Table 1. Original sources of mycorrhizal fungi used in inoculation of orchid seed.

Isolate	Host orchid	Host/collection information
BNR 8-3	*Dactylorhiza urvilleana*	Terrestrial; collected 8 June 2002 from Barhal Valley, Artvin
BNR 8-6	*Dactylorhiza saccifera*	Terrestrial; collected 8 June 2002 from Barhal Valley, Artvin
BNR 10-8	*Dactylorhiza umbrosa*	Terrestrial; collected 8 June 2002 from Barhal Valley, Artvin
BNR 14-4	*Serapias vomeracea* subsp. *vomeracea*	Terrestrial; collected 20 June 2002 from Kafkasor, Artvin
BNR 14-8	*Serapias vomeracea* subsp. *vomeracea*	Terrestrial; collected 20 June 2002 from Kafkasor, Artvin
BNR 15-2	*Serapias vomeracea* subsp. *vomeracea*	Terrestrial; collected 20 June 2002 from Kafkasor, Artvin
BNR 17-4	*Serapias vomeracea* subsp. *vomeracea*	Terrestrial; collected 20 June 2002 from Kafkasor, Artvin
RS 22-2	*Orchis palustris*	Terrestrial; collected 18 June 2002 from Ilica, Erzurum
BNR 24-3	*Orchis palustris*	Terrestrial; collected 18 June 2002 from Kandilli, Erzurum
BNR 26-1	*Orchis palustris*	Terrestrial; collected 18 June 2002 from Askale, Erzurum
BNR 27-1	*Orchis palustris*	Terrestrial; collected 18 June 2002 from Tercan, Erzincan
BNR 28-7	*Orchis palustris*	Terrestrial; collected 18 June 2002 from Altinkent, Erzincan
BNR 30-1	*Dactylorhiza umbrosa*	Terrestrial; collected 20 June 2002 from Borcka, Artvin
BNR 31-6	*Dactylorhiza saccifera*	Terrestrial; collected 20 June 2002 from Borcka, Artvin
BNR 33-4	*Orchis palustris*	Terrestrial; collected 20 June 2002 from Yesildere Village, Erzurum
BNR 35-1	*Orchis palustris*	Terrestrial; collected 20 June 2002 from Gokceyamac Village, Erzurum

In this paper, we describe seed germination techniques for *Orchis palustris* Jacq. and *Serapias vomeracea* subsp. *vomeracea* (Burm.f.) Briq. *in vitro* using orchid mycorrhizal fungi obtained from different areas in the Northeast Anatolia Region of Turkey.

2. Materials and Methods

Seed and fungal collection

Mature, undehisced yellowing capsules of *Orchis palustris* Jacq. from Erzurum and *Serapias vomeracea* subsp. *vomeracea* (Burm.f.) Briq. from Artvin districts in Turkey were collected in June 2002. After collection, seeds were stored in sealed, sterile glass vials at 2°C in total darkness for 5 months.

Sixteen mycorrhizal fungus isolates from the root-like organs of native *Orchis*, *Dactylorhiza* and *Serapias* species in Northeast Anatolia region of Turkey were used to inoculate seeds (Table 1). This is the first study for these isolates used in orchid seed germination. Leaf-bearing specimens with intact root systems were collected, placed in plastic bag and transported to the laboratory at 8–10°C. Small root pieces were washed in running tap water, surface disinfected in 0.5% NaOCl for 1 min, rinsed in sterile distilled water, then placed on water agar (WA) containing streptomycin sulfate (50 mg/l) and incubated at 25°C for 2 to 5 days (Demirci and Döken, 1993). Cultures of *Rhizoctonia*-like fungi were hyphal tipped from water agar to potato dextrose agar (PDA), incubated 10 days at 25°C and then stored at 10°C on PDA slants. Isolates of *Rhizoctonia* obtained in this manner were identified on the basis of characteristics of their vegetative hyphae (Ogoshi, 1975); nuclear conditions (binucleate or multinucleate) were determined using safranin O and 3% KOH according to Bandoni (1979). The isolates were named as BNR (Binucleate *Rhizoctonia*) and RS (*Rhizoctonia solani*).

Seed sowing and fungal inoculation

Seeds were disinfected (surface-sterilized) for 1 min in absolute EtOH: 5.25% NaOCl: deionized (DI) water (1:1:1) followed by three 1-min rinses in sterile DI water were then sown according to the procedure described by Dixon (1987). Approximately 100–250 disinfected seeds were spread onto a 1 × 4 cm filter paper strip (Whatman No. 1). The strip was placed in a Petri dish containing 20 ml of oatmeal agar (2.5 g l^{-1} rolled oats, 7.0 g l^{-1} agar, 1 l DI water), pH of 6.0, autoclaved at 121°C for 15 min and each plate was then inoculated with ca. 1 cm^3 block of agar containing fungal mycelium of a single fungal isolate (5 replicate plates per isolate) and plates without fungal inoculation served as control (5 replicates plates). Petri plates were sealed with Parafilm and exposed to a white light photoperiod (L:D 16h:8h, 30 µmol m^{-2} s^{-1}) at 22±1°C day and night for 90 days. Seed germination was monitored weekly. Seed germination and subsequent development were scored for duration of 5 weeks on a scale of 0–5, where: 0, no germination; 1, production of rhizoids (i.e. germination); 2, rupture of testa of the enlarged embryo; 3, appearance of

promeristem; 4, appearance of first true leaf; and 5, elongation of true leaf and formation of branch roots (Zettler and Hofer, 1998). Data of germination percentage were evaluated by analysis of variance (ANOVA), and multiple comparisons were made using Duncan's multiple range test.

3. Results

Seeds of *O. palustris* and *S. vomeracea* germinated within 21 days of sowing. In general, seeds of *O. palustris* had significantly higher ($P \leq 0.001$) mean percent germination (27.3%) than seeds of *S. vomeracea* (22.9%). Moreover, significantly differences ($P \leq 0.001$) in percent germination were observed among the mycorrhizal fungal isolates. BNR 8-3 (42.5%) and BNR 15-2 (42.1%) gave equally the highest percent germination among the isolates tested, followed by BNR 14-4 (38.4%), RS 22-2 (37.4%) and BNR 8-6 (37.1%), respectively. The lowest seed germination was obtained from uninoculated control (0.7%). Few control seeds incubated in the absence of mycorrhizal fungi germinated, and none of the resulting protocorm developed beyond stage 1.

In *O. palustris*, germination was highest when seeds were inoculated with the RS 22-2 isolate (52.1%), followed by BNR 8-3 (45.3%), BNR 28-7 (38.7%) and BNR 8-6 (37.9%), respectively ($P \leq 0.001$, Table 2). In the control treatment, only 0.85% of *O. palustris* seeds germinated. Advanced seedling development (>stage 3) was achieved for the species with BNR 8-3, BNR 10-8 and RS 22-2 (Fig. 1). Of the stage 3 protocorms observed, all were between 1 and 2 mm in length, spherical in shape and contained numerous encircling rhizoids except in the vicinity of the shoot (Fig. 1). *O. palustris* seedlings harboring three isolates eventually developed leaves suitable for soil transfer *ex vitro* 90 days after sowing (Table 2). Of the three, isolate BNR 8-3 was most effective by boosting 5.9% of seeds to stage 5 (versus 2.3% and 2.1% for BNR 10-8 and RS 22-2, respectively) (Table 2). During incubation under photoperiod, leaves of *O. palustris* seedlings appeared green in color and had presumably initiated photosynthesis.

Seed germination was significantly affected by the inoculation of mycorrhizal isolates in *S. vomeracea* ($P \leq 0.001$, Table 3). The highest initial percent germination (49.1%) was achieved after seeds were inoculated with BNR 15-2, followed by BNR 14-4 (43.6%) and BNR 8-3 (39.7%), respectively. Germination percentages were lower (<10%) for seeds inoculated with BNR 24-3, BNR 27-1 and BNR 31-6 compared with the other isolates. The lowest germination percentage was obtained from uninoculated control treatment (0.5%). None of the fungus isolates promoted development beyond stage 2.

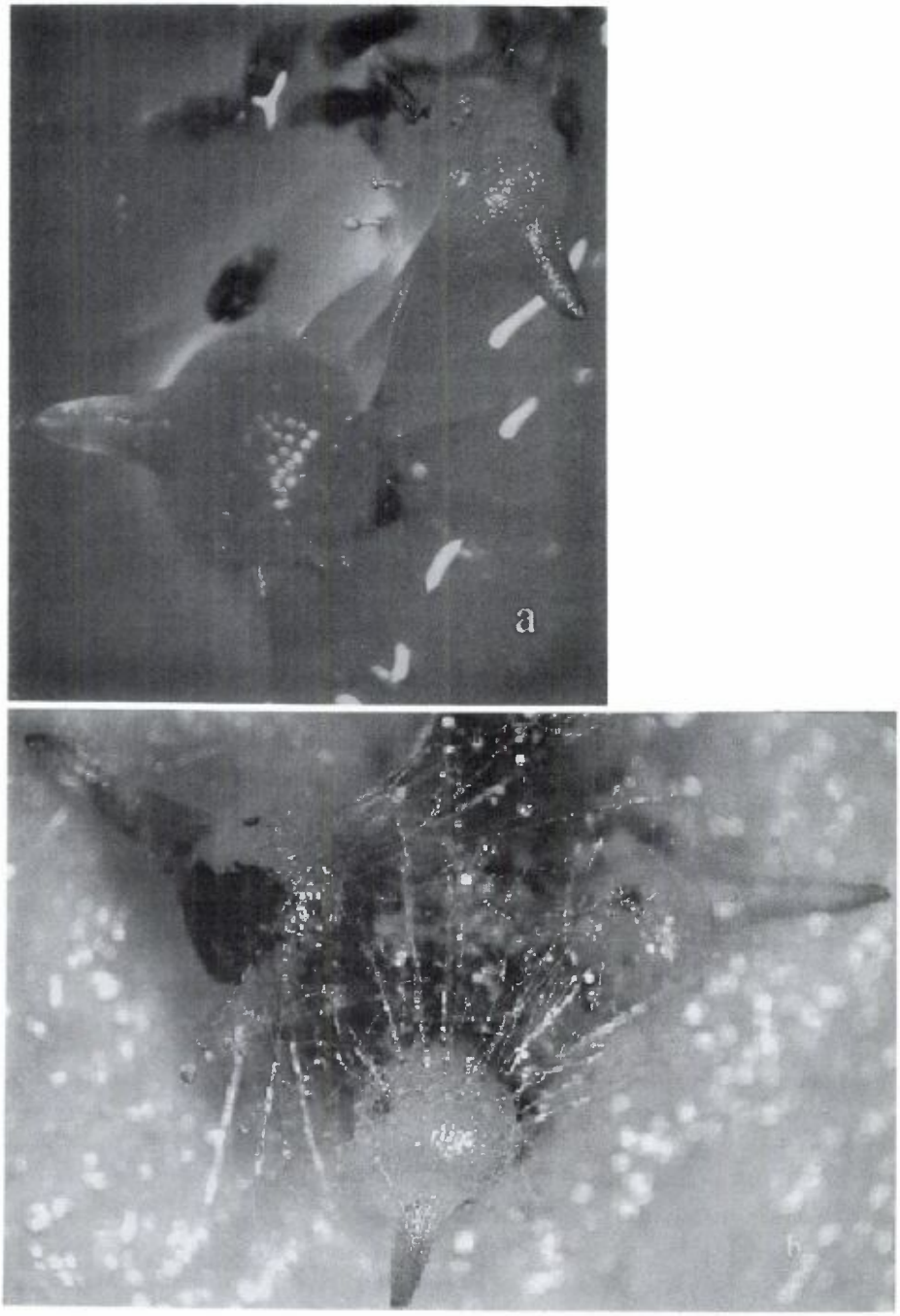

Figure 1. *Orchis palustris* protocorm development in association with a) BNR 8-3 (50×) and b) RS 22-2 (40×), 33 days after sowing.

Table 2. Effects of mycorrhiza isolates on seed germination and development of *Orchis palustris* 90 days after sowing.

Fungal isolates	Total seeds	Stage						% germination	(±SE)
		0	1	2	3	4	5		
BNR 8-3	658	360	12	128	61	58	39	45.28	0.04
BNR 8-6	417	259	2	156	0	0	0	37.88	0.06
BNR 10-8	726	474	0	153	37	45	17	34.71	0.03
BNR 14-4	841	564	4	273	0	0	0	32.94	0.05
BNR 14-8	537	339	0	198	0	0	0	36.87	0.05
BNR 15-2	479	311	7	161	0	0	0	35.07	0.07
BNR 17-4	683	493	9	181	0	0	0	27.81	0.03
RS 22-2	514	246	0	145	44	68	11	52.14	0.06
BNR 24-3	729	653	8	68	0	0	0	9.88	0.02
BNR 26-1	838	739	0	99	0	0	0	11.38	0.03
BNR 27-1	913	787	14	112	0	0	0	13.80	0.03
BNR 28-7	594	364	3	227	0	0	0	38.72	0.05
BNR 30-1	843	734	0	109	0	0	0	12.93	0.05
BNR 31-6	574	453	0	121	0	0	0	21.08	0.04
BNR 33-4	553	382	12	159	0	0	0	30.92	0.06
BNR 35-1	659	513	7	139	0	0	0	22.15	0.03
Control	589	584	5	0	0	0	0	0.85	0.02

4. Discussion

This is the first study to recover mycorrhizal fungi from *Orchis*, *Dactylorhiza* and *Serapias* growing in the Northeast Anatolia Region of Turkey, and the first to successfully utilize these isolates to germinate orchid seeds *in vitro*. Although previously some *Rhizoctonia* isolation was carried out on different plants (Demirci and Döken, 1995; Demirci, 1998; Eken and Demirci, 2003), the present study appears to be the first in which the mycorrhizal fungi isolation from orchids and use it on orchids seed germination for this region. The use of mycorrhizal fungi in our study was found to be an effective means to investigate *in vitro* seed germination and protocorm development of two terrestrial orchids. We found differences among the mycorrhizal fungus isolates on the symbiotic seed germination of *O. palustris* and *S. vomeracea*. Zettler and Hofer (1998) and Stewart and Zettler (2002) used similar methods to investigate seed germination and found that *in vitro* orchid seed germination was highly variable.

Table 3. Effects of mycorrhiza isolates on seed germination of *Serapias vomeracea* subsp. *vomeracea* 90 days after sowing.

Fungal isolates	Total seeds	Stage						% germination	(±SE)
		0	1	2	3	4	5		
BNR 8-3	421	254	0	167	0	0	0	39.67	0.07
BNR 8-6	716	456	1	259	0	0	0	36.31	0.02
BNR 10-8	543	432	3	108	0	0	0	20.44	0.05
BNR 14-4	968	546	5	417	0	0	0	43.59	0.05
BNR 14-8	376	287	0	89	0	0	0	23.67	0.06
BNR 15-2	633	322	3	308	0	0	0	49.13	0.04
BNR 17-4	818	695	0	123	0	0	0	15.04	0.03
RS 22-2	527	408	6	113	0	0	0	22.58	0.03
BNR 24-3	612	558	0	54	0	0	0	8.82	0.06
BNR 26-1	443	336	2	105	0	0	0	24.15	0.05
BNR 27-1	756	689	0	167	0	0	0	8.86	0.07
BNR 28-7	487	332	7	148	0	0	0	31.83	0.04
BNR 30-1	839	683	0	156	0	0	0	18.59	0.04
BNR 31-6	649	595	5	49	0	0	0	8.32	0.03
BNR 33-4	513	408	0	105	0	0	0	20.47	0.05
BNR 35-1	560	463	0	97	0	0	0	17.32	0.06
Control	627	624	3	0	0	0	0	0.48	0.02

In our study, all of the mycorrhizal isolates promoted seed germination in both species; however, protocorm development occurred only in *O. palustris* using the BNR 8-3, BNR 10-8 and RS 22-2 isolates. Studies into the association of orchids with mycorrhizal fungi have revealed that there is a higher degree of specificity between the symbionts than previously assumed (Masahura and Katsuya, 1994; Perkins et al., 1995; Zettler and Hofer, 1998; Markovina and McGee, 2000; Zettler et al., 2001; Stewart and Zettler, 2002). For that reason, the most effective symbiont(s) on seed germination and protocorm development of a given orchid species must be identified so that propagation can be made most effective (Hadley, 1982; Zettler et al., 2001).

Many fungi that form mycorrhizae in orchids belong to the form genus *Rhizoctonia* (Sneh et al., 1991). In this research, we found that a pathogenic strain of *Rhizoctonia solani* (RS 22-2) had mycorrhizal effects on the both species, with isolate RS 22-2 promoting protocorm development in *O. palustris*. Pathogenic *R. solani* isolates have been reported to be mycorrhizal with some orchid species *in vitro* in previous studies (Hadley, 1982; Warcup, 1985).

From this study, we conclude that a number of potential mycorrhizal fungi exist in Northeast Anatolia Region of Turkey, that are capable of supporting

orchid seed germination and protocorm development. This information may be critical for synthesizing a self-perpetuating orchid population. Our successful culture of *O. palustris* and *S. vomeracea* using different fungal isolates (BNR 8-3, BNR 10-8 and RS 22-2) is noteworthy for two reasons. First, these isolates can now be utilized as a means to enhance habitat restoration and conservation. Second, our results suggest that one isolate (BNR 8-3) may be more effective than the others at promoting seed germination and protocorm development.

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