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Fungal diversity and *ex vitro* symbiotic germination of *Serapias vomeracea* (Orchidaceae)

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Abstract – Conservation of orchids can be possible with effective seed germination and seedling growth methods. In this context, *ex vitro* symbiotic seed germination and seedling growth of orchid seeds may be convenient and advantageous. In this study, both the diversity of the root endophytic fungi in *Serapias vomeracea* (Burm.f.) Briq. and the *ex vitro* effects of these fungi on seed germination, seedling development and tuber formation were revealed. The fungi were isolated monthly for two years from *S. vomeracea* roots and the isolates were identified based on morphological characters and internal transcribed spacer (ITS) region of nuclear ribosomal DNA (rDNA) sequences. All of the *Rhizoctonia*-like isolates that joined the mycorrhizal association were closely related to *Tulasnella calospora* (thirty isolates). Non-*Rhizoctonia* isolates are closely related to *Fusarium tricinctum* (two isolates), *Aspergillus spelaeus* (one isolate) and *Talaromyces pinophilus* (Pezizales) (one isolate). The viability rate of the seeds was 90.32%. The seed packs were placed in soils containing fungus and the germination process was followed. All isolates associated with *Tulasnella calospora* promoted germination and seedling development. Isolate Svl 21 (*Tulasnella* sp.) was found to have the highest germination rate (98%) but isolate Svl 4 developed seedlings with advanced leaves (stage 4 (S4): seedlings with advanced leaves and/or rooted, 13.67%). All seedlings at S4 were transferred to the natural environment; the first tubers were observed seven months after. In this study, for the first time, a tuberous European orchid, *S. vomeracea* developed from seed to adult plant in a natural environment.

Keywords: Endophytic fungi, orchid conservation, symbiotic cultivation, *Serapias vomeracea*

Introduction

Orchids are of great importance as medicinal, food and ornamental plants in the world. They are under threat of extinction as they are over-collected from nature and their habitats are destroyed (Rasmussen 2002). In addition, orchid tubers are collected too much for salep production and medical purposes in Greece, Iran and Turkey (Sezik 2002, Ghorbani et al. 2014, Kreziou et al. 2016). Although they produce a large number of seeds it is not easy to propagate them in the natural environment by seed germination (Rasmussen 2002). However, seed germination is the most important factor for both medical, commercial and *in situ*/*ex situ* conservation and reintroduction. Orchids propagated from seeds are used successfully in reintroduction, especially in orchid protection projects (Paul et al. 2012). Previous research results indicate that more than one fungus joins the mycorrhizal association continuously or seasonally in the roots of orchids (Tondello et al. 2012). Inoculation with appropriate fungus is the most important factor for the orchid to adapt to the natural environment and survive because the population size of orchids and the number of individuals are associated with the presence and abundance of suitable fungi in the soil. The decrease in numbers of particularly rare and threatened species results from the loss of mycorrhizal fungi due to habitat change or destruction (Rasmussen 2002). In this context, understanding the relationship between orchids and fungi is important for the protection of orchids, their reintroduction and production for agricultural purposes. Most orchid mycorrhizal fungi belong to *Rhizoctonia*-like fungi, a diverse polyphyletic group of pathogens, endophytes, saprophytes and mycorrhizal fungi (Bayman and Otero 2006). This group includes the anamorphic (asexual) genera, *Ceratorhiza*, *Epulorhiza*, *Moniliopsis*, and *Rhizoctonia* (Moore 1988). The teleomorphs (sexual stages) of these genera are *Ceratobasidium*, *Tulasnella*, *Sebacina and Thanatephorus*, respectively (Bayman and Otero 2006).

Tropical and temperate orchids can also be produced by *in vitro* asymbiotic methods. However, the procedure re-

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quires complete axenic conditions and expensive laboratory equipments. Also, asymbiotic seedlings are difficult for *in situ* or *ex situ* adaptation (Aewsakul et al. 2013). Alternatively, successful results have recently been obtained from *ex vitro* symbiotic seed germination studies. However, there are very few studies in which this method has been applied. Quay et al. (1995) and Aewsakul et al. (2013) germinated some epiphytic orchids symbiotically in *ex vitro* conditions.

There is not enough research on *ex vitro* symbiotic seed germination and seedling development of temperate orchids (especially Euroasian tuberous orchids). Due to the glucomannan content of their tubers, orchids have a considerable economic importance as an additive in ice cream, salep and other foods (Sezik 2002). Despite their economic importance, they have not yet been cultured and are under serious threat of extinction due to the collection of thousands of tubers every year. In order to prevent the destruction and extinction of temperate orchids, it is essential to produce them with a fast, easy and inexpensive method. Due to anthropogenic effects and global climate change many orchids will be under a threath of extinction in the near future.

There are five species included in the *Serapias* genus in Turkey, and *Serapias vomeracea* (Burm.f.) Briq. is one of the most collected orchid species because they have 1-5 tubers and a high glucomannan content. A large number of studies have been conducted on *in vitro* asymbiotic and symbiotic germination of the seeds (Ozkoc and Dalcı 1993, Acemi and Ozen 2019). However, temporal fungal diversity in *S. vomeracea* roots and the effects of these fungi on symbiotic germination and seedling growth in *ex vitro* conditions have not been investigated.

Hence, the main objectives of this study are: (i) to establish the diversity of fungi participating in mycorrhizal association in the roots of *S. vomeracea*, (ii) germination of *S. vomeracea* seeds and seedling formation in the presence of root endophytic fungi in *ex vitro* conditions, and (iii) to determine whether an *ex vitro* symbiotic method is suitable for orchid production. It is also hoped that the data obtained from this study will be useful for further reproduction and conservation of this orchid species.

Materials and methods

Study site

Serapias vomeracea is distributed in the coastal area of the Black Sea and Aegean regions of Turkey. The research area of this study is Ondokuz Mayıs University campus area. The habitats of the species are the open spaces located next to oak forests at about 50 m a.s.l. The species of the genera *Hordeum*, *Avena* and *Melilotus* are present in these habitats and over 100 *S. vomeracea* individuals are also represented in the research area. They bloom in May and the seeds maturate in July. In 2015, seasonal avarage temperatures and amount of precipitation were 12.2 °C and 176 mm in spring, 23.9 °C and 85 mm in summer, 16.8 °C and 134 mm in autumn. In 2016, they were: 13.8 °C and 171 mm in spring, 24.8 °C and 69 mm in summer, and 15.1 °C and 133 mm in autumn.

Physical and chemical properties of the soil

For soil analyses, an about 2 kg sample was taken from 0-15 cm depth of 1 m^2 area after removing leaves and other debris from the surface of soil in research area where *S. vomeracea* is commonly distributed. The soil was dried at room temperature in laboratory and used for all analyses.

In the soil saturated with water, a direct pH reading was taken with a glass electrode pH meter (Aciego Pietri and Brookes 2008). Electrical conductivity was determined with an EC – meter in saturation extract (Rhoades 1996), organic matter using the Smith-Weldon method (Nelson and Sommers 1982). Total sand, silt, lime and clay were determined by the densimeter method (Bowman and Hutka 2002). Exchangeable cations (calcium, magnesium, and potassium) were determined with the Mehlich-3 method (Mehlich 1984). Available phosphorus was analysed using the molybdate blue method (Murphy and Ridley 1962).

Fungus isolation

Before root samples were collected, *S. vomeracea* phenological stages were followed for one year in their habitats (Campus area of Ondokuz Mayis University). It was determined that the leaves formed in February to March and the roots completely dried in July.

The roots for fungi isolation were taken through two years (2015-2016). Every month, complete roots of a plant were collected. Cross-sections were then taken from the roots and examined under a microscope for the presence of fungi. All the roots containing fungal coils were used for fungi isolation, performed according to Clements et al. (1986). The roots were sterilized in 1.5% NaOCl solution for 5 minutes and washed in sterile distilled water. Under aseptic conditions, root pieces (1-2 cm) were placed in petri dishes containing fungi isolation medium (FIM) (Clements et al. 1986) and incubated at 27 °C for 2 days in the dark. Following stereo microscopy examination, any fungal colonies were transferred to FIM and purified. Pure fungus cultures were stored at 4 °C.

Morphological and molecular identification of the fungi

Isolates for preliminary identification were grown in FIM plates and thin agar blocks of 48-h-old cultures were viewed under a microscope for the mycelial and branching characteristics of the isolates. The following distinct morphological characters were observed: mycelia branched at acute to right angles, constrictions at or near the point of branching and septum formation near the branching point. Isolates showing rhizomorph and asexual spore (conidia) structure were defined as non–*Rhizoctonia*. The isolates were grown in potato dextrose agar (PDA) at 25 ± 2 °C for

7-8 days to determine the color and texture of the colonies. The color of the colony was defined according to the color chart of the Royal Horticultural Society of London. The hypha diameter of the fungi was measured under light microscope. To determine the number of nuclei in the cell, isolates were grown for three days at 25 °C in Petri dishes containing water-agar (WA), stained with Safranine-O solution and nuclei were counted under the microscope (Bandoni 1979).

DNA isolation from fungal mycelia was performed using the CTAB (cetyltrimethyl ammonium bromide) method described by Pascual et al. (2000) with some modifications (e.g., 50 mg of mycelium was crushed in a sterile mortar in 1 mL extraction buffer and incubated for 30 min.) For each isolate, the internal transcribed spacer (ITS) region of the nuclear ribosomal rDNA was amplified by PCR. ITS1 and ITS2 regions, including the ribosomal 5.8S RNA gene, were amplified using the universal primers ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5' TCCTCCGCTTATTGATATGC 3') (White et al. 1990). PCR amplification reactions were performed in a 50 µl reaction containing 1 µl genomic DNA (1 ng μl^{-1}), 1 µl (2.5 mM) dNTP mix (Sigma), 0.25 µl Taq DNA polymerase (5 U/µl) (Promega, Go-TaqFlexi DNA Polymerase), 1 µl each of primers (25 pmoles), 10 µl 5×PCR buffer supplied by manufacturer (Promega, Go-Taq Green Buffer) and 3 µl $MgCl_2$ (1.5 mM) (Sigma) and 32.75 µl sterile ddH20. PCR amplification was carried out as follows: an initial denaturation at 94 °C for 3 min followed by 30 cycles of 94 °C for 1 min, 49 °C for 2 min, 72 °C for 3 min and a final extension at 72 °C for 7 min (Salazar et al 1999).

PCR products of the rDNA-ITS region were sequenced by Macrogen (Macrogen Inc., Seoul, Republic of Korea) by using ABI 3730 XL DNA sequencer with ITS1 and ITS4 primers. For each PCR product, sequences of both strands were combined to generate a consensus sequence by using BioEdit version 7.2.5 software (Hall 1999). The consensus sequences of the rDNA-ITS region were compared with the sequence data in GenBank (National Center for Biotecnology Information) by using BLASTn tool. We used 97 to 100% sequence identity to delimit fungal species and genera. One sequence for each isolate was deposited in the GenBank database. DNA polymorphism was determined for the ITS gene sequences using DNA Sequence Polymorphism software (DNASP), version 6.0 (Rozas et al. 2017). Haplotype groups were formed according to the nucleotide polymorphism of the sequences. The data set formed for *Tulasnella* species including the sequence of 15 fungal isolates and the 24 reference sequences obtained from NCBI.

Genetic distances for data sets were calculated using the MEGA 6 software package (Tamura et al. 2013). The tree (*Rhizoctonia*-like) was constructed using Maximum Likelihood (ML) analysis with 1000 bootstrap replications using MEGA 6.

Collection of the seeds and seed viability test

Capsules produced by natural pollination were collected from the individuals grown in the meadows next to oak forests in 2015. The seeds were removed from the capsules, kept in room conditions for a few days in the laboratory to lose moisture, and then stored at 4 °C. Pre-treatments for viability test and TTC (2,3,5 triphenyl tetrazolium chloride) test were conducted according to Kömpe et al. (2020) and Van Waes and Deberg (1986), respectively. Approximately 100-150 seeds were placed in each pack. The seeds were incubated in moist cocopeat for 7 days so that the seed coat could crack. Thus, TTC would be allowed to enter into the embryo. The seeds were incubated in TTC (1%) solution for 12 hours at 28 °C, followed by 12 h incubation in sterile distilled water. The viability experiments were performed with six repetitions. The seeds with red-pink embryos were evaluated as live. Viability rates (%) = number of stained embryos /total seed number × 100.

Germination of the seeds and development of the seedlings under *ex vitro* condition

The soil samples were taken from 3-5 cm depth and 30 cm around the adult orchids individuals. A mixture of 2:1 soil: perlite was prepared and sterilized in autoclave at 121°C for 20 min. The sterile soil mixture was filled into pots (20 x 31 x 13 cm) to a depth of 15 cm. The seeds were placed between sheets of water-resistant nylon mesh (45 µm pore size). Approximately 400-500 seeds (10 mg) were placed in each pack. Ten packs of seeds were placed in each pot. Fifteen *Rhizoctonia*-like (*Tulasnella*) and four non-*Rhizoctonia* isolates (*Fusarium*, *Aspergillus*, *Talaromyces*) were used in germination tests. For each fungal isolate, two independent pots containing sterile soil mixture were prepared. Fungi were grown on FIM for 7 days and fungus discs (1-2 mm in diameter) obtained from then were placed in each corner of the pots. Seed packs were placed in control pots not inoculated with fungus. *Ex vitro* germination and growth experiments were performed with six repetitions. The pots were incubated at 25 ± 2 °C in the climate chamber with a 16/8h light/dark photoperiod and 33 PAR (photosynthetic active radiation). It was irrigated with sterile distilled water once a week. Three months after the seeds were embedded in the pots, 3 seed packs were randomly selected from each pot to calculate germination and seedling growth rates and evaluated according to the scale of Clements et al. (1986).

The extent of seed germination and development was divided into stages: S0, S1, S2, S3 and S4. These stages are represented as follows: S0: No germination (seed); S1: Protocorm; S2: Leaf premordium; S3: The first photosynthetic leaf; S4: Seedling with advanced leaves (and/or rooted).

Germination percentage was calculated as number of seeds in stages 1-4 divided by the total number of seeds (Clements et al. 1986). Germination rates (%) = number of germinated seeds /total seed number X 100.

Microscopic observations of mycorrhizal associations

For showing that mycorrhizal association had been established, transverse sections were taken from experimental protocorms produced *ex vitro*, from the roots of experimen-

tal seedling and from the roots of adult plants in natural habitat, and 0.1% lactophenol (RAL Diagnostic) was dropped and incubated for 10 min at 25 °C. The sections were then washed with distilled water to remove residual staining. Each section was examined under a microscope for presence of fungal coil and photographed.

Statistical analysis

The viability tests and the effects of fungal isolates on *ex vitro* germination and growth were analyzed using one way-ANOVA. Results were compared using the SD (standard deviation) of means, and the post-hoc Duncan's multiple range test. Statistical significance was set at $P < 0.05$. All analyses were performed in SPSS 15.0 (SPSS Inc., Chicago, USA).

Results

The soil properties were as following: electrical conductivity (EC, dS m^{-1}) 0.896, pH 6.88, organic matters 7%, clay 12.43%, silt 21.79, sand 65.78%, lime: 3.17, Ca + Mg (cmol kg^{-1}) 25.04 + 18.40, P (ppm) 6.21, K (cmol kg^{-1}) 1.76.

The fungi of *Serapias vomeracea*

In the first year of the experiment, the phenological stages continued from March to July. In the second year they took place between February and June. Fungal isolations were made from March to July in 2015 and from February to June in 2016. From the roots of *S. vomeracaea* 18 isolates (Svl 1-18) were found in the first year and 16 in the second year (Svl 19-34). Applying the pre-identification procedure, two main isolate groups were determined: binucleate *Rhizoctonia*-like group with 30 isolates and multinucleate non-*Rhizoctonia* group with 4 isolates. *Rhizoctonia*-like (*Tulasnella* sp.) isolates were found every month when fungal isolation was carried out. Among non-*Rhizoctonia* isolates, two isolates of *Fusarium* sp. were isolated in March 2015, one isolate of *Aspergillus* sp. in June 2015 and one isolate of *Talaromyces* sp. in February 2016 (Fig. 1). Morphological features of the isolated fungi in both groups (hyphae diameter, number of nuclei, colony color, colony appearance) were determined. Three different colony colors of *Rhizoctonia*-like isolates were determined after growth on PDA medium at 25 ± 2 °C in the dark for two weeks. The most frequently observed colony color was grayish yellow, while orange-white and yellowish white were observed less frequently. Colony appearance was frequently submerged and powdery. Vegetative hyphae color of all the isolates was transparent. Hyphae diameter varied between 2.5 and 4.4 µm. Vegetative hyphae color of all isolates was transparent. Colony color of the non-*Rhizoctonia* isolates were determined as red and orange (Svl 10, Svl 11, respectively), white (Svl 13), grayed yellow (Svl 20), while the colony appearance was determined as submerged aerial hyphae and powdery. Vegetative hyphae color of all isolates was transparent. Hyphae diameter varied between 2.5 and 4.4 µm.

The PCR products of the ITS regions of all the fungal isolates were sequenced and aligned. Obtained consensus sequences were uploaded to GenBank and compared with other sequences from that database. Accession numbers for our fungal ITS regions and identity percentages with most closely related sequences from GenBank are given in Tab. 1. According to BLASTn results, 2 of the 4 non-*Rhizoctonia* isolates were found to show 100% identity to *Fusarium tritinctum* (Svl 10 – Svl 11) (GenBank acession number

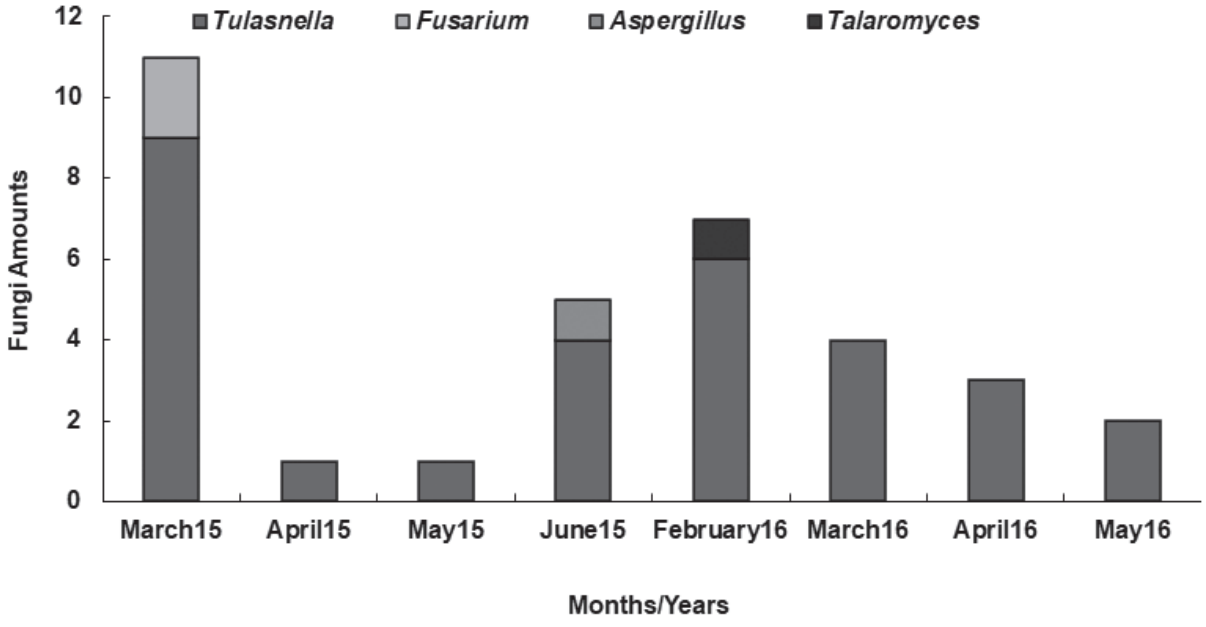

Fig. 1. Numbers of endophytic root fungi of *Serapias vomeracea* during the isolation months at 2015 and 2016. *Rhizoctonia*-like (*Tulasnella* sp.) isolates were obtained every month when fungal isolation was carried out. Among non-*Rhizoctonia* isolates, two isolates of *Fusarium* sp. were isolated in March 2015, one isolate of *Aspergillus* sp. in June 2015 and one isolate of *Talaromyces* sp. in February 2016.

Tab. 1. Molecular identification of mycorrhizal fungi isolated from *Serapias vomeraceae* roots based on the closest match in the GenBank. BLAST results show the top hit matching the sequencing. Two *Fusarium tritinctum* (MK250655 – MK250515) one *Aspergillus spelaeus* (MK250156) and *Talaromyces pinophilus* (MK255324) isolates were found. The other isolates had identity rates to uncultured *Tulasnella* (eg. MK250064, MK250062, MK250060 etc.). Length per base pair of DNA (bp), GenBank Accession Number (AN), Close relatives (a unique identifier assigned to records in the NCBI databases), the highest percent identity for fungi sequences (% ID) and references are given).

Isolate designation	bp	GenBank (AN)	Close relatives (AN)	%ID	Reference
Svl 3	567	MK250064	Uncultured Tulasnellaceae (KC243935.1)	98	Těšitelová et al. 2013
Svl 4	547	MK250062	Uncultured *Tulasnella* (JF926504.1)	100	Girlanda et al. 2011
Svl 5	583	MK250060	Uncultured Tulasnellaceae (JX649082.1)	98	Bailarote et al. 2012
Svl 9	603	MK250058	Uncultured Tulasnellaceae (JX649082.1)	99	Bailarote et al. 2012
Svl 10	548	MK250655	*Fusarium tricinctum* (JX045791.1)	100	Unpublished
Svl 11	609	MK250515	*Fusarium tricinctum* (HQ703409.1)	100	Unpublished
Svl 12	573	MK256219	Uncultured Tulasnellaceae (JX649082.1)	99	Bailarote et al. 2012
Svl 13	609	MK250156	*Aspergillus spelaeus* (MG976863.1)	97	Unpublished
Svl 14	603	MK250061	Uncultured Tulasnellaceae (JF926504.1)	99	Girlanda et al. 2011
Svl 15	608	MK250075	Uncultured Tulasnellaceae (JX649082.1)	99	Bailarote et al. 2012
Svl 18	6 10	MK250519	Uncultured Tulasnellaceae (JF926504.1)	99	Girlanda et al. 2011
Svl 19	510	MK250524	Uncultured Tulasnellaceae (JX649082.1)	99	Bailarote et al. 2012
Svl 20	499	MK255324	*Talaromyces pinophilus* (LT558963.1)	100	Guevara-Suarez et al. 2016
Svl 21	450	MK281614	Uncultured Tulasnellaceae (JF926504.1)	99	Girlanda et al. 2011
Svl 22	615	MK250526	UncultrednTulasnellaceae (JX024734.1)	99	Jacquemyn et al. 2012
Svl 29	496	MK250522	*Tulasnella* sp (KF537647.1)	99	Ding et al. 2014
Svl 30	310	MK250656	Uncultured Tulasnellaceae (JX649083.1)	99	Bailarote et al. 2012
Svl 31	406	MK250520	*Tulasnella* sp. (JQ713578.1)	99	Unpublished
Svl 34	603	MK250530	Uncultured Tulasnellaceae (JF926504.1)	100	Girlanda et al. 2011

MK250655, MK250515 respectively), while one isolate was found to show 99% identity to *Aspergillus spelaeus* (Svl 13) (GenBank accession number MK2505615) and one isolate was found to show 99% identity to *Talaromyces pinophilus* (Svl 20) (GenBank acession number MK255324). All of the *Rhizoctonia*-like isolates had identity rates to uncultured *Tulasnella* (eg. Svl 1, Svl 3, Svl 4 etc.) of between 97-100% (GenBank acession number MK249887, MK250064, MK250062, respectively) (Tab. 1). A phylogenetic tree was constracted to reveal the phylogenetic relationship between *Tulasnella* isolates of *S. vomeracea* and *Tulasnella* sp. from other orchids (Fig. 2).

Among the ITS sequence data set, 15 haplotypes were detected based on sequence analysis of 30 *Tulasnella* isolates. ITS sequences exhibited a haplotype diversity of 87%. Haplotype 5 exhibited the highest frequency within the ITS sequences, being generated from 12 sequences. Haplotype 4 and haplotype 7 exhibited low frequencies within the ITS sequences, and were generated respectively from 4 and 2 sequences. Other haplotypes exhibited the lowest frequencies among the ITS sequences, and were generated from only one sequence each. *Tulasnella* ITS sequences from our study grouped with *T. calospora* clade. In the phylogenetic tree, *Tulasnella* isolates (e.g. Svl 3, Svl4, Svl5, Svl9 etc.) isolated from *S. vomeracea* roots were therefore found to be closely associated with *T. calospora*. (GenBank accession no: AY373298.1, FJ613176.1, GU166421.1, HQ889722.1 etc.) (Fig. 2).

The seed viability test

The embryos of seeds incubated in cocopeat for 1, 2 and 3 days were not stained. The viability rates of the seeds incubated for 5 days and 7 days were found to be 40.00% ± 9.27 and 90.32% ± 1.30, respectively.

***Ex vitro* symbiotic germination and symbiotic association with the seeds**

Ex vitro germination tests were evaluated for total germination and development stages after three months of incubation. Developmental stages (S1-4) are shown in Fig. 3A. *Fusarium tricinctum* (Svl 10, Svl 11), *Aspergillus spelaeus* (Svl 13) and *Talaromyces* (Svl 20) isolates did not promote germination.

There was no germination in the control pots without fungi (Fig. 3B). All the *Tulasnella* isolates on the phylogenetic tree promoted germination and growth at varying rates (Tab. 2, Fig. 3C-E). According to the counts performed at the end of three months, 98% of seeds germinated in the packages inoculated with the isolate Svl 21 and the seeds inoculated with this isolate developed until to S3. The seeds placed in the pots inoculated with Svl 4, Svl 14 and Svl 34 germinated at the rates of 93.2%, 94% and 90% respectively, and these isolates supported the development of the seedling up to the S4 (13.67%, 10.8%, 9.8%, respectively) (Fig. 3D, E). The percentage of seedlings that reached the S4 of development (13.67%) inoculated with the isolate Svl 4 was

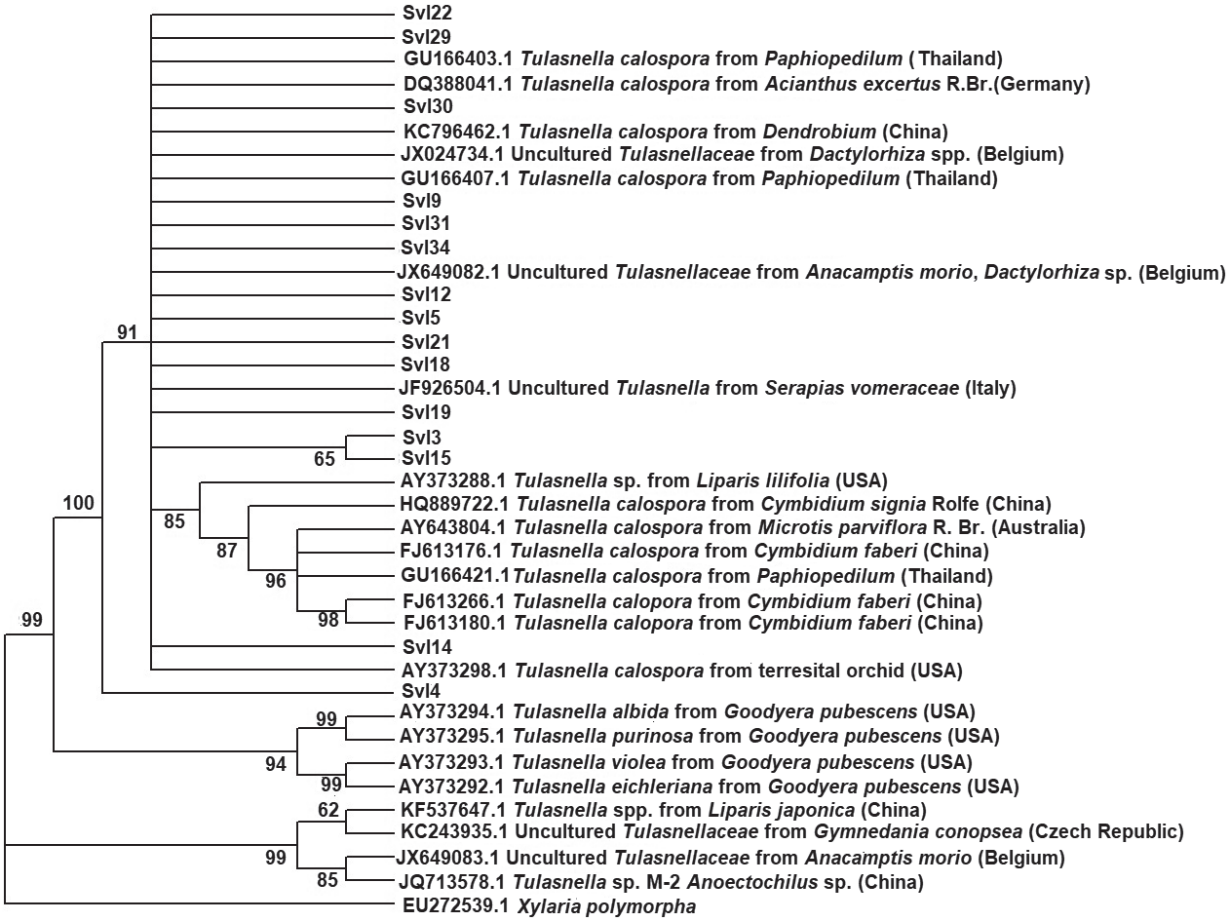

Fig. 2. Maximum-Likelihood (ML) tree based on an alignment of ITS-5.8 sequences, showing relationship of fifteen *Tulasnella* species. Node tips show NCBI accession numbers followed by fungal species name, host species and country name. The tree was rooted with the sequence of *Xylaria polymorpha* (accesion number EU272539.1) Numbers above branches are maximum likelihood bootstrap probalities (>50%).

found to be statistically significantly high when compared with seedlings reaching the same stage of development inoculated with other fungi.

Thirty seedlings grown in pots (S4- seedlings with advanced leaves (and/or rooted)) were planted in their natural habitats and the first real tubers occurred at 5 months after

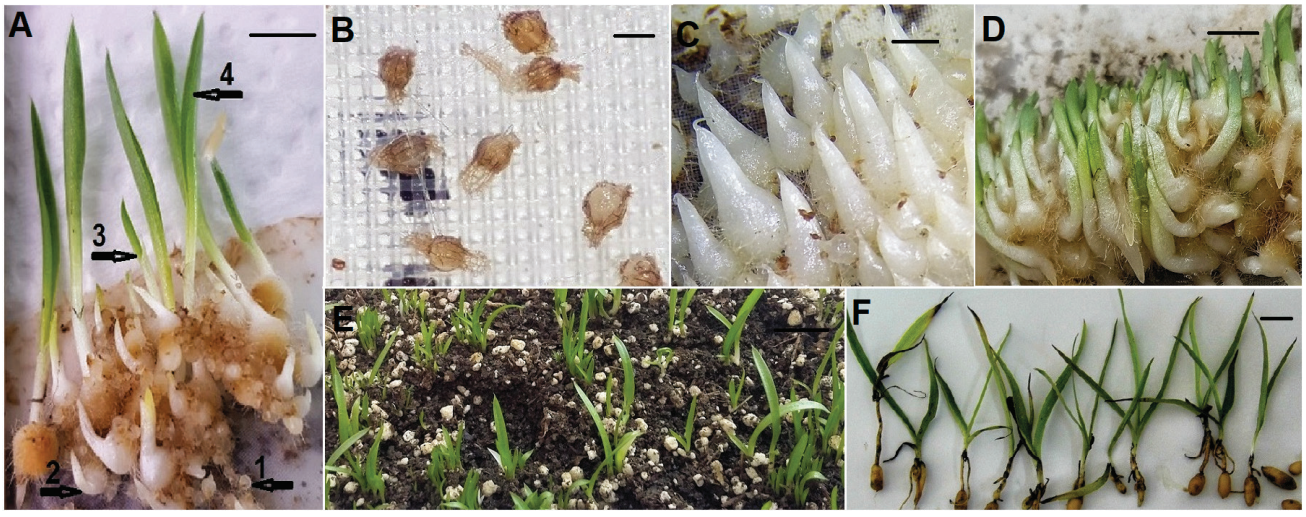

Fig. 3. From the seed to the seedlings of *Serapias vomeracea*. A – developmental stages (S1-4). Arrows and numbers show the developmental stages. B – control (no fungal isolate). Seed coats ruptured but, no advanced development (S0). C – protocorms and leaf primordiums, D – leafy plantlet, E – the seedling in natural area, F – the first tuberous adult plant. Scale bars: A – 10 mm, B – 0.3 mm, C – 0.2 mm, D – 10 mm, E – 10 mm, F – 10 mm.

Tab. 2. Germination and development rates after three months from inoculation of *Serapias vomeracea* seeds with *Rhizoctonia*-like fungi in *ex vitro* conditions. Development of the seedlings was divided into stages: S0, S1, S2, S3 and S4. S0: No germination (seed); S1 – protocorm, S2 – leaf primordium, S3 – the first photosynthetic leaf, S4 – seedling with advanced leaves (and/or rooted). The seed packs in control pots were not inoculated with fungal isolate. The effects of fungal isolates on *ex vitro* germination and developmental stages were analyzed using one way-ANOVA. Results were compared using the SD (±: standard deviation) of means, and the post-hoc Duncan's multiple range test. Statistical significance was set at $P < 0.05$. There is no statistically significant difference between groups with the same letters, N = 6.

Fungi	%Germination	S0	S1	S2	S3	S4
Control	0.00 ± 0.00e	100 ± 0.00a	0.00 ± 0.00g	0.00 ± 0.00h	0.00 ± 0.00e	0.00 ± 0.00d
Svl 3	94.00 ± 2.54ab	6.00 ± 2.54de	4.40 ± 1.67fg	78.00 ± 3.16a	11.60 ± 2.50d	0.00 ± 0.00d
Svl 4	93.20 ± 2.38ab	6.80 ± 2.38de	11.97 ± 8.79e	39.27 ± 8.07ef	27.94 ± 7.88a	13.67 ± 5.35a
Svl 5	87.00 ± 3.39cd	13.00 ± 3.39bc	4.80 ± 0.83fg	59.40 ± 3.28b	17.80 ± 3.49bcd	5.20 ± 1.64c
Svl 9	94.00 ± 3.16ab	6.00 ± 3.16de	7.60 ± 1.14ef	65.80 ± 4.14b	15.20 ± 2.94cd	5.40 ± 3.20c
Svl 12	87.00 ± 4.06cd	13.00 ± 4.06bc	19.00 ± 2.23d	52.00 ± 4.63c	10.60 ± 2.60d	5.40 ± 2.07c
Svl 14	94.00 ± 3.74ab	6.00 ± 3.74de	0.60 ± 0.89g	63.60 ± 5.59b	19.00 ± 5.47bcd	10.80 ± 2.16b
Svl 15	82.40 ± 3.28d	9.60 ± 6.84cd	22.80 ± 3.56d	42.40 ± 0.64de	12.00 ± 1.87d	4.80 ± 1.64c
Svl 18	87.00 ± 4.06cd	13.00 ± 4.06	19.00 ± 2.23d	52.00 ± 4.63c	10.60 ± 2.60d	5.40 ± 2.07c
Svl 19	87.00 ± 3.08cd	13.00 ± 3.08bc	32.8 ± 6.76b	43.60 ± 7.95de	10.20 ± 3.27d	0.40 ± 0.54d
Svl 21	98.00 ± 2.00a	2.00 ± 2.00d	21.80 ± 10.40d	47.40 ± 8.04cd	28.8 ± 17.81a	0.00 ± 0.00d
Svl 22	90.00 ± 4.69bc	10.00 ± 4.69cd	25.00 ± 0.09cd	41.00 ± 6.40de	24.00 ± 5.65ab	0.00 ± 0. 00d
Svl 29	94.60 ± 3. 97ab	5.40 ± 3.97de	60.40 ± 5.12a	20.60 ± 3.28g	10.40 ± 1.32d	0.00 ± 0. 00d
Svl 30	93.20 ± 3.56ab	6.80 ± 3.56de	34.20 ± 6.64b	33.20 ± 3.49f	27.80 ± 3.16a	0.00 ± 0. 00d
Svl 31	83.60 ± 3.84d	16.40 ± 3.84b	36.80 ± 2.86b	24.00 ± 5.33g	22.80 ± 2.85abc	0.00 ± 0. 00d
Svl 34	90.00 ± 3.16bc	10.00 ± 3.16cd	30.40 ± 2.96bc	39.00 ± 2.23ef	12.80 ± 1.49bc	9.80 ± 2.281b

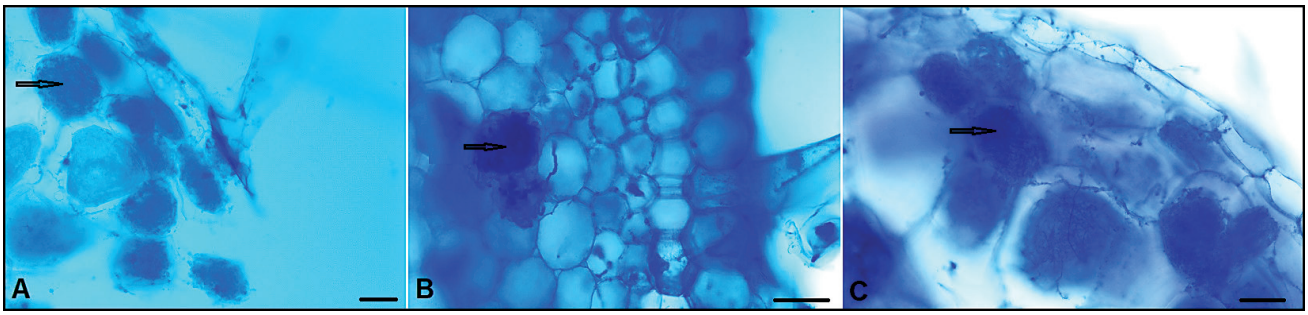

Fig. 4. Fungal pelotons stained with lactophenol cotton blue. A – in the cortical cells of adult *Serapias vomeracea* roots, B – in the *ex vitro* protocorm cells, C – in the cortical cells of the roots of *ex vitro* seedlings. The arrows indicate fungal coils. Scale bars: 50 µm.

the seedlings were transferred to the soil. All of the seedlings transferred to soil developed tubers (Fig. 3F).

The presence of symbiotic association was shown in cross-sections from the roots of adult plants, the *ex vitro* experimental protocorms and the *ex vitro* experimental seedling roots (Fig. 4A, B, C, respectively).

Discussion

Orchids are in danger of extinction due to severe destructive factors such as destruction of natural habitats, excessive tuber harvesting for medical or commercial purposes, and global climate change (Ghorbani et al. 2014). Symbiotic propagation of orchids with suitable root endophytic fungi is a very advantageous method for reintroduction to the habitats of endangered orchids and for agricultural cultivation (Aewsakul et al. 2013).

During the annual life cycle of the plant, various fungi can join the mycorrhizal association, and a fungus obtained during isolation from roots, especially in the flowering period, may not encourage the germination of the seeds of the same orchid (Girlanda et al. 2011). For this reason, all fungi participating in the mycorrhizal association were obtained by culture-dependent isolation method performed monthly for two consecutive years. It was determined that all our *Rhizoctonia*-like isolates are closely related to *Tulasnella calospora*. Girlanda et al. (2011) reported that members of *Ceratobasidium* as well as *Tulasnella* joined the mycorrhizal association of *S. vomeracea*. However, *Ceratobasidium* was not isolated in our study. All the *Tulasnella* isolates supported the development at various rates. The differences in development to S4 may be due to the test period being limited to 3 months. Differences in the effects of the fungal isolates on germination and development indicate that isolates re-

lated to *Tulasnella* may be different at species level. Therefore, their precise identification is needed to make using molecular techniques.

It was shown by Fracchia et al. (2014) and Vujanovic et al. (2000) that some *Fusarium* species promoted germination of tropical orchid seeds. None of the following species, *Fusarium tricinctum* (Svl 10 and Svl 11), *Aspergillus spelaeus* (Svl 13) and *Talaromyces pinophilus* (Svl 20), all isolated in our study, stimulated germination of the seeds.

In recent years, it has been indicated that the members of Pezizales are also members of the mycorrhizal association (Stark et al. 2009). Also, it has been determined that fungi included in the order of *Pezizales* also join the mycorrhizal association in the roots of *Dactylorhiza* (Kömpe and Mutlu 2017), *Anacamptis* and *Orchis* species (Mutlu and Kömpe 2020). Although non-*Rhizoctonia* fungi in orchid roots join the mycorrhizal association, it has been reported that they do not establish a symbiotic relationship with seeds (Stark et al. 2009). Our study also indicates that non-*Rhizoctonia* fungi do not establish a symbiotic relationship with *S. vomeraceae* seeds.

Orchid tubers are economically important in Turkey, Iran, and Greece. Therefore, large-scale cultures should be set up for their commercialization. *Ex vitro* symbiotic propagation of orchids has certain advantages over *in vitro* asymbiotic propagation since the asymbiotic method requires complex laboratory equipment, expensive chemicals and sterile laboratory conditions. In addition, studies on germination and seedling growth in natural conditions (*in situ*, *ex situ*) are also not suitable. Structure of the soil, other microorganisms, various insect larvae or nematodes make the efficiency of this method difficult (McCormick et al. 2013). Otherwise, it is stated that *ex vitro* production is easy, cheap and the most suitable method for mass production (Aewsakul et al. 2013). Soil or a substrate is sterilized to eliminate biotic factors, while superficial, surface sterilization of seeds and other complex laboratory methods and materials are not required.

There are very few studies on *ex vitro* germination and seedling development of orchid seeds to date and they are about epiphytic orchids (Quay et al. 1995, Aewsakul et al. 2013). Hence, this research is the first study in which the seeds of *S. vomeracea*, a temperate tuberous orchid, were germinated in *ex vitro* conditions and the tuber formation in natural conditions was observed. This orchid is one of the most collected orchids because it is one of the orchids with highest glucomanan content (Ozkoç and Dalcı 1993, Acemi and Özen 2019).

According to the results of this study, *ex vitro* seedlings of *S. vomeracea* under heavy destruction may establish new populations after being transferred to nature and this may contribute to the rehabilitation of destroyed areas. The most important indicator of the adaptation of tuberous temperate orchids to their natural conditions is the occurence of the first tuber, because it remains in the soil and develops as a new individualin the following year (Sezik 2002). Production of tubers by all seedlings transferred to soil indicates that the reintroduction to natural conditions after *ex vitro* symbiotic germination is succesful. Thus new populations may be established by applying this method to the other orchids under threat of extinction.

Tetrazolium test showed that viability of the seeds of *S. vomeracea* (90.32% ± 1.30) was lower than that observed in germination test with Svl21 (98% ± 2). Several studies indicated that the tetrazolium test was not a reliable indicator for orchid seeds (Vujanovic et al. 2000, Kömpe et al. 2020). There may be several reasons for this, which suggests that the viability test is not a good indicator for the germination of orchid seeds. Incubation period may not be sufficient for the breakage of all the seed coats. Water may not reach equally to all seeds in a seed package, or seed coats did not break in a way that the dye can penetrate evenly. For this reason, viability tests must be supported by seed germination tests.

While viability test is not always a reliable indicator of germination potential for orchid seeds, performing a viability test on seeds for which germination potential is not known may be useful to prevent loss of time and materials.

Conclusions

Rhizoctonia-like fungi isolated from *S. vomeracea* roots are closely related to *Tulasnella*, therefore it can be said that the dominant fungus of the mycorrhizal association in *S. vomeracea* is generally *Tulasnella*. In this research, the life cycle of *S. vomeracea* from seed to tuberous seedling stage was presented for the first time in *ex vitro* conditions. With our method, it is possible to produce orchids on a large scale and to prevent orchid destruction and help reintroduction to their natural habitats.

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