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Symbiotic and Asymbiotic Germination of Endangered *Spiranthes spiralis* (L.) Chevall. and *Dactylorhiza osmanica* (Kl.) Soó var. *Osmanica* (Endemic)

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Abstract: This study reports symbiotic and asymbiotic germination of the seeds of endangered species *Dactylorhiza osmanica* (Kl.) Soó var. *osmanica* (endemic) and *Spiranthes spiralis* (L.) Chevall. Mycorrhizal fungus (Fosm1-*Rhizoctonia solani*) that was isolated from the roots of *D. osmanica* var. *osmanica* was stimulated germination of the seeds of *D. osmanica* var. *osmanica* but caused rowing and browning of protocorms. Whereas the symbiotic association between Fosm1 and the seeds of *S. spiralis* was fully compatible. It was determined that *Fsp. sp. (R. repens)* didn't compatible fungus for germination of the seeds of examined orchids, because of further development of protocorms was no observed. F1 (Binucleate *Rhizoctonia* AG A) was established successfully symbiotic association with the seeds of both *D. osmanica* var. *osmanica* and *S. spiralis* and it was stimulated germination and further development of these orchids protocorms. It was determined germination of the seeds of *D. osmanica* (K.I) Soó' var. *osmanica* and *S. spiralis* (L.) Chevall in two asymbiotic culture media and the effect of inorganic nitrogen on the germination and protocorm development of this orchids seeds. Inorganic nitrogen was enhanced the germination and development of *S. spiralis* seeds in the Van Waes Deberg culture medium but the rate of germination of the seeds of *D. osmanica* var. *osmanica* were reduced. The germination and protocorm development were reduced in Knudson C culture medium.

Key words: *Dactylorhiza osmanica*, *Spiranthes spiralis* symbiotic and asymbiotic seed germination, mycorrhiza

INTRODUCTION

Orchidaceae is one of the largest angiosperm families and the many species occupied variety of habitats globally. Orchids have minute seeds that have tiny undifferentiated embryo lacking of a cotyledon and endosperm (Arditti, 1982). The symbiotic association is relatively unique in the plant kingdom in that the symbiotic fungi of orchid plants seem to acquire no benefits (Smreciu and Currah, 1989; Peterson *et al.*, 1998).

Fungal infection to germinating seeds occurs from suspensor or rhizoid. The infected hyphae form dense coils of mycelium called pelotons in cortical cells (Williamson and Hadley, 1970). There is direct evidence that carbon and other nutrients are transported from fungus to the orchid cell (Rasmussen, 1995).

Most of the fungal partners of terrestrial orchids have been classified as the genus *Rhizoctonia*. The fungi have sterile mycelia with very little tendency to form spores in cultures, are sorted into anastomosis group (Sneh *et al.*, 1991).

Moore (1987) distributed the species within *Rhizoctonia* into several form genera based on numbers

of nuclei per cell, septa pore ultrastructure and suspected teleomorph connection: *Ceratorhiza*, *Epulorhiza* and *Monliopsis*. Several other fungi have also been suggested to be important symbionts of certain orchid species (Zelmer and Currah, 1995).

A delicate balance exists in the symbiotic association between orchids and fungi. In some orchid species, a fungus that once can be a symbiont or parasitic and symbiosis results in mortality of protocorms, when the association is not fully compatible (Hadley, 1970; Ozkoc and Dalci, 1992; Zettler *et al.*, 1999; Shimura and Kocla, 2005). Recently some molecular genetic investigations on orchids and fungi were showed this is not simply a correlation between one orchid and one or several fungi (Taylor and Bruns, 1999).

D. osmanica var. *osmanica* is an endemic species. Unfortunately, 300 000 tuber probably collected from wild habitat and as a result of this species are threatened with extinction.

In a previous study (Ozdener and Ozkoc, 1996), it was determined that the successful symbiotic association between Fosm1 and the seeds of *S. spiralis* were occurred and healthy seedling were observed.

To preserve the rare and threatened orchids, it is necessary to practice propagation methods. Using different media and nutrients, seed germination in a large number of orchid species has been accomplished (Arditti 1982, Arditti and Ernst 1984, Van Waes *et al.*, 1986b). However, methods which are appropriate for one species may not be applicable to others (Arditti, 1982).

It is possible that the differences in responses to inorganic nitrogen are due to the species used in the experiments. Germination was generally much lower on media with inorganic nitrogen. Some investigations were indicated that exogenous nitrogen is not necessary for the first germination stage although it was needed for further development (Van Waes and Deberg, 1986b).

We report here the investigation of a compatible fungus or fungi and successful symbiotic germination of the seeds of *D. osmanica* var. *osmanica* and *S. spiralis*.

Furthermore it was determined that the germination of the seeds of *D. osmanica* var. *osmanica* and *S. spiralis* in two asymbiotic culture media (Knudson C and Van Waes Deberg) and the effect of inorganic nitrogen sources in the Van Waes Deberg and Knudson C media on the germination and protocorm development.

MATERIALS AND METHODS

Plant material: Ripe seeds from desiccated fruits of *S. spiralis*, *D. osmanica* var. *osmanica* were collected from North Anatolia. Seeds were air-dried and then stored in small bottles at 4°C until use. The seeds of these orchids were examined under a light microscope (Leika).

Isolation of fungi from the roots: Healthy root parts of mature plants were selected and were thoroughly washed in running water and section from each root were examined under a microscope for presence of endophytes. Then root segments were surface sterilized for 7 min with 1.5% solution of sodium hypochloride and rinsed thoroughly with sterile distilled water. The roots segments were cut into approximately 0.5 mm sections and placed in a petri dish that contained Warcup's isolating medium described by Clements and Ellyard (1979). The petri dishes were kept in the dark at 25°C. After subculturing on the same medium for pure strains of fungi and maintained on Potato Dextrose Agar (PDA).

Fungi isolation from seed surface: The seeds of *S. spiralis* were packed with silky material and covered with perforated nylon. Twelve packet were prepared and buried to soil at a depth of 2-3 cm. One packet were taken out and investigated microscopically every month. The seeds were placed to isolation medium of Clement's without surface sterilization. *Rhizoctonia* like fungi were

isolated. The resulting fungal isolates were maintained as pure cultures and were evaluated for their ability to induce symbiotic germination.

Identification of *Rhizoctonia*: Nuclei were stained with safranin-O according to Bandoni (1979). *Rhizoctonia*-like fungi were identified according to Sneh *et al.* (1991).

Seed viability test: Seed viability was established according to the tetrazolium method of Van Waes and Deberg (1986a). Orchid seeds were soaked for 20 min in 5% (w/v) Ca(OCl)₂ and 1% Tween 80 and then rinsed three times in sterile distilled water and allowed to stand in water and for 24 h. Seeds were then soaked in 1%TTC (2,3,5-triphenyltetrazoliumchloride)for 24 h and rinsed three times in sterile water to remove excess TTC. Seeds were observed under a Nikon stereo microscope and red-pink embryos were scored as viable.

Seed surface sterilization: Seed lots of approximately 30 mg of each species were pretreated with 1.5% solution of sodium hypochloride containing 0.1% Tween 80 during 10, 15, 20, 30, 40, 50 and 60 min then were rinsed three times with sterile distilled water.

Symbiotic seed germination: At appropriate condition of surface sterilization, surface sterilized seeds of *D. osmanica* var. *osmanica* and *S. spiralis* were sown in culture tubes that contained 25 mL modified oat medium (Clements *et al.*, 1985). After sowing, each tube was inoculated with a 5 mm diameter block of fungal inoculum.

Three tubes were prepared for each fungus treatments and three replicates were treated for each fungal isolate. Control tubes were not inoculated with any fungal isolate. All culture tubes were kept at 25°C in dark for one month and then incubated under 16 h photoperiod from daylight fluorescent lamps (120 µM) and 25±2°C for 3 months.

Asymbiotic seed germination: In this study, Van Waes Deberg BM and Knudson C media were used for to determine the effect of culture media on the germination. The effect of inorganic nitrogen on the germination and further protocorm development was determined by removed inorganic nitrogen sources [(NH₄NO₃(NH₄)₂SO₄ and KNO₃ from VWDBM;(NH₄)₂SO₄ from Knudson C] (Arditti, 1982; Van Waes and Deberg, 1986b). The sterilise seeds were sown on culture tubes containing 10 mL of Knudson C and Van Waes Deberg media and modification without inorganic nitrogen of these media.

The tubes were incubated at 25±2°C and in dark during 3 months. Observation on germination were made each week.

Throughout all experiments, 25 mm × 150 mm culture tubes containing 15 mL solid medium were used. Each tube contained 50-200 seeds because regulated sowing was impossible on account of very minute seeds. In all experiments each treatment was replicated five times.

The some protocorms that occurred in VWDBM were transferred to fresh prepared VWDBM and VWD without inorganic nitrogen (VWD-N). Furthermore the protocorms in VWD-N were transferred to WVD. BM and VWD-N. Three protocorms were placed to each tube and this application was also replicated five times. Cultures were subjected to 16 h of light, 8 h of dark at 25°C during 3 months.

Seed germination, protocorm and seedling development were recorded after incubation for month as: (1) = no germination; (2) = top shaped protocorm; (3) = elongation of the leaf primordium and enlargement protocorm; (4) = formation of root and leaflet.

Statistics: For evaluation of seed germination and developmental stages General Linear Model ANOVA procedure was used. (SPSS 10.0 for Windows: Anon, 1998).

RESULTS

Seed morphology and viability: The seeds of *S. spiralis* and *D. osmanica* var. *osmanica* were shown in Fig. 1A and B, respectively.

The seed viability, stained by TTC was found as 64% in the seed of *D. osmanica* var. *osmanica* and 96% in those of *S. spiralis*. The results of viability were agreed with those of germination of the seeds of each orchids.

Fungal isolation and identification: In cross sections, in cortical cells of the roots of *S. spiralis* were observed alive and digested pelotons. This fungal isolate was coded as Fsp. sp. and it was determined that Fsp. sp. is *R. repens* (Fig. 2A).

Fosm1 was identified as *R. solani* previously but in this study no anastomous group of this isolate was determined (Fig. 2B).

F1 which was isolated from surface of seeds was identified as binucleate *Rhizoctonia*, AG A

Surface sterilization: The effect of surface sterilization duration on germination was examined by sterilizing the seeds for 10, 15, 20, 30, 40, 50, 60 min in solution of 1.5% sodium hypochloride. The highest rate of germination was received at 10 and 30 min. *S. spiralis* and *D. osmanica* var. *osmanica* and the rate of germination were 86% and 15.6%, respectively.

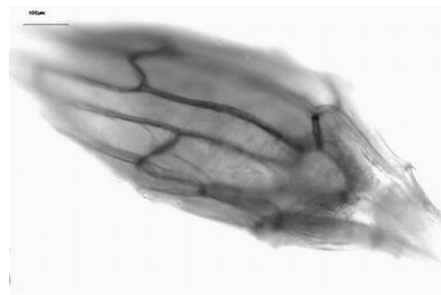


Fig. 1A: The seed of *S. spiralis*

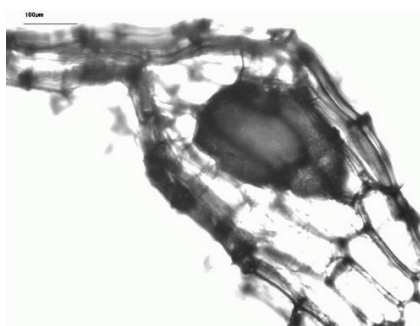


Fig. 1B: The seed of *D. osmanica* var. *osmanica*

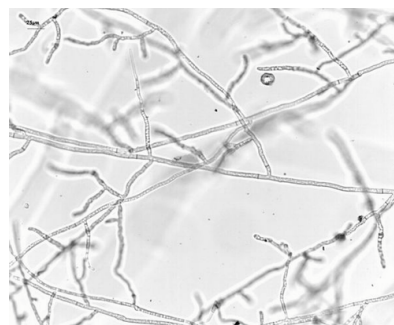


Fig. 2A: Fungal hyphae of F sp.sp. (*R. repens*) from *S. spiralis*

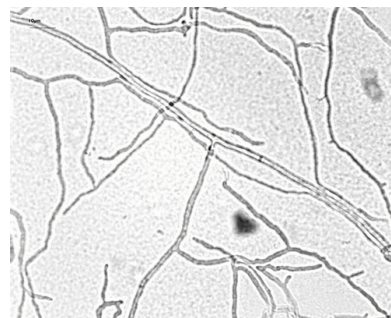


Fig. 2B: Fungal hyphae of Fosm1 (*R. solani*) from *D. osmanica* var. *osmanica*

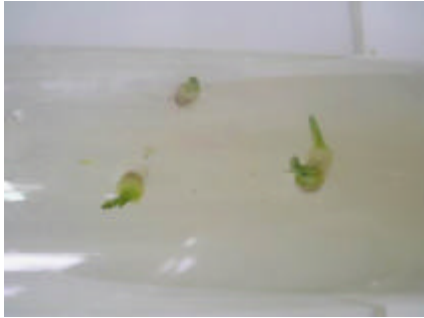


Fig. 3: The seedling developed from the seeds of *D. osmanica* var. *osmanica* which was inoculated with F1. (The seedling's height on the left measure 1 cm)

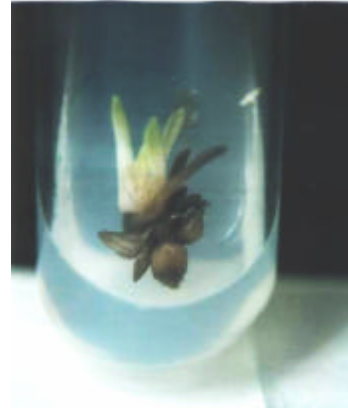


Fig. 5A: The seedling from the protocorm of *D. osmanica* var. *osmanica* which was transferred from VWD-N from VWDBM (the seedling is 2.6 cm)

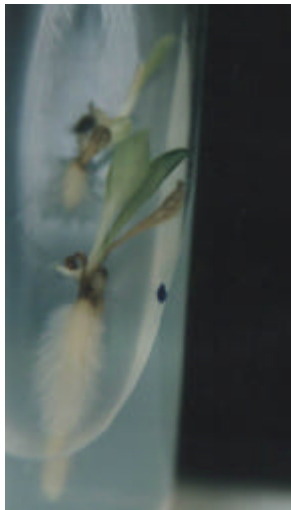


Fig. 4A: The seedling from the protocorm of *S. spiralis* which was transferred from VWD-N to VWDBM. (The longest seedling is 4.5 cm)



Fig. 5B: The seedling from the protocorm of *D. osmanica* var. *osmanica* which was transferred from VWD-N from VWD-N (The seedling is 0.7 cm)

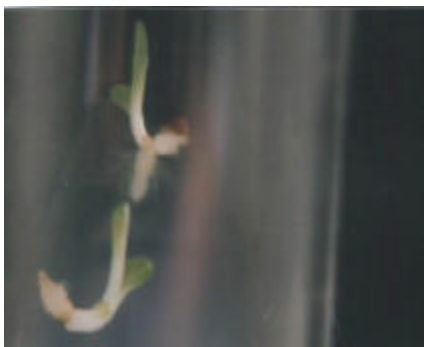


Fig. 4B: The seedling from the protocorm of *S. spiralis* which was transferred from VWD-N to VWD-N. (The seedling on the upper is 1 cm)

(Data are not evaluated statistically). Prolonged sterilization is rather detrimental for the germination of *S. spiralis* and *D. osmanica* var. *osmanica*.

Symbiotic germination: Fosm1 that isolated from *D. osmanica* var. *osmanica* was stimulated the germination of the seeds of these orchids but protocorms were no further developed and browning and rot of the protocorms were occurred.

In a previous work, it was determined that Fosm1 was stimulated germination and further development of the seeds of *S. spiralis*, as compared to the effect of Fsp. sp. and F1, it was repeated with *R. solani*, in this study. The rate of germination was also found as 86% in this study (Ozdener and Ozkoc, 1996).

Fsp. sp. was stimulated the germination of the seeds of both *S. spiralis* and *D. osmanica* var. *osmanica* but no further protocorm development were observed (Table 1 and 2).

F1 (Binucleate *Rhizoctonia* AG A) and Furv (*Rhizoctonia repens*) were established successful

Table 1: Statistical analysis of species × fungal interaction

Source	Sum of squares	df	Mean square	F	Sign.
Species	5877.000	1	5877.200	526.725	0.000**
	1705.548	1	1705.548	245.367	0.000
	2048.480	1	2048.480	354.306	0.000
	110.592	1	110.592	148.247	0.000
Fungi	5980.085	4	1495.021	133.986	0.000
	8947.218	4	2236.804	321.796	0.000
	4704.505	4	1176.126	203.423	0.000
	226.638	4	56.660	75.951	0.000
Species × Fungi	7375.148	4	1843.786	165.243	0.000
	3169.282	4	792.320	113.987	0.000
	5010.228	4	1252.557	216.643	0.000
	226.638	4	56.660	75.951	0.000

There were statistically significant differences between the studied species and fungal isolates. In addition to this species × fungal isolate interaction was also statistically significant (**p<0.01)

Table 2: The germination and developmental stages of the seeds of *S. spiralis* 3 replicate were tested for each treatment and each replicate contained 3 tubes and each tube 50-100 seeds.

Fungi	Developmental stages			
	Stage 1	Stage 2	Stage 3	Stage 4
Control	100.0±0.875a	0.0±0.832e	0.0±0.839e	0.00
Fosm1	14.0±0.875d	20.0±0.832d	65.86±0.839a	0.00
Furv	24.0±0.875c	44.0±0.832c	32.0±0.839b	0.00
F1	29.7±0.875bc	55.8±0.832b	14.5±0.839c	0.00
Fsp.sp	38.0±0.875b	58.0±0.832a	4.0±0.839d	0.00

Mean followed by the same letter are not significantly different at the 0.05 level Tukey's HSD test

Table 3: The germination and developmental stages of the seeds of *D. osmanica* var. *osmanica* 3 replicate were tested for each treatment and each replicate contained 3 tubes and each tube 50-100 seeds.

Fungi	Developmental stages			
	Stage 1	Stage 2	Stage 3	Stage 4
Control	79.96±0.850a	20.03±0.484c	0.00±0.259c	0.00±0.315c
Fosm1	0.00±0.850d	0.00±0.484e	0.00±0.259e	0.00±0.315c
Furv	62.90±0.850b	9.00±0.484d	14.00±0.259b	14.10±0.315a
F1	54.83±0.850bc	21.33±0.484b	19.73±0.259a	5.00±0.315b
Fsp.sp	47.96±0.850c	52.03±0.484a	0.00±0.259d	0.00±0.315c

Mean followed by the same letter are not significantly different at the 0.05 level Tukey's HSD test

Table 4: Analysis of variance of species × culture media interaction

Source	Type III sum of squares	df	Mean square	F	Sig.
Culture media	23690.05	3	7896.683	447.540	0.000**
Species	7640.802	1	7640.802	433.038	0.000**
Culture medium × Species	1909.837	3	636.612	36.080	0.000**

There were statistically significant differences between the studied species and culture media. In addition to this species × culture media interaction was also statistically significant (** p<0.01)

symbiotic association with the seeds of *D. osmanica* var. *osmanica* and *S. spiralis*. F1 and Furv were stimulated germination of the seeds of *D. osmanica* var. *osmanica* and the protocorms were grown to developmental stage 4 (Fig. 3). Also *S. spiralis* protocorms were developed to stage 3 with Furv and F1 (Table 1 and 2).

Asymbiotic germination: The germination of the seeds of each orchid were higher rate in Van Waes Deberg medium than Knudson C medium. The protocorms that occurred in KCBM an KC-N were poor, very small and unhealthy. There were statistically significant differences between culture media and orchid species (Table 3). Furthermore significant differences were also observed among culture media (between VWDBM; VWD-N; KCBM and KC-N) (Table 4).

Inorganic nitrogen was stimulated both germination and protocorm development of the seeds of *S. spiralis*.

On the contrary the germination of the seeds of *D. osmanica* var. *osmanica* was reduced in VWD-N. Inorganic nitrogen was also stimulated the protocorm development of *D. osmanica* var. *osmanica*.

The protocorms of *S. spiralis* and *D. osmanica* var. *osmanica* that transferred from VWD-N to VWDBM were developed to healthy seedlings (Fig. 4A and B).

When the protocorms of these orchids in VWD-N were transferred to fresh prepared VWD-N was not developed to healthy seedlings (Fig. 5A and B).

DISCUSSION

There were related with seed scored as viable and the percentage of symbiotic germination for each orchid species.

In spite of Fosm1 (*R. solani*) was isolated from *D. osmanica* var. *osmanica* once, it was stimulated

germination of the seeds of this orchid, then browning and rot of the protocorms was occurred. Fosm1 is not a compatible fungus for germination of the seeds of *D. osmanica* var. *osmanica*. Similarly Fsp. sp. is also not a compatible fungus for *S. spiralis* seeds. Because $4\pm 0.839\%$ of protocorms were grown to developmental stage 3, whereas the higher rate of protocorms were developed stage 3 with Fosm1, Furv and F1 (65.86 ± 0.839 , 32.0 ± 0.839 , 14.5 ± 0.839 , respectively Table 2). Isolates from one species of orchid do not necessarily stimulate seed germination or further development of protocorms of the same species. In some orchid species, a fungus may parasitic or ineffective and resulted in high mortality of germinated seeds, when the association is not compatible (Hadley, 1970; Beyrle *et al.*, 1995; Zettler *et al.*, 1999; Shimura and Koda, 2005).

It is possible that Furv (*R. repens*) and F1 (Binucleate *Rhizoctonia* AG A) is also compatible for germination of the seeds of *S. spiralis*.

Furv and Fsp. sp. was identified as *R. repens* but Furv was more pronounced the rate of germination and protocorm development than Fsp. sp. (Table 2 and 3). It is possible that anastomous groups of Furv and Fsp. sp. is distinct.

We think that Furv and F1 are fully compatible fungi for *D. osmanica* var. *osmanica* seeds because of additional protocorm growth was observed after inoculation with these isolates (Table 3).

A successful symbiotic association between same orchid and fungus or that isolated from the roots of an orchid or from soil in the surrounding of the plant is required for growth and survival of transplants of orchid seedlings in wild habitat (Rasmussen, 1995; Sharma and Tandon, 1987; Shimura and Koda, 2005).

In asymbiotic cultures culture medium and inorganic nitrogen were effected the germination of the orchid seeds. Knudson C medium was not appropriate for *D. osmanica* var. *osmanica* and *S. spiralis* seeds. In this medium (basal medium or without inorganic nitrogen), the weak, unhealthy protocorms were occurred and no further development was occurred. Knudson C basal medium was found better for asymbiotic germination of some epiphytic orchids (Sharma and Tandon, 1987). The nutritional status requirements of orchid seeds for germination varies due to their physiological state (Kumaria and Tandon, 1991).

Inorganic nitrogen were stimulated both germination and protocorm development of the seeds of *S. spiralis*. Inorganic nitrogen was stimulated the protocorm development of *D. osmanica* var. *osmanica*, but the germination were reduced in VWD-N. Several investigators attribute the low germination of European orchids to the presence of inorganic nitrogen in the culture medium (Mead and Bulard, 1979; Van Waes and

Deberg, 1986b). The very high germination percentage on the media with contains no inorganic nitrogen notable, indicating that exogenous nitrogen is not necessary for the first germination stage although it was needed for further development (Van Waes and Deberg, 1986b).

Our results of germination and protocorm transfers (between culture media with or without inorganic nitrogen) were showed inorganic nitrogen was necessary for further protocorm development. In asymbiotic germination studies the best culture medium or media and the necessity of inorganic nitrogen for seed germination and protocorm development should be determined.

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