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Diversity of endophytic fungi from various Aegean and Mediterranean orchids (saleps)

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Abstract: The diversity and host specificity of endophytic and *Rhizoctonia*-like fungi were investigated in orchids from the Aegean and Mediterranean regions. Endophytic fungi from various Aegean and Mediterranean orchids (*Anacamptis pyramidalis* (L.) L.C.M.Richard, *Orchis sancta* L., *Ophrys fusca* Link., and *Serapias vomeracea* subsp. *orientalis* Greuter) were isolated and identified partially. Surface sterilisation of roots and tubers was carried out in laminar airflow under aseptic conditions. Several modified methods for the isolation of symbiotic fungi from orchid roots and tubers were used. Most of the orchid endophytes isolated was found to be *Fusarium* Link ex Fr. spp. A total of 47 isolates, having genus characterisations as 44 (94%) isolates belonging to the genus *Fusarium*, 2 (4%) isolates belonging to the *Rhizoctonia* DC. ex Fr.-like fungi, and 1 (2%) isolate belonging to the genus *Papulaspora* Preuss, were found from the orchid root and tubers. Endophytic *Fusarium* spp. were isolated from Aegean and Mediterranean orchids *Anacamptis pyramidalis, Orchis sancta, Ophrys fusca,* and *Serapias vomeracea* subsp. *orientalis. Rhizoctonia*-like fungi were only isolated from *Orchis sancta* whereas *Papulaspora* sp. was only isolated from *Anacamptis pyramidalis.*

Key words: Aegean and Mediterranean orchids, orchid endophytes, *Rhizoctonia*-like fungi, *Fusarium, Papulaspora*

Çeşitli Ege ve Akdeniz orkidelerindeki (salep) endofitik fungus çeşitliliği

Özet: Bu çalışmada, Ege ve Akdeniz orkidelerinden (salep) izole edilen endofitik ve *Rhizoctonia*-benzeri fungusların konak spesifitesi ve dağılımı ilk kez incelenmiştir. Ege ve Akdeniz orkidelerindeki (*Anacamptis pyramidalis* (L.) L.C.M.Richard, *Orchis sancta* L., *Ophrys fusca* Link., *Serapias vomeracea* subsp. *orientalis* Greuter) endofitik fungusların izolasyonu ve kısmi identifikasyonu yapılmıştır. Kök ve yumruların aseptik koşullar altında laminar flow içerisinde yüzey sterilizasyonu yapılmıştır. Orkid kök ve yumrularından simbiyotik fungusların izolasyonu için bazı modifiye yöntemler kullanılmıştır. İzole edilen orkid endofitlerinin çoğunun *Fusarium* Link ex Fr. spp. olduğu bulunmuştur. Orkid kök ve yumrularından elde edilen toplam 47 izolatın, 44'ü *Fusarium* genusu (% 94), 2'si *Rhizoctonia* DC. ex Fr. -benzeri fungus (% 4) ve 1'i *Papulaspora* Preuss genusu (% 2) olarak tanımlanmıştır. Endofitik *Fusarium* türleri Ege ve Akdeniz orkidelerinden *Anacamptis pyramidalis, Orchis sancta, Ophrys fusca, Serapias vomeracea* subsp. *orientalis*'den izole edilmiştir. *Rhizoctonia*-benzeri funguslar sadece *Orchis sancta*'dan izole edilirken *Papulaspora* sp. sadece *Anacamptis pyramidalis*' den izole edilmiştir.

Anahtar sözcükler: Ege ve Akdeniz orkideleri, orkid endofitleri, *Rhizoctonia*-benzeri fungi, *Fusarium, Papulaspora*

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Introduction

Orchids are one of the largest families of monocotyledonous plants and can be found in diverse habitats. Some peculiarities of their biology, e.g. their ability to live in symbiosis with fungi, help them to adapt to different environmental conditions. Interactions between orchids and mycorrhizal fungi are essential for germination of their tiny seeds (Benzing & Friedman, 1981; Rasmussen et al., 1991; McCormick et al., 2006; Tsavkelova et al., 2008). The rapid loss of native orchid habitat throughout ecologically important areas has prompted researchers to develop appropriate plans for the propagation and reintroduction of many native orchid species (Stewart & Kane, 2006, 2007).

Orchid mycorrhizas are endomycorrhizas formed between plants of the *Orchidaceae* and basidiomycetous, or rarely ascomycetous, fungi often of the form-genus *Rhizoctonia*. The fungi form intracellular coils or less regular hyphal aggregates within host tissue and these structures are known as pelotons. There is no doubt that heterotrophic orchid seedlings and adult plants of achlorophyllous species are dependent on a mycorrhizal association for supply of carbohydrate and possibly other nutrients. Under natural conditions, orchid seeds do not successfully germinate and the protocorm does not develop without being infected by the mycorrhizal fungus. Early in the photosynthesis phase this transfer of carbon ceases and, with respect to carbon, mature green plants appear, independent of their fungal partner (Alexander & Hadley, 1983, 1984; Clements, 1988; Zemleret et al., 1996).

Rhizoctonia spp. are well known as widely distributed pathogens, saprophytes, and mycorrhizal fungi of orchids. The species have been isolated from plant roots, leaves, and stems, and have been found in various habitats including cultivated land and natural forests (Masuhara & Katsuya, 1994). Non-pathogenic representatives of the ascomycetous genus *Fusarium* have been also reported as endophytes. Isolates of the species *F. aquaeductuum* and *F. solani* were found in the roots of the tropical palm tree *Licuala ramsayi* (Rodrigues & Samuels, 1990), *F. solani* and *F. oxysporum* isolates in the epiphytic orchid *Epidendrum stangeanum*, and *F. oxysporum* isolates in the roots of the terrestrial orchid *Platanthera praeclara* (Arditti & Pridgeon, 1997; Tsavkelova et al., 2008).

Symbiotic seed germination techniques represent an efficient way to promote the orchid–fungus association under in vitro conditions and to study in vitro orchid–mycobiont specificity (Kulinov & Filippov, 2001; Stewart & Kane, 2007). For symbiotic germination studies it is required to determine mycorrhizal specificity. It is very important for the conservation of threatened orchid taxa.

The present study aimed to investigate both the diversity and the host specificity of *Rhizoctonia*-like fungi for the first time from orchids of the Aegean and Mediterranean regions. Within the scope of this study, other endophytic fungi from orchids in the Aegean and Mediterranean regions were also isolated and partially characterised.

Materials and methods

Orchid species and study sites

Eighty-three orchid samples belonging to 5 species were collected from the Aegean and Mediterranean regions (Figure 1). The samples originated from 3 different locations, with flowering time in April and May in 2002-2003 (Table 1) and were stored in polyethylene bags at +4 °C. Identification of orchid species was done according to Davis (1984) at Ege University Botanical Garden Herbarium Research and Application Centre, Turkey.

Isolation of endophyte and mycorrhizal fungus

Roots and tubers were washed under water to remove the soil remnants and the surface was sterilised with 70% ethanol for 2-3 min and then with 20% of a sterilisation solution (20 mL of 5% NaOCl and 0.01% Tween 20 and sterile distilled water) for 2 min. All the sterilisation protocols were carried out in laminar air flow under aseptic conditions (Bayman et al., 1997; Otero et al., 2002). The pieces of each root were plated on Fungal Isolation Medium (FIM) in petri plates (Clements et al., 1986) and they were also plated on Murashige and Skoog media (MS) in a specific jar of plant tissue culture. The conditions of tissue culture were a photoperiod of 16 h light/16 h dark, and the light intensity was 3500-4000 lux (Murashige & Skoog, 1962). After the incubation, the fungal colonies chosen both from the petri plates and from the jars were transferred to Malt Extract Agar (MEA, Oxoid) slants and the pure cultures of fungal isolates were preserved +4 °C for further investigation.

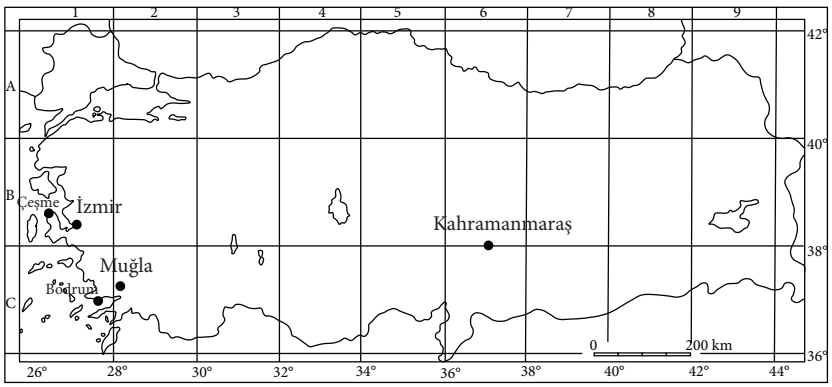

Figure 1. Collection areas of orchid species in Turkey.

Table 1. Orchid species, study sites, and number of samples of Aegean and Mediterranean orchid (salep) species.

Location	Orchid species	Number of Samples
Çeşme-İzmir	*Anacamptis pyramidalis*	6
Çeşme- İzmir	*Orchis sancta*	19
Çeşme-İzmir	*Ophrys fusca*	30
Bodrum-Muğla	*Serapias vomeracea* subsp. *orientalis*	4
Kahramanmaraş	*Orchis sancta*	24
Total	**5**	**83**

Cultural and morphological characteristics of the isolates

MEA, Potato Dextrose Agar (PDA), and Corn Meal Agar (CMA) were used for the determination of cultural and morphological characteristics of the isolates at genus level. Colony colour (both front and reverse), conidial morphology (if existing), hyphal morphology, presence or absence aerial mycelia, and hyphal width were determined according to Barnett (1960), Domsch et al. (1980), Currah et al. (1987), Hasenekioğlu (1991), Sneh et al. (1991), and Shan et al. (2002).

The possible *Rhizoctonia*-like isolates thus separated were further analysed by Olympus (CX-31) light microscope. The number of nuclei per cell, hyphal branching, appearance of sclerotia, and monilioid cell morphology were determined in the possible *Rhizoctonia* cells (Athipunyakom et al., 2004). The cells were stained with safranin O-KOH using Bandoni's method (Bandoni, 1979) and with lactophenol cotton blue (Ma et al., 2003).

Growth rates of *Rhizoctonia* spp. isolates

Growth rates (mm/h) were determined according to the technique of Currah et al. (1987). Six millimetre diameter agar discs from the edge of colonies growing on PDA were removed and were inoculated onto the middle of PDA, CMA, and MEA plates. Radial increments in colony diameter were measured in 2 directions every 48 h over 2 weeks. All the growth rate determinations were done in triplicate (Shan et al., 2002).

Results

Isolates of endophyte and mycorrhizal fungus

Most of the isolated orchid endophytes were found to be *Fusarium* spp. A total of 47 isolates, of which 44 (94%) isolates carried the *Fusarium* genus characteristics, 2 (4%) isolates belonged to the *Rhizoctonia*-like fungi, and 1 (2%) isolate belonged to the genus *Papulaspora* (Table 2), were found from orchid root and tubers. *Rhizoctonia*-like fungi could only be isolated from MS in a specific jar for plant tissue culture and it was the *Orchis sancta* collected from Kahramanmaraş. Endophytic fungi and many unidentified fungi (data not shown) were isolated from the roots of *Anacamptis pyramidalis, Orchis sancta, Ophrys fusca*, and *Serapias vomeracea* subsp. *orientalis* collected from İzmir. All the other endophytic fungi, namely *Fusarium* sp. and *Papulaspora* sp., were isolated from FIM in petri plates.

Cultural and morphological characteristics of endophytic fungi (*Fusarium* sp. and *Papulaspora* sp. isolates)

Based on colony morphology, pigmentation, and conidia morphology, 44 of the strains were considered to belong to the genus *Fusarium*. One strain was considered to belong to the genus *Papulaspora* based on the presence of papulaspores.

Cultural and morphological characteristics of *Rhizoctonia*-like fungi

Rhizoctonia sp. EGE-K-I and *Rhizoctonia* sp. EGE-K-II did not produce sclerotia on all media when incubated at 28 °C for 2 months**.** Monilioid cells, aerial mycelia, and concentric zones on colonies were observed in cultures of *Rhizoctonia*-like fungi isolates (Figure 2). Table 3 summarises the significant features observed in the *Rhizoctonia*-like fungi isolates.

Discussion

The most common endophytic fungi isolated from the Aegean and Mediterranean orchids' (saleps) roots and tubers were *Fusarium* spp., uni (UNR) and binucleate *Rhizoctonia*-like fungi (BNR), and *Papulaspora* sp.

Eighty-three orchid plant samples were studied and among them the most investigated orchid was *Orchis sancta* (Çeşme and Kahramanmaraş) (Table 1). Only 2 of the *Rhizoctonia*-like fungi were isolated from *Orchis sancta*.

There are many problems related to studies of orchid root endophytes and they all give low success rates. Firstly, many endophytic fungi do not sporulate in pure culture. Since traditionally fungi are classified by their spores and spore-bearing structures, nonsporulating fungi are very difficult to identify. For this reason, unidentifiable fungi are often grouped into 'morphospecies' on the basis of colony colour, morphology, and growth rate on agar media. DNA sequencing studies have shown that this technique is quite successful at grouping related fungi together. Secondly, many endophytic fungi are undescribed and do not fit well into previously described taxa. Thirdly, some endophytes do not grow in culture. Culturing of

Table 2. Isolates of endophytes and mycorrhizal fungi from Aegean and Mediterranean orchids.

Orchid species	The endophyte isolates				Total
	Fusarium spp.	Uninucleate *hizoctonia* sp.	Binucleate *Rhizoctonia* sp.	*Papulaspora* sp.	
Orchis sancta (Kahramanmaraş)	-	1	1	-	2 (4.3%)
Anacamptis pyramidalis	6	-	-	1	7 (14.9%)
Orchis sancta (İzmir)	14	-	-	-	14 (29.8%)
Ophrys fusca	20	-	-	-	20 (42.6%)
Serapias vomeracea subsp. *orientalis*	4	-	-	-	4 (8.5%)
Total	**44**	**1**	**1**	**1**	**47**

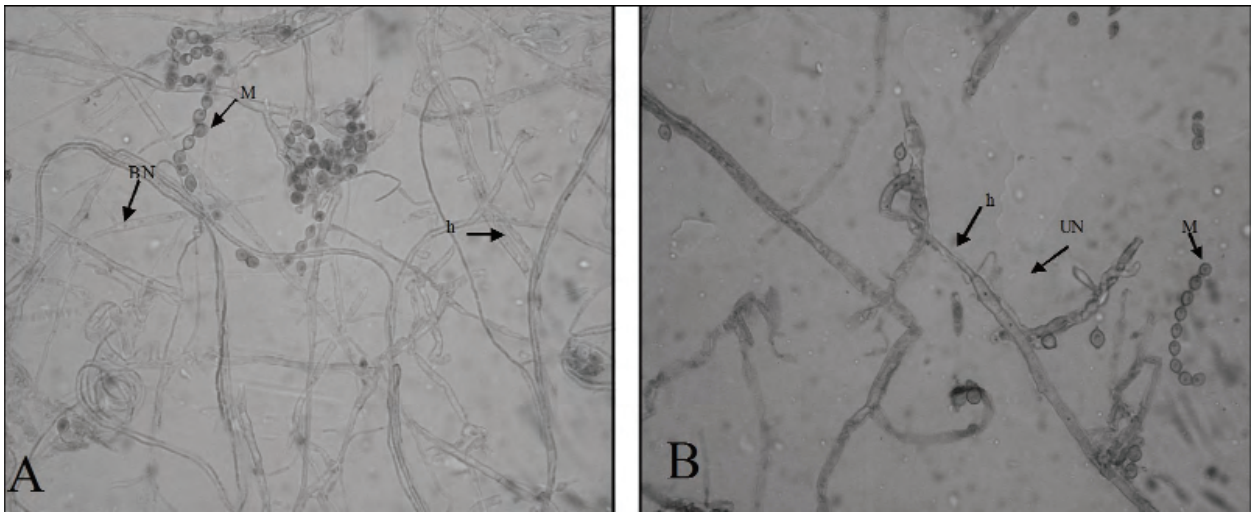

Figure 2. A. *Rhizoctonia* sp. EGE-K I; binucleate (BN), vegetative hyphae (h) and monilioid cells (M). B. *Rhizoctonia* sp. EGE-K II uninucleate cells (UN), vegetatitive hyphae (h) and monilioid cells (M).

Table 3. The significant features observed in the *Rhizoctonia*-like fungus isolates.

Characterisation	***Rhizoctonia*-like fungi**	
	Rhizoctonia sp. EGE-K-I	*Rhizoctonia* sp. EGE-K-II
Colony colour	White to light brown, turned dark brown with age with concentric zonation	White to cream when young turned orange- brown to dark brown at maturity with concentric zonation
Hyphal width (µm)	2-5	2-5
Angle of hyphal branching	90°	90°
Monilioid cell morphology	Ellipsoid	Ellipsoid
Monilioid cell width (µm)	3-4.5	3.5-5.5
Number of nuclei per cell	Binucleate	Uninucleate
Growth rate (mm/h)	0.2	0.2
Appearance of sclerotia	-	-

microorganisms from plant tissues provides a skewed picture of the organisms that grow there. One solution to this problem is to use PCR-based methods to amplify DNA directly from orchid roots using fungal-specific primers. So far, such techniques have been used to study orchid mycorrhizal fungi but not non-mycorrhizal endophytes (Bayman & Otero, 2006).

The success rate of isolation of *Rhizoctonia*-like fungi from *Orchis sancta* was 4.3% (Table 2). This low success rate of isolation is in accordance with the findings of Bayman et al. (1997). In their study, they reported that the frequency of mycorrhizas in adult plants of epiphytic, tropical orchids varied from very low to very high.

One morphological feature that has helped in the classification of *Rhizoctonia* is the number of nuclei present in the young cells. Multinucleate, binucleate, and uninucleate cells have been observed and *Rhizoctonia* species can be divided into 3 groups based on the nuclear condition of vegetative cells: uni-, bi-, and multinucleate (UNR, BNR, MNR) (Hietala, 1997; Otero et al., 2002). In this study, the *Rhizoctonia* sp.

EGE-K-I was found to be binucleate while the *Rhizoctonia* sp. EGE-K-II was found to be uninucleate. Studies indicated that there were variations among some binucleate *Rhizoctonia*-like fungi isolates and some were nonpathogenic or some were weakly pathogenic to cultivated plants (Otero et al., 2002). Since the *Rhizoctonia*-like fungi isolates of this study were binucleate they can be considered nonpathogenic.

Some cultures of *Rhizoctonia* were left for at least 6 weeks to allow development of monilioid cells with different dimensions and shapes and the formation of sclerotia (Shan et al., 2002; Stewart & Kane, 2006, 2007). Both isolates of *Rhizoctonia*-like fungi formed monilioid cells in culture.

Many other genera were isolated from the roots of various orchid species. These fungi were reported as either non-*Rhizoctonia* fungi or endophytes from orchid roots (Bayman & Otero, 2006). Non-*Rhizoctonia* fungi *Cylindrocarpon* sp., *Papulaspora* sp., *Phialophora richardsiae*, and *Ulocladium* sp. were reported from terrestrial photosynthetic orchid roots of *Spiranthes magnicamporum* in Canada (Zelmeret et al., 1996; Otero et al., 2002). Root endophytic fungi commonly occurred in Spitsbergen but only *Olpidium brassicae, Pleospora herbarum, Papulaspora, Microdochium bolleyi*, and *Rhizoctonia solani* were identified with reasonable certainty (Väre et al., 1992). In this study, *Papulaspora* sp. was only isolated with reasonable certainty from *Anacamptis pyramidalis.*

In this study, we obtained 44 *Fusarium* spp. and only 1 *Papulaspora* sp. from Aegean and Mediterranean orchid roots as endophytes. *Fusarium* spp. have the ability to induce orchid seed colouration and germination as reported by Vujanovic et al. (2000). However, the importance of the non-mycorrhizal *Fusarium* fungus in promoting germination seems to be relatively minor compared to that of specific *Rhizoctonia* orchid mycorrhizas. Several orchid-associated fungi were isolated from symptom-less surface-sterilised roots of epiphytic tropical orchids *Dendrobium moschatum* and *Acampe papillosa* grown in a greenhouse. Endophytic *Fusarium* isolates were obtained from Aegean and Mediterranean orchids such as *Anacamptis pyramidalis, Orchis sancta, Ophrys fusca*, and *Serapias vomeracea* subsp. *orientalis*.

Recently, most studies have concentrated on greenhouse or transplant crops, where seedlings are grown in non-competitive substrates containing an endophytic non-pathogenic isolate of *Fusarium oxysporum* (NP*Fo*) where it has the advantage of root colonisation prior to the pathogen in the field (Dhingra et al., 2006). *Fusarium* isolates obtained from roots and tubers (in the Aegean and Mediterranean orchids (saleps)) have the potential for use in symbiotic germination. Effective in situ conservation of rare and threatened orchids required an understanding of the complex interaction between the orchid and its mycorrhizal associate. A successful symbiotic association between orchid and orchid mycorrhizal endophytes or that isolated from the roots of an orchid or from soil around the plant is required for the growth and survival of transplants of orchid seedling in wild habitats (Rasmussen, 1995; Stewart & Zettler, 2002; Shimura & Koda, 2005; Stewart et al., 2007).

In view of the findings obtained in our study, we conclude that it is very important to investigate the diversity, specificity, and distribution of fungal endophytes associated with Aegean and Mediterranean orchids such as *Anacamptis pyramidalis, Orchis sancta, Ophrys fusca,* and *Serapias vomeracea* subsp. *orientalis*. Further research in this field should be carried out on optimisation of the protocols for the reintroduction and establishment of orchid seedlings to field sites.

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