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Spiranthes spiralis (L.) Chevall'de mikorizal çeşitlilik

Vildan AKIN MUTLU[1], İbrahim ÖZKOÇ[1*]

Öne Çıkanlar:
- *Spiranthes spiralis* köklerinin fungal florası.
- Baskın endofitik grup *Tulasnella* üyeleridir.

Anahtar Kelimeler:
- Fungi
- Orchids
- Tohum çimlenmesi
- Simbiyotik kültür
- *Tulasnella*

ÖZET:

Tehdit altındaki orkidelerin etkili bir şekilde korunması için kökle ilişkili fungusların tanımlanması ve tohum çimlenmesinde etkinliğinin değerlendirilmesi koruma protokolleri oluşturmak için önemlidir. Bu nedenle çalışmamızda *Spiranthes spiralis* (L.) Chevall 'in kök-ilişkili funguslarının çeşitliliği araştırılmıştır.. Kültüre bağımlı yaklaşımına göre, 'in köklerinden 37 endofitik fungus izole edilmiş, morfolojik ve moleküler tanımlamaları yapılmıştır. kökünde baskın fungus türünün *Tulasnella* cinsi olduğu belirlenmiştir. Türkiye'de ilk kez köklerinden *Thanatephorus fusisporus* türü izole edilmiştir. İzole edilen fungusların *S. spiralis* tohumlarını simbiyotik kültürde çimlendirme etkinlikleri değerlendirilmiştir. Nisan ayında izole edilen VY 25 (*Tulasnella*) izolatı en yüksek çimlenme oranını (%73.77) göstermiştir. VY 1, VY 4, VY 8, VY 16, VY 25, VY 30, (*Tulasnella*) izolatları çimlenmeyi ve fide gelişimini teşvik etmiştir. *Thanatephorus* (%46.79) ve *Ceratobasidium* (%32.42) cinslerinin ise tohumlarını çimlendirmede etkili olmadığı görülmüştür. Araştırma, fungal partnerin gelişim evrelerine ve/veya aylara göre değiştiğini ortaya koymuştur. Bu çalışma, Türkiye'de köklerinden izole edilen organizmalar için ilk moleküler verileri içermektedir. Aynı zamanda bu sonuçlara göre, tohum çimlenmesini ve bitki büyümesini destekleyen funguslar, nesli tükenmekte olan ılıman orkidelerin korunması ve doğaya yeniden kazandırılması için önerilebilir.

Mycorrhizal diversity in *Spiranthes spiralis* (L.) Chevall

Highlights:
- Fungal flora of *Spiranthes spiralis*
- The dominant endophytic group is members of *Tulasnella.*

Keywords:
- Fungi
- Orchids
- Seed germination
- Symbiotic culture
- *Tulasnella*

ABSTRACT:

For effective conservation of threatened orchids, identifying root-associated fungi and assessing their activity in seed germination is important for establishing conservation protocols. Therefore, our study investigated the diversity of *Spiranthes spiralis*'s root-associated fungi. According to the culture-dependent approach, 37 endophytic fungi were isolated from the roots and morphologically and molecularly identified. It was determined that the dominant fungal species in the roots was the genus *Tulasnella*. For the first time in Türkiye, *the Thanatephorus fusisporus* species was isolated from roots. The germination efficiency of the isolated fungi in the symbiotic culture of *S. spiranthes* seeds was evaluated. VY 25 (*Tulasnella*) isolate isolated in April showed the highest germination rate (73.77%). VY 4, VY 18, VY 25, (*Tulasnella*) isolates promoted germination and seedling development. *Thanatephorus* (46.79%) and *Ceratobasidium* (32.42%) were not effective in germinating seeds. The study revealed that the fungal partner varied according to developmental stages and months. This study contains the first molecular data for organisms isolated from roots in Türkiye. According to these results, fungi that promote seed germination and plant growth can be recommended for the conservation and reintroduction of endangered temperate orchids.

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INTRODUCTION

Orchidaceae is one of the largest families of flowering plants, accounting for about 8% of the Angiosperm variety of species (Chase et al. 2015; Willis, 2017). Only 1000 types have been assessed for the IUCN Global Red List so far (Rankou, H.2011) and 56.5% of those evaluated have been identified as entering one of the threat categories (in critical danger, in danger, and vulnerable). Global climate change, drought, fires, and anthropogenic effects rapidly destroy orchid habitats, requiring the protection of many orchid species. Due to these threats and complex life cycles, orchids are thought to be particularly vulnerable to the impact of global environmental change (Fay et al. 2009; Swarts & Dixon 2009; Gale et al. 2018; Kömpe & Mutlu 2021). For future generations, not only the tropic orchids need effective protection, but many terrestrial orchids have to be protected because they will inevitably be destroyed (Swarts & Dixon 2017). The fungal partners are necessary for all orchids to get nutrient elements from the soil at least in the early stages of development. This relationship continues even in the adult phase (Rasmussen & Rasmussen, 2009; Smith & Read, 2008). The orchid endomycorrhizal fungi improve nutrient element absorption from the soil and regulate the relations between other organisms and the habitats (Smith & Read, 2008; Dighton, 2009). The majority of orchid endomycorrhizal fungi are often members of the genus *Rhizoctonia,* which is included in Basidiomycota (Smith & Read, 2008; Dearnaley et al. (2012). *Rhizoctonia*-like fungi include members of Ceratobasidiaceae, Tulasnellaceae, and Sebacinales (Dearnaley et al. (2012). Previous studies have revealed that terrestrial orchids are associated with a wide variety of fungi (Herrera et al. (2017). Studies have been done in Europe on the fungal diversity of terrestrial photosynthetic orchids (usually *Anacamptis*, *Himantoglossum*, *Ophrys*, *Orchis* and *Serapias*) (Girlanda et al. 2011; Jacquemyn et al.,2012; Mutlu & Kömpe, 2020). Determination of orchid endomycorrhizal fungi is the first and the most essential step for orchid conservation and reintroduction (Shao et al. 2017). The next step is to identify the fungi that promote seed germination to conduct *in vitro, ex vitro* or *in situ* propagation studies (Shao et al. 2017). The Species of *Spiranthes* have spread across Europe, North-West Africa, Syria, Iran and all regions of Türkiye. It contains about 40 species worldwide. *S. spiralis* (L.) Chevall, which is classified as having the "Least Concern" status according to the European IUCN Red List (Rankou, H.2011) is perennial or tuberous plant. *S.spiralis* which is called the pearl orchid (Güner et al 2012) is one of the two *Spiranthes* species in Türkiye (Davis, 1967). Unlike other temperate orchids, plants flowers during autumn. The roots of other orchids often dry out and die after fruit formation, but the roots are napiform. These napiform roots remain underground all year. Previously, in Türkiye, the isolation of fungi from roots and *in situ* seed surfaces was done by Sazak & Ozdener (2006). According to morphological properties, *Rhizoctonia repens*, isolated from the roots, was classified as *Rhizoctonia solani* AG A at the stage of *in situ* seeds. The isolation of fungi from the roots of during the flowering phase for two years (2006 and 2007) showed a variety of mycorrhizal fungi (Tondello et al., 2012). However, the effects of these isolates on germination were not tested. It is not known whether there is a specific relationship between mycorrhizal fungi and the seed germination. However, a different result was shown with *Spiranthes brevilabris*, where it was determined that there is a specific relationship between the fungus isolated from *S. brevilabris* roots and the seeds' germination (Stewart & Kane, 2007). The rhizosphere microbiome represents a dynamic and complex network of relationships concerning the microbial community in plant roots. This structure, which is generally considered the second genome of the plant, has very important roles in plant health. Since it is known that the plant microbiome collectively contributes to plant growth and development, revealing and developing this structure will also be important for orchid cultivation (Afridi et al., 2022).

In light of this information, the main purpose of this study is to determine (i) the diversity of mycorrhizal fungi in the roots of plants throughout the year, (ii) the effect of root endophytic fungi on germination and seedling development of seeds under *in vitro* conditions.

MATERIALS AND METHODS

Study Site

S. spiralis is distributed in all regions of Türkiye. The research area was chosen as Ondokuz Mayıs University campus in Samsun province located in the central part of the Black Sea region of Türkiye. The habitat is open areas near oak forests.

Isolation of Fungi from Roots

Fungal isolation started in September 2013 when the plant was in blossom and continued until July 2014 when the above-ground parts of the plants were dry. Old and young roots of plant individuals were taken every month. Roots were examined under the microscope for fungus infection and all roots containing fungal coils were used for fungal isolation. Fungal isolations were carried out according to the method of Clements et al. (1986). Then the roots were surface-sterilized in 1.5% NaOCl solution for 1-2 minutes and washed in sterile distilled water. Root pieces of 1–2 cm in length were placed in isolation medium under aseptic conditions and then incubated at 25 °C in the dark for 1–2 days. Hyphae developing from root fragments were pre-identified under a Leica light microscope at ×10 magnification. *Rhizoctonia*-like fungi were defined as rhizomorph and asexual fungi that did not form conidia in young vegetative hyphae, showing steep branching, narrowing at or near the branching point, and septum formation close to the branching point (Clements et al., 1986). Each *Rhizoctonia*-like colony was transferred to fungus isolation medium (FIM) (0.50 g/L $Ca(NO_3)_2$, 0.20 g/L KH_2PO_4, 0.10 g/L KCl, 0.10 g/L $MgSO_47H_2O$, 0.10 g/L Yeast extract, 5.0 g/L sükroz, 10.0 g/L agar) and purified (Clements et al., 1986). Pure fungal cultures were stored at 4 °C until the study's next step.

Morphological Identification of Fungal Isolates

Rhizoctonia-like isolates were incubated in the dark at 26 ±2°C for 10 days in Potato Dextrose Agar (PDA) medium (Oxoid, CM0139) to determine colony color, colony appearance, rate of colony growth, sclerotia presence, and sclerotia diameter (Carling et al., 1987; Sneh et al., 1991). Colony color and colony appearance were determined according to the Royal Horticultural Society color catalogue (RHS, 1995). The isolates were incubated for 3 days at 26 ±2°C in a petri dish containing Water agar (WA) to determine the hyphae diameter and the number of nuclei, staining with Safranin O and 3% KOH (Bandoni, 1979). Nuclei were counted in 15 randomly selected cells.

Molecular Identification of Fungal Isolates

The isolates to be molecularly characterized were first transferred to 250 mL Erlenmeyer flasks containing Potato dextrose broth (PDB) medium (Oxoid, CM0962) and placed in a shaking incubator at 180 rpm and 24°C for 10 days in the dark. Developed fungal mycelia were collected by filtration, washed three times with sterile distilled water, dried, crushed to powder in liquid nitrogen and placed in sterile 1.5 ml Eppendorf (Carling et al., 1987).

Approximately 50 mg of mycelial powder per isolate was used to extract the genomic DNA according to the CTAB (cetyltrimethylammonium bromide) method (Pascual et al., 2001). The DNA template was dissolved in 1 x TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0). DNA concentration was measured using a nanospectrophotometer (DS-11 FX+, Denovix Inc., Wilmington, DE, USA). The

DNA obtained for each isolate was diluted to 10 ng/µL with 1 x TE solution and used for PCR experiments.

The ITS4 (TCCTCCGCTTATTGATATGC) / ITS5 (GGAAGTAAAAGTCGTAACAAGG) primer pair was used to amplify the ITS region of the genomic rDNA of *Rhizoctonia* isolates (White et al., 1990). A 50 µl reaction mixture was prepared for amplification. Each reaction mix contained: 1µl template DNA, 5µl 10XPCR buffer, 1µl dNTP (2.5 mM) mix, 3µl MgCl2, 1µl Primer ITS-4 (25 mM) and 1µl Primer ITS-5(25 mM), 0.25µl Taq polymerase (5U), 37.75µl sterile ddH20. The negative control contained the same PCR reagents but not genomic DNA. The PCR reaction was performed using the following conditions: initial denaturation at 94°C for 3 minutes followed by 30 cycles, denaturation at 94°C for 1 minute, adhesion at 52°C for 1 minute, elongation at 74°C for 3 minutes, and final elongation at 72°C for 7 minutes (Salazar et al., 1999).

PCR products were purified and bidirectionally sequenced by the Macrogene Sequencing Service (Seoul, Korea). Sequences of each isolate were generated using the BioEdit version 7.2.5 software to produce the consensus sequence (Hall, 1999). Consensus sequences were compared with sequence data in GenBank using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) network services. A sequence for each isolate was stored in the GenBank database. Multiple sequence alignments were performed with Clustal X version 2 (Larkin et al., 2007). DNAsp (DNA Sequence Polymorphism) 6.0 software programs were used for haploid analysis of the isolates (Rozas et al., 2017). A data set was created with the determined representative sequences of each haplotype group. The jModel test program was used to correctly make the model selection in the formation of phylogenetic trees (Posada, 2008). In order to reveal the identity of the isolates and the phylogenetic relationships between them, a tree was created with the Maximum likelihood (ML) algorithm in the MEGA 6 software program (Tamura et al., 2013). The software automatically generated the Maximum Likelihood (ML) tree and the reliability of the phylogeny was evaluated using 1000 bootstraps.

In vitro Symbiotic Seed Germination

All obtained isolates were tested on a modified oat medium for their seed germination efficacy (Clements et al., 1986). 250 ± 50 (0,001 g) seeds were surface-sterilized in NaOCl (1.5%) for 10 minutes (Clements et al. 1986). Sterile seeds were sown on the surface of test tubes containing Modified Oat Medium (MOM) (0.2g/L of $Ca(NO_3)_2$, 0.2g/L of KH_2PO_4, 0.1g/L of $MgSO_47H_2O$, 0.1g/L of KCI, 0.1g/L of Yeast Extract, 2g/L sucrose, 3.5g/L grounded oat and 10g/L of Agar) (Clements et al. 1986) and one of the fungal isolates was inoculated. Two independent experiments and three replicates were performed for each fungal isolate. Test tubes were then incubated at 25 ± 2 °C in a climate chamber with a 16/8 hour light/dark photoperiod and 33 PAR (photosynthetic active radiation). Three months (90 days) after incubation, the germination effect of each fungal isolate was evaluated numerically (Clements et al., 1986).

Germination rate was calculated by dividing the number of seeds in stages S1-S4 by the total number of seeds (Clements et al., 1986). Germination rates (%) = number of germinated seeds / total number of seeds X 100.

Statistical Analysis

Data analyses were performed in SPSS software 27.0 (SPSS Inc., Chicago, USA). Following the ANOVA (Analysis of variance), Tukey's HSD (Honestly significant difference) test was used to analyze the statistically significant effects of fungal isolates on seeds' viability, in vitro germination and growth.

The level of statistical significance was set at $p < 0.05$. Germination and seedling development were evaluated three months after fungus inoculation. The rates of germination at the developmental stage for the control group (no inoculated fungus) and the seeds inoculated with fungal isolate were calculated by taking the averages of six repeated experiments and calculating the standard deviation.

RESULTS AND DISCUSSION

Fungal Isolation and Identification

Over the course of a year, 37 Rhizoctonia-like fungi were isolated from roots. Fungal isolations were made between September (2013) and August (2014). Tulasnella sp. isolates were obtained every month except February, two isolates of Ceratobasidium sp. were obtained in December (2013) and February (2014), and one isolate of Rhizoctonia sp. was obtained in September 2013 (Figure 1). Some researchers reported that they isolated fungi belonging to similar genera from S.spiralis plants except Tulasnella. (Tondello et al., 2012; Fujimori et al., 2018).

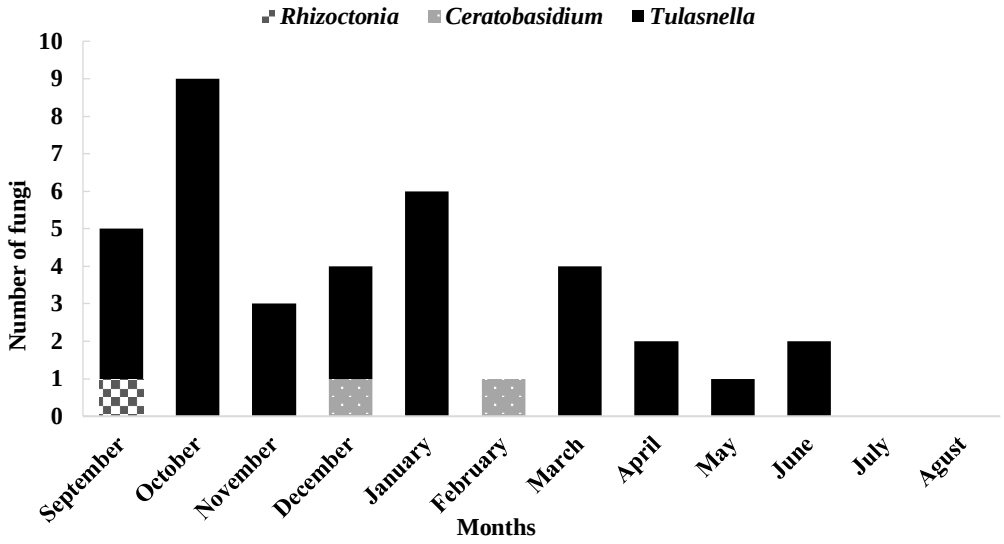

Figure 1. Monthly distribution of *Rhizoctonia*-like fungal isolates from *S. spiralis* (L.) Chevall roots

Morphological Identification of Fungal Isolates

Rhizoctonia-like isolates were initially divided into two groups (binucleate and multinucleate) according to the number of nuclei. Binucleate (BN) and multinucleate (MN) isolate groups were divided into two subgroups according to colony type and hyphal width.

For the BN group, VY 1, VY 3, VY 4, VY 6, VY 8, VY 9, VY 11, VY 12, VY 13, VY 14, VY 15, VY 16, VY 18, VY 19, VY 25, VY 26, VY 28, VY 30 and VY 32 isolates were divided into two subgroups. The mean hyphae diameter of (VY 13, VY 19) isolates found in the first subgroup was determined as 2 µm, and the colony color was yellowish white. While air hyphae were not observed, ellipsoidal rod-shaped monilioid cells were observed. Scleroid formation was not observed. The hyphae diameters of the second subgroup isolates were determined as 2.5 µm and no sclerotia, or air hyphae formation was observed. Monilioid cells were more spherical than cells in the first subgroup.

Among the MN isolates included in the second group, VY 2 was isolated in September 2020 and VY 5 in October 2020. The colony color of the VY 2 isolates in the PDA medium was grayish yellow and hyphal width was determined as 2.5 µm. Ellipsoidal and barrel-shaped monilioid cell formation and aerial hyphal development were observed. Irregularly shaped grayish-yellow sclerotia (4–9.5 µm)

formation was observed. The colony color of VY 5 found in the second subgroup changed from grayish orange to brownish over time and no formation of air hyphae was observed. The width of the hyphae was determined as 4.5 µm. Monilioid cells were ellipsoidal and barrel-shaped. Elliptical irregularly shaped dark brown sclerotia (0.87–5.2 µm) was observed in a month-old PDA medium. It was determined that the VY 5 isolate was the fastest-growing isolate (within 3 days), and the other isolates reached the level that covered the entire petri dish in 10 days (Figure 2).

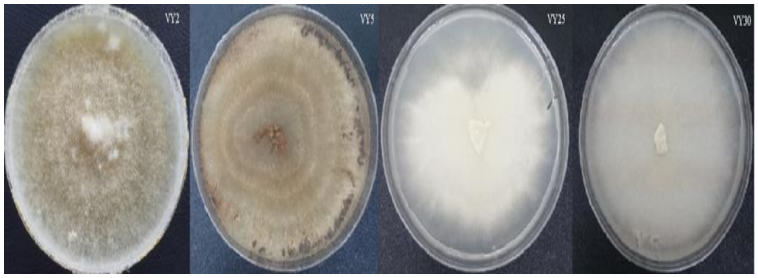

Figure 2. Colony appearance of *Rhizoctonia*-like fungal isolates isolated from *S. spiralis* (L.) Chevall roots

Molecular Identification of Fungal Isolates

The ITS consensus sequences of all isolates (excluding missing and messy regions) were identified into 21 haplotype groups based on DNA sequence similarities using the DNAsp program. Representative sequences of each haplotype group were identified in the GenBank database with 81-100% similarity to their closest relatives (Table 1). According to the match result in the database (NCBI), VY 1, VY 3, VY 6, VY 8, VY 9, VY 11, VY 12, VY 14, VY 15, VY 16, VY 17 VY 18, VY 25, VY 30, VY 32) isolates was defined as *Tulasnella* (anamorph: *Epulorhiza*) VY 5, VY 13 ve VY 19 isolates were defined as *Ceratobasidium* (anamorph: *Ceratorhiza*) genus of VY 5, VY 13 and VY 19 isolates. VY 2 (98%) *Rhizoctonia fusispora* (synonym: *Thanatephorus fusisporus*) matched with VY 4 *Tulasnella calospora* (anamorph: *Epulorhiza repens*) and with VY 25 *Tulasnella deliquescens* (synonym: *Tulasnella calospora*). On the other hand, the VY 26 isolate showed 81% similarity with the uncultured fungus isolated from *Liparis loeselii* habitat soil in the database and did not match at the taxon level (Ding et al., 2014). It has been determined that the fungus that is frequently seen in the roots belongs to *Tulasnella* genus (Table 1).

Table 1. Details of the data set of ITS sequences analyzed in this study

Isolate no	Month of isolation	**Access number of GenBank**	Base number **(bp)**	İdentification	Host	Identity **(%)**	Closest match (Access no)	References
VY1	October	MT775595	544	*Tulasnella*	*Serapias vomeracea*	97	JF926502	(Girlanda et al.,2011)
VY2	October	MT775823	678	*Rhizoctonia fusispora*	specimen_voucher="KC833	98	HQ441575	(Roberts, 1999)
VY3	October	MT775834	459	*Tulasnella*	*Serapias vomeracea*	99	JF926503	(Girlanda et al.,2011)
VY4	September	MT775836	628	*Tulasnella calospora*	*Bletia punctata*	99	MG008683	GenBank
VY5	September	MT775837	627	*Ceratobasidiaceae* sp.	*Anacamptis pyramidalis*	97	MK951653	GenBank
VY6	October	MT790748	647	*Tulasnella* sp.	*Anacamptis morio*	88	KJ789933	(Ercole et al.,2015)
VY8	October	MT776392	444	*Tulasnella* sp.	*Anacamptis palustris*	100	MG762605	(Mutlu & Kömpe, 2020)
VY9	October	MT776848	822	*Epulorhiza* sp	*Cymbidium goeringii*	98	FJ613228	GenBank
VY11	November	MT776556	640	*Tulasnella* sp	*Anacamptis laxiflora*	100	MG762604	(Mutlu & Kömpe, 2020)
VY12	December	MT776562	547	*Epulorhiza* sp.	*Cymbidium goeringii*	100	FJ594926	GenBank
VY13	December	MT776667	649	*Ceratobasidium* sp.	*Anacamptis coriophora*	100	MG762693	(Mutlu & Kömpe, 2020)
VY14	May	MT785875	620	*Epulorhiza* sp.	*Cymbidium goeringii*	85	FJ613252	GenBank

VY15	March	MT785874	550	*Tulasnella* sp.	*Liparis japonica*	92	KF537647	(Ding et al.,2014)
VY16	January	MT776722	544	*Epulorhiza* sp	*Geodorum densiflorum*	96	KJ765990	GenBank
VY17	February	*MT779803*	753	*Epulorhiza* sp.	*Cymbidium goeringii*	83	FJ613212	Submission
VY18	January	MT776901	528	*Epulorhiza* sp.	*Cymbidium faberi*	98	FJ613264	GenBank
VY19	January	MT776903	661	*Ceratobasidium* sp.	*Orchis simia*	100	MG762694	(Mutlu & Kömpe, 2020)
VY25	April	MT785876	567	*Tulasnella deliquescens*	*Spiranthes sinensis*	86	LC175329	(Fujimori et al. (2018)
VY26	April	MT790743	449	Uncultured fungus	Dune slack soil	81	MK737482	GenBank
VY30	June	MT778068	581	Uncultured Tulasnellaceae	*Dactylorhiza* sp	99	JX024734	(Herrera et al. (2017)
VY32	December	MT778803	509	*Tulasnella* sp.	*Anacamptis papilionacea*	98	MG762601	(Mutlu & Kömpe,2020)

According to the phylogenetic analysis results, the isolates were divided into two genera (*Tulasnella* and *Ceratobasidium*) in the Maximum Likelihood (ML) tree (Fig. 2). *Tulasnella* was represented by clade A-B-C-D-E-F-G. In the A clade, VY 1, VY 3, VY 6 and VY 11 isolates showed a low similarity (52%) to *Tulasnella bifrons* (AY373290.1). In clade B, the VY 4 isolate showed a high identity (BS =100), with the species *Tulasnella calospora* (MG008683).

In the C clade, VY 9 isolate (FJ613264.1) was associated with *Epulorhiza* sp. with 52% of similarity. In the D clade, VY 8 isolate was associated with *Tulasnella. deliquencens* (syn. *T. calospora*) species (BS=72). In the E clade, VY 12, VY 18, VY 14, VY 25, VY 17, VY 26 isolates were clustered with uncultured fungi (MK737402.1) isolate (Figure 2). In the F clade VY 30 isolate and VY 15, VY 16, and VY 32 isolates in the G clade were associated and extremely related to the *Tulasnella* genus. *Ceratobasidium* genus was strongly supported (BS = 100) and represented by the H-J-K-L clades; in the H clade, the VY 2 isolate showed a high similarity with *Thanatephorus fusisporus* (HQ441575.1) (BS=98). In the J clade, VY 19 *Ceratobasidium* (MG762694.1) showed a high identity supporting it to belong to the OS 1(*Orchis simia*) isolate (BS =99). In the K clade, VY 5 was clustered with *R.solani* isolates and in the L clade VY 13 isolate was found to be closely related to the *Rhizoctonia fraxini* (MH4855687.1) (BS =78) (Figure 3).

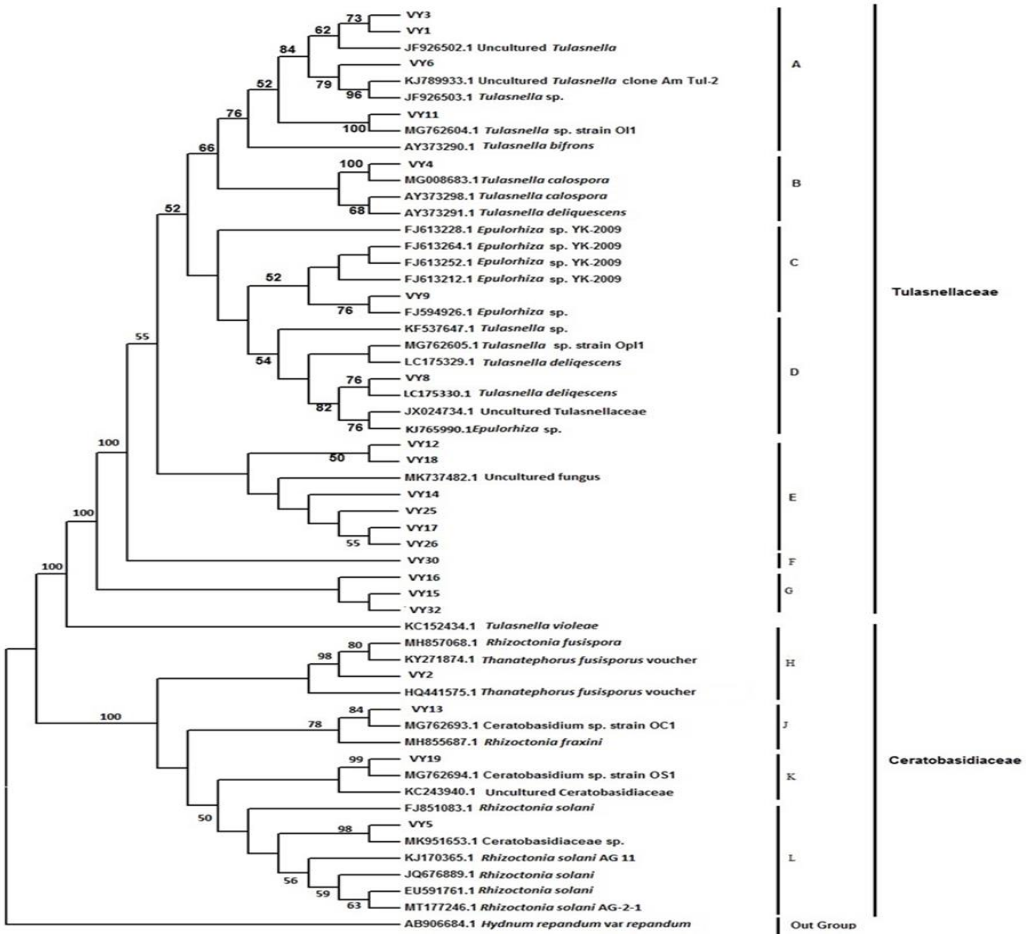

Figure 3. rDNA ITS phylogeny of *Tulasnella*, *Ceratobasidium*, *Rhizoctonia* and their associated mycorrhizal fungi. The tree was rooted with sequences of the fungal species *Hydnum repandum* var. *repandum.* Sequences downloaded from GenBank are indicated by accession numbers. In the tree, bootstrap values greater than 50% from ML analysis

Seed Germination

Symbiotic germination was performed on representative isolates of 21 haplotype groups according to their DNA sequence similarities. After two months of incubation, their total germination status was evaluated. The effects of fungi on germination were significantly different compared to the control. There was a significant difference between the groups in terms of germination rate, F(20.42)= 9.69, p= 0.000). When fungal isolates were compared, it was determined that the VY 25 isolate was very effective on both germination and development compared to other isolates, and the effect on germination was statistically significant. (Table 2).

Symbiotic germination was performed on representative isolates of the haplotype group (Stewart & Kane, 2007). After two months of incubation, their total germination status was evaluated. The effects of the fungi on germination were significantly different compared to the control. There was a significant difference in germination rate between the groups, F(20.42)= 9.69, p= 0.000). When the fungal isolates were compared, it was determined that isolate VY 25 was very effective in both germination and growth compared to the other isolates and its effect on germination was statistically significant (Table 2). VY 4 (October), VY 18 (January) and VY 25 (April) isolates showed germination rates of 50.07%, 50.31%, 73.77%, respectively and supported seedling development (Figure 4). VY 25 isolates supported development until the first photosynthetic leaf, while the remaining isolates supported development until the leaf primordium (Figure 4).

Table 2. *In vitro* symbiotic germination of seeds with *Tulasnella*, *Ceratobasidium* and *Rhizoctonia* isolates. ±: Standard deviation, n=6

Fungus isolate	Germination (%)
C	0.00±0.00d
VY1	40.86±11.39bc
VY2	46.79±8.64bc
VY3	43.84±13.54bc
VY4	50.07±14.89ab
VY5	24.71±2.10c
VY6	26.27±6.33bc
VY8	24.55±5.30c
VY9	48.59±8.13bc
VY11	32.42±0.77bc
VY12	32.16±7.6bc
VY13	42.77±3.15bc
VY14	36.92±7.23bc
VY15	31.54±6.44bc
VY16	39.55±2.01bc
VY18	50.31±9.12ab
VY19	33.60±9.07bc
VY25	73.77±11.25a
VY26	39.49±4.15bc
VY30	34.99±3.02bc
VY32	37.44±6.04bc

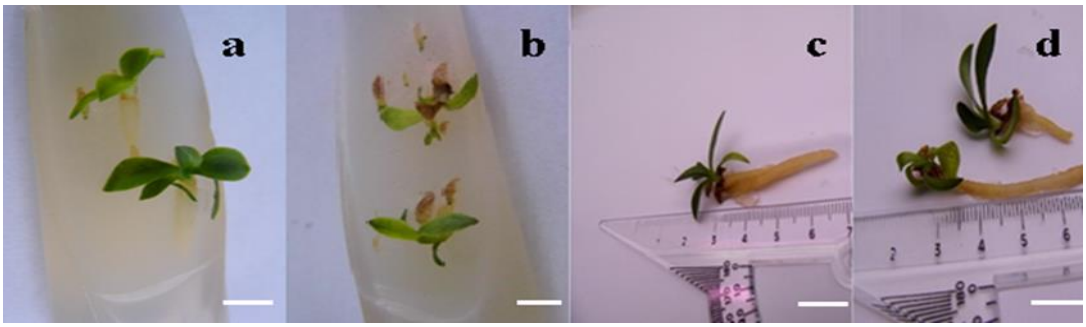

Figure 4. *In vitro* symbiotic seed germination. From Seed to seedlings of. (a); developmental stages (VY25) (b); developmental stages (VY4). (c); Seedling with developed leaves and roots (VY4), (d); Seedling with developed leaves and roots (VY25), Scale bars: 10 mm (a), 10 mm (b), 4 cm (c), 4 cm (d)

It has been generally accepted that fully photosynthetic orchids that grow in a sunny meadow are not mycorrhiza specific because the form genus *Rhizoctonia* has been widely isolated from the roots of these plants (Warjup, 1971; Warjup, 1981; Rasmussen, 2002; MacCormick et al., 2004; Attri L.K. 2022).

Orchid mycorrhizal symbionts play an essential role in the restoration and cultivation of orchids (Oja et al., 2020). For the restoration and cultivation of threatened and endangered orchid species in their natural habitats, their seeds must encounter suitable mycorrhizal fungi and germinate symbiotically (Stewart & Kane, 2006; Aggarwal & Zettler 2010; Sathiyadash et al., 2014; Decruse et al., 2018).

It was stated that most green orchids are associated with polyphyletic *Rhizoctonia* group (Serendipitaceae (Sebacinales), Tulasnellaceae and Ceratobasidiaceae) fungi. (Batty et al., 2006; Valaderes et al., 2011; Zhang et al., 2020). It has been reported in the studies that seasonal change may be effective in the mycorrhizal fungus diversity of orchids (Jumpponen & Jones, 2001; Kohout et al., 2013). Tondello et al. (2012) have reported that it is associated with fungi of the genus *Tulasnella* and *Ceratobasidium* - seasonally different mycorrhizal associations from the roots of *A. morio* (Ercole et al.,

2015). Our study aimed to reveal the variety of fungi participating in mycorrhizal association with the roots throughout the year and the effects of these fungi on seed germination. Our isolations showed that *Rhizoctonia* group isolates were inhabitants of roots throughout the year. It was determined that three genera (*Rhizoctonia*, *Ceratobasidium*, *Tulasnella*) occupied the roots in September-October-November, two genera (*Ceratobasidium* and *Tulasnella*) in December-January-February, and only one genus (*Tulasnella*) hosted the roots in March-April-May and June. In July and August, the above-ground parts were completely dry and no host was found on the roots. Our results revealed that was invaded by more than one fungal group during its development. Sazak & Özdener (2006) isolated *Rhizoctonia* fungi, Tondello (2012) isolated *Ceratobasidium* and *Rhizoctonia* fungi from roots. The results of our study are consistent with these results. Previous studies reported that *Tulasnella* genus is predominantly found in the majority of orchid roots (Jacquemyn et al., 2010; Jacquemyn et al. 2011; Lievens et al., 2010).

It was reported that tulasnelloid (*Tulasnella*) and sebacinoid (Sebacinales) fungi were isolated from *S.spiralis* roots, as well as ceratobasidioid (*Ceratobasidium*) fungi (Caleova et al., 2021). In our study, 91% of the genus *Tulasnella* was isolated from plant roots throughout the year. This result supports the idea that the primary mycorrhizal symbiont of *S. spiralis* is *Tulasnella* as in the study of Ling-Ling et al., (2019).

VY 4 (October) isolates were identified as *Tulasnella calospora*. In our study, *T. fusisporus* (VY 2) strain was isolated from the roots of for the first time in Türkiye. At the same time, VY 11, VY 13 and VY 19 isolates isolated from *Anacamptis sp*. showed 100% homology with the ITS sequence of the isolates isolated from the roots (Kömpe et al., 2020).

It was reported that various fungi can participate in the mycorrhizal association throughout the annual life cycle of the plant and that a fungus obtained during isolation from the roots, primarily during flowering, does not support the germination of the same orchid seeds (Girland et al., 2011).

Some mycorrhizal fungal species interact with the plant only during seed germination, but others continue to interact in the adult stage (Peterson et al., 2004; Zettler et al., 2011). In our study, VY 4 and VY 5 isolates promoted seed germination at the protocorm stage but VY 4 (October) stimulated seedling growth up to stage 4, while VY 5 (October) did not. The best seedling growth was stimulated by VY 25 isolated in April. This result is consistent with the result reported by Girland et al. 2011. In addition, our study showed that the choice of mycorrhizal partner during development is random, which is accordant with the study of Rasmussen (2002).

CONCLUSION

Four isolates belonging to the genus *Tulasnella* (VY 4, VY 18, VY 25) were observed to promote germination and seedling growth (at different rates). According to these results, *Tulasnella* was the most suitable fungus for seed germination among the fungi isolated from roots. Moreover, adult plant roots continue to interact with more than one mycorrhizal fungi to obtain nutrients and adapt to their habitat. However, a comprehensive molecular characterization of the Tulasnella fungi associated with could help restore and cultivate the threatened orchid in its natural habitat.

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Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contributions

İbrahim ÖZKOÇ designed the research plans, read them and made important suggestions, Vildan AKIN MUTLU carried out the experiments and wrote the article.

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