Specific detection of Tirmania pinoyi in roots of artificially infected several perennial Cistaceae by Nested-PCR

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Research Article

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Abstract

Truffle farming does not exist in Iran, and no formal studies have been conducted on its culture. For commercial production of *Tirmania pinoyi*, it is necessary to accurate detection of it in the roots of the host plants. In this study, for the first time, mycorrhizal association of *T. pinoyi* with several perennial Cistaceae including *Helianthemum lippii*, *H. almeriense*, *Cistus albidus*, and *Cistua ladanifer* were investigated. In all plant species inoculated with *Tirmania pinoyi*, an ectendomycorrhizal association with varying degrees of sheath development was observed. Our results shows that *H. lippii* is suitable plant host for *Tirmania pinoyi*, and mycorrhization rate and the relative mycorrhizal dependency (RMD) in this native plant was about 90% and 57.85%, respectively, and higher than the other three plant species. The effect of the mycorrhizal fungus on host growth (root weight, root height, shoot weight, shoot height, plant height, and plant weight) was statistically significant compared to non-inoculated plants. To detect the roots colonized by *T. pinoyi*, a PCR based diagnostic method was developed with the species-specific primer pairs FTiPi and RTiPi designed from the sequence of the internal transcribed space regions (ITS1 and ITS2). The specificity of the primers was verified by PCR analysis of DNA from *T. pinoyi* specimens and other desert truffles. The method detected *T. pinoyi* in artificialy infected root plants, and no cross-reactions were observed with any other tested desert truffles. This species-specific PCR method is suitable for quick, simple, and reliable detection of *T. pinoyi* mycorrhizas.

Introduction

Desert truffles are edible hypogeous fungi that establish mycorrhizal symbiosis with annual and perennial shrubs belonging to Cistaceae (Kagan-Zur et al. 2013). Member of Cistaceae family adapted to arid and semiarid ecosystems of Middle East and Mediterranean region that aridity index (AI = annual precipitation/potential evapotranspiration) in these areas is lower than 0.5 (UNEP 1992). In the arid and semiarid ecosystems, mycorrhizal symbioses promote growth and help their host plants to thrive by increasing the supply of nutrients, especially P from nutrient-stressed soils (Morte et al. 2000), reducing water stress (Morte et al. 2010), and improving soil aggregation in eroded soils (Caravaca et al. 2003). Among desert truffles, *Terfezia* and *Tirmania* are well known delicacies and largely marketed (Bouzadi et al. 2017) and play an important role in the maintenance of rangelands by preventing erosion and desertification (Moreno et al. 2000a). Many research showed that desert truffles markedly increased ability of root system for nutrient capture and cycling in soils with low nutrient availability (Jeffries and Barea 2012), increased plant tolerance to environmental stresses such as acidity, drought, heavy metals, and salinity (Morte et al. 2010, Bermaki et al. 2017), improved the photosynthesis rate (Morte et al. 2000), and promoted the vegetative growth. Therefore desert truffles are of considerable interest for agroforestry, productivity and diversity of natural plant ecosystems, and commercial purposes. To date, only *Terfezia claveryi* and *Terfezia boudieri* have been successfully cultivated, the former in Spain (Honrubia et al. 2001, Morte et al. 2009), and the latter in Tunisia and Israel (Slama et al. 2010, Kagan-Zur et al. 2013). The almost nonexistent scientific papers on desert truffle cultivation in Iran. Cultivation of desert truffles is usually done through obtaining mycorrhizal seedlings grown under controlled nursery conditions and transplanting under field conditions. The mycorrhizal status of these plants is a crucial step. The majority of ectomycorrhizal plants are members of the Cistaceae such as *Helianthemum almeriense, H. kahiricum, H. ledifolium, H. lippii* (L) Pers., *H. salicifolium, H. sessiliflorum, Fumana procumbens Cistus albidus, C. incanus, C. ladanifer*, and *C. salviifolius* (Morte et al. 1994, Zaman et al. 2009, Sbissi et al. 2011, Turgeman et al. 2011, Slama et al. 2012, Hamza et al. 2013, Zitouni-Haouar et al. 2014, Alheeti et al. 2021, Louro et al. 2021). Three *Tirmania* species are recorded in the Index Fungorum: *T. honrubiae* Morte, Bordallo & Ant. Rodr, *T. pinoyi* (Maire) Malencon, and *T. nivea* (Desf) Trappe (Kirk 2008).

Based on anatomical description, *Tirmania pinoyi* has been observed to form endomycorrhiza lacking hartig net and mantle but displaying undifferentiated intracellular hyphae with *H. ledifolium* and *H. salicifolium* (Awameh and Alsheikh 1979, Alsheikh 1985, Ismahene and Zohra 2014). Leduc et al. (1986) demonstrated the *T. pinoyi* can with *Helianthemum* and *Cistus* form an ectendomycorrhiza lacking a mantle and, depending on the phosphorus content of the soil, with or without a Hartig net (Leduc et al. 1986). Fortas and Chevalier studied *Helianthemum guttatum* colonized by *T. pinoyi* and found a shift from ectomycorrhizae at high phosphate (P) concentration to endomycorrhizae at low concentration (Fortas and Chevalier 1992).

In Iran, desert truffles are present in arid, semi-arid, and rangland regions and are represented by six species including *Terfezia boudieri* (Ammarellou and Saremi 2008), *T. claveryi*, *Tirmania pinoyi*, *T. nivea*, *Picoa juniperi*, and *P. lefebvrei* (Jamali and Banihashemi 2012). In recent years, truffle harvest have declined in Iran and the world due to a number of factors such as acid rain, deforestation, global warming, loss of host plants due to pests and thermotolerant/thermophilic fungal pathogens, overharvesting, and most importantly, ground-water scarcity (Hall et al. 2003). Thus, desert truffles are becoming a promising, alternative crop in regions with hot climate, where desertification will cause an alarming increase in dry lands (Marqués-Gálvez et al. 2021).

Tirmania pinoyi from semi-arid and rangeland regions with hot climate, calcareous soil, and low altitude in Iran are mainly associated with *H. lippii*. *Helianthemum lippii* is a dominant nationally protected plant of the Cistaceae family in tropical regions of Iran, which is associated with *T. pinoyi* desert truffle. The cultivation of this perennial Cistaceae mycorrhizal by *T. pinoyi* under controlled conditions would preserve the two partners in the symbiosis which are of ecological and economic interest and play an important role in the fight against desertification. During the symbiotic phase, correct identification of desert truffle species are essential for tracking the persistence of them in artificially colonized plants in the field. Fungal structures can be detected in roots, but the morphological features of mycorrhizal roots are in general very similar and not sufficient for identification at the species level (Demir et al. 2011, Jamali and Banihashemi 2013a, Jamali and Banihashemi 2013b; Guevara-Guerrero et al. 2022). Biophysical, biochemical, immunological, and olfactory approaches have previously been used as markers to identify and characterize truffles (Papa et al. 1987, Pacioni and Pomponi 1989, Gandeboeuf et al. 1997, Neuner-Plattner et al. 1999), but these techniques are limited by the amount of biological material required. In contrast, PCR-based DNA method can be used for specific detection of fruitbodies, mycelium, and ectomycorrhizae with minute amounts of tissue (Séjalon-Delmas et al. 2000). Most of the previous studies aimed at truffles producing ectomycorrhiza with forest trees (e.g. *Tuber*) rather than those of desert truffles. Only few studies have been focused on the direct identification of desert truffles in actively colonized root using nested PCR (Kovács et al. 2007, Jamali and Banihashemi 2013a, Jamali and Banihashemi 2013b). The main objective of this study were to the greenhouse production of mycorrhizal four perennial Cistacea plants with *T. pinoyi* under controlled conditions and develop a relatively simple, fast, specific and sensitive PCR-based method to detect *T. pinoyi* in artificially inoculated plants and confirmation

of the efficiency of artificial inoculation of seedlings that would be of use in commercial production. Furthermore, this is the first report on the PCR-based approach to detect *T. pinoyi* in host plants.

Materials And Methods

Sampling and seed collection

Ripe fruits (capsules) containing mature seeds of *H. lippii* were handpicked in March 2020 from natural rangeland population in Lamerd region, Fars province, Iran (27°46′N, 53°57′E; Altitude: 944). The seeds of three plants, *H. almeriense*, *Cistus laurifolius*, and *C. ladanifer* were purchased from a Spanish Company (SEMILLAS CANTUESO). The seeds were manually cleaned, surface sterilized with 0.5% sodium hypochlorite for 1-2 min, followed by washing three times with sterile distilled water for 5 min. Mechanical scarification of the seed coat was achieved by moderate abrasion the seeds for 10 sec between two sandpaper (Zaidi et al. 2010). Five seeds were planted at a depth of approximately 1 cm in each section of the plastic seedling tray containing sterile peat-perlite (1:1 v/v). After seeding the well-developed seedlings were transplanted into 1000-cm^3 polyethylene pots (20cm high and 12cm diameter) containing a mixture of sterilized peat-vermiculate-sand (2:1:1 v/v) in a greenhouse with 25±1°C temperature, 50-60% relative humidity and 12 h light/12 h dark and irrigated twice a week with tap water (Slama et al. 2010, Jamali and Banihashemi 2013a).

Soil analysis

Soil samples taken from desert truffles producing sites under fruiting bodies of *T. pinoyi*, are processed in a laboratory to determine the physico-chemical characteristics of the soil, by analyzing several parameters: pH level, electrical conductivity, organic carbon, soil texture, potassium and total phosphorus and nitrogen, total limestone (%), and the presence of mineral salts (Walkley and Black 1934, Bremner and Mulvaney 1982, Loeppert and Suarez 1996, Thomas 1996).

Morphological identification

T. pinoyi fruiting bodies were collected in March 2020, from South and Southeast of Iran (Table 1).Microscopic examinations were carried out in tap water, 5% aqueous KOH, Melzer's reagent and cotton blue in lactic acid. The photomicrographs were made with an Olympus model BX-51 microscope to capture images. Scanning electron microscopy (SEM) images were taken with an AIS2300C microscope. Samples were mounted in concentrated NH□OH 30% and allowed to soak for 10 min. They were then dehydrated with increasing concentrations of ethanol (10, 20, 40, 60, 80 and 100%) for 10 min each. After dehydration, the samples immersed in pure acetone for 30 min. The dried samples were sputter-coated with gold-palladium (Moreno et al. 2000b).

Inoculation

A spore suspension of the well matured *T. pinoyi* ascocarps was prepared by blending the finely-chopped fruit bodies with a blender, until the spores were released (Zitouni-Haouar et al. 2014). Two months after transplanting, twenty plants of each plant species were inoculated with the spore suspension containing 0.1 g of fruit body in 5 ml of sterilized distilled water and with a concentration of approximately 1×10^7 spores (Morte et al. 2006). The ascospore suspension was directly applied in contact with the roots. The inoculated plants grew in the greenhouses with 25±1°C temperature, 50-60% relative humidity and 12 h light/12 h dark for 6 to 9 months. Control plants without mycorrhizal inoculums were grown under the same condition.

Microscopic analysis of roots

Fungal colonization was assessed in the roots of the inoculated greenhouse-grown plants. Mycorrhizal roots sampled from four host plants were washed and examined using stereomicroscope to determine the presence and pattern of the mycorrhizal system. Subsequently, root pieces (1 cm) cleared according to Phillips and Hayman (1970) method with 10 % potassium hydroxide solution, and stained with 0.1 % anilin blue in lactic acid (Grace and Stribley 1991). Fifty stained root pieces were selected randomly from each mycorrhizal plant, mounted in lactoglycerol (v/v), and examined under light microscope (Trouvelot et al., 1986). The percentage of root colonization was determined using the F %=100 N□/N formula. Where N□ is the number of positive root segments and N is the total number of root of segments observed (Biermann and Linderman 1981).

Plant growth

For each host plant, 20 randomly sampled seedlings were harvested, of which 10 were not inoculated and 10 were inoculated with *T. pinoyi*. The growth of inoculated and non-inoculated seedlings was estimated by measurements of their plant fresh weight, plant dry weight, root fresh weight, root dry weight, shoot fresh weight, shoot dry weight, plant height, root height, and shoot height. For the measurements of the dry weight of root and shoot, the samples were oven-dried 60 °C for 72 h. Chlorophyll contents were measured by a chlorophyll meter (SPAD-502 Minolta, Japan). The relative mycorrhizal dependency (RMD) were also measured according to Plenchette et al. (1983).

Extraction of DNA

Total genomic DNA was extracted from 50 mg of *T. pinoyi* ascocarps using the Cetyl- rimethyl-ammonium-bromide (CTAB) method (Gardes and Bruns 1993). Infested root samples (100 mg) from greenhouse inoculated plants were ground in liquid nitrogen, and DNA was extracted using the CTAB-based protocol of Lekgari (2010) with some modification. DNA purity and quality of the DNA extracts were determined spectrophotometrically (MaestroNano Pro) and by agarose gel electrophoresis according to standard procedures (Sambrooke et al. 1989).

Designing of *Tirmania pinoyi* specific primers

The primers were designed using ITS sequence of *T. pinoyi*(GenBank accession No. OM439630). The target sequence was compared with that from other desert truffles species including *T. pinoyi*by using software BioEdit v7.0.5 (Hall 2005). The forward primer (FTiPi) 5'-CCATCTTCCAAAACCTTGATTG-3' (nucleotide positions 112-133), and the reverse primer (RTiPi) 5'-GAATTTCTGAAGGACGACTCTG-3' (nucleotide positions 390-411) were designed to amplify 5.8S ITS region of rDNA of *T. pinoyi*. Selected primers were checked *in silico* by searching against the non redundant sequences from other organisms *via* Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/ tools/primer-blast) of National Center for Biotechnology Information (NCBI) with default parameters (Ye et al. 2012) in GenBank. Primers then evaluated for criteria such as G-C content, hairpin formation, melting temperature, self-annealing, and self-dimerization using OLIGO 7 Primer Analysis Software (Rychlik 2007). These primers were used for the second round of amplification during nested PCR.

Nested PCR

First round of nested PCR was performed with universal primers ITS1 (5´-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5´-TCCTCCGCTTATTGATATGC-3') (White et al. 1990). The amplification was performed in PCR tube containing 2.5 µl of 10× polymerase reaction buffer (CinnaGen, Iran), 2.5 mM of MgCl2, 20 pmol of each of ITS1 and ITS4 primers, 1.25 nmol of each deoxynucleotide, 5 U Taq DNA polymerase and 2.5 µl of a diluted genomic DNA. The final volume of the reaction mixture was made up to 25 µl with sterile distilled water. The optimized thermocycler conditions for the reaction were initial denaturation at 95 °C for 2 min, 30 cycles at 95 °C for 30 s, 55 °C for 40 s, 72 °C for 60 s and final extension at 72 °C for 10 min. The second round of amplification was carried out using same final concentration of the reagents as described above, except replacing the DNA template with 0.5 µl PCR product from the first round of amplification. The thermocycler conditions were also kept the same program. The amplification results were visualized through electrophoresis using 1% agarose gel in 1× TAE buffer for 45 min at 90 V with 100 bp DNA ladder (CinnaGen, Iran). The controls, with no DNA, were included in every set of amplification to check the DNA contamination in reagents and reaction buffers.

Specificity and Sensitivity Assay

Specificity of the FTiPi and RTiPi primer pair for the detection of *T. pinoyi* was determined by using the genomic DNAs isolated from *T. pinoyi* and various morphologically and moleculary characterized desert truffles species and some other fungi (*Ascotricha cartarum*, *Paecilomyces formosus*, *Neosctalidium novahollandiae*, *Biscogniauxia mediterranea*, *Fusarium oxysporum*). The genomic DNAs isolated from these fungi were used as template for the nested PCR assay as described above. The sensitivity of the nested PCR for the detection of *T. pinoyi* was evaluated by using the different concentrations (100 $ngml^{-1}$ to 10 $fgml^{-1}$) of genomic DNA as template.

Detection of *T. pinoyi* in the inoculated plants

In order to detect *T. pinoyi*, the genomic DNA was isolated from the artificially inoculated and healthy plants by using the above mentioned method and amplified using FTiPi and R TiPi primers both directly and as internal primers for nested-PCR. The nested PCR was performed as described above. The genomic DNA from the *T. pinoyi* was used as positive control in all the experiments. In negative control, genomic DNA was replaced with sterile distilled water.

Results

Study Sites

In Iran, the distribution of *T. pinoyi* is limited to the South and Southeast, which is close to the Persian Gulf and located between 51° and 61° E longitude and 25° and 29° N latitude. These areas in the Köppen-Geiger classification are divided into areas with hot desert climates. Altitude of these areas are low and between 850 to 1,100 m above sea level. The annual mean temperature between 1985 and 2015 was 19 °C, and the annual mean precipitation for the same period was 30 mm.

Physico-chemical analysis

The results of the physico-chemical analyzes showed that the soils of the *T. pinoyi* sites of the South and Southeast of Iran are sandy loamy, with an alkaline pH (between 7.5 and 8.7), unsalted, calcareous, and poor in organic matter, phosphorus, and potassium (Table 1).

Morphological and molecular identification

In this study, 30 specimens of *Tirmania* associated with roots of *H. lippii* were collected with the following features: ascocarps subglobose, pyriform, irregular, 5-12 cm in diameter, and whitish to yellowish to light brown at maturity; gleba solid, white to light brown, and spongy; and immature asci octospored, subglobose, ellipsoidal, 50-62 × 72-98 µm, and amyloid with Meltzer's reagent. Ascospores globose, hyaline, with wall finely roughened, and 16-22 µm. Based on morphological characteristics these specimens identified as *T. pinoyi* (Hawker 1954; Trappe 1971; Jamali and Banihashemi 2012). The ITS region of the specimens has been amplified using ITS4 and ITS1 universal primers, and a single PCR product of about 600 bp was obtained as measured by agarose gel. Based on a BLASTn search of NCBI GenBank nucleotide database (http:// blast.ncbi.nlm.nih.gov/blast.cgi), *T. pinoyi* specimens found in this study (accession numbers: OM439630 to OM439632) revealed 99.89 % identity with valid sequences of *T. pinoyi* deposited in GenBank (GQ231540, MK478852, MH084954, MK478864, MK967455). According to DNA sequence analyses and morphological characters, these specimens could be assigned to *T. pinoyi*. The phylogenetic analysis of the sequenced ITS fragment positioned our *T. pinoyi* specimens within the *T. pinoyi* clade (Fig. 1).

Primer design and nested PCR

In the present work, the nested PCR method has been developed for the detection of *T. pinoyi* in the four artificially inoculated perennial Cistaceae plants. In order to design the specific primers, ITS-5.8S-ITS2 sequence of *T. pinoyi* (GenBank accession No. OM439630) was used (Fig. 1). The target sequence was compared with ITS regions of other desert truffles using BioEdit v7.0.5 software. The aligned sequences were used to design the FTiPi and FTiPi primers. In

the first round of amplification, universal primer pair ITS1 ⁄ ITS4 was used. Whereas, in the second round of amplification, a predicted 300-bp DNA fragment was successfully amplified using FTiPi and FTiPi primers (Fig. 2).

Specificity and sensitivity of the assay

The specificity of the primers was tested by using genomic DNAs of desert truffles and some other fungi. An expected 300 bp DNA fragment was amplified using the FTiPi/FTiPi primers only from *T. pinoyi*. No PCR procuts were obtained from the other tested desert truffles and fungal isolates. The amplification of PCR product exclusively from the genomic DNA of *T. pinoyi* indicated that the designed primers were especially specific for the *T. pinoyi*. The sensitivity of the primer set FTiPi/FTiPi was tested by using different concentrations of genomic DNA of *T. pinoyi* fruiting body as a template in the individual nested PCR assays. In the first step, the conventional PCR reaction was carried out using FTiPi and FTiPi primers. The PCR product analysis indicated that the lower limit for the detection of *T. pinoyi* was 30 ng of DNA per 25 µl of PCR mixture. To increase sensitivity, the nested PCR protocol was performed using ITS1/ITS4 primers and FTiPi/RTiPi primer pairs. This enhanced the sensitivity of the assay and the detection of the pathogen with 50 pg of DNA was obtained (Fig. 3). Thus, nested PCR increased the lower detection limit of genomic DNA from 30 ng to 50 pg.

Detection of *T. pinoyi* in artificially infected plants

The nested PCR was performed to detection the *T. pinoyi* in artificially infected plants. The genomic DNAs were isolated from artificially infected and healthy control plants and subjected to the nested PCR assay. All artificially infected plants were found to be positive for *T. pinoyi* as a 300-bp PCR product was obtained on the agarose gel. Whereas, no PCR products were amplified with DNA from the control plants (Fig. 4). Two random samples from each plant that had 300 bp PCR products amplified by FTiPi/RTiPi had 100% DNA sequence identity with previously published DNA sequences of *T. pinoyi* specimens.

Growth attributes

Microscopic examination of the root fragments showed that *T. pinoyi* formed ectendomycorrhizae with varying degrees of sheath development, with all four plant species (i.e., *C. ladanifer*, C. *laurifolius*, *H. almeriense*, and *H. lippii*). The root systems of *H. lippii* plants inoculated with *T. pinoyi* are well mycorrhized and form a Hartig network with a well-developed sheath (Fig. 5 a-d). The results of the mycorrhizal association between *T. pinoyi* and *H. lippii* show that the inoculated plants are significantly more developed than the control plants. The ANOVA for plant parameters growth shows the significant effects of mycorrhization on the fresh and dry weight of entire plants, fresh and dry weight of roots, fresh and dry weight of shoots, plant height, root height, and shoot height (Table 2). In *H. almeriense*, the *T. pinoyi* form a Hartig network with a rudimentary sheath (Fig. 5 e-h). The height of the inoculated plants and shoot height were significantly affected by *T. pinoyi*, but mycorrhiza had no significant effect on plant weight and root height (Table 2). In *C. laurifolius*, *T. pinoyi* form ectomycorrhizae with a less-developed sheath and a Hartig network (Fig. 5 i-l). Fresh and dry weight of shoots, shoot height and the height of the inoculated plants is greater than that of the non inoculated plants (Table 2). In C. *ladanifer*, *T. pinoyi* form ectendomycorrhizae with a developed sheath and a Hartig network (Fig. 5 m-p). Except for root fresh weight and shoot height, a significant difference was observed in other parameters compared to the non-inoculated plants (Table 2).

Discussion

In Iran, *T. pinoyi* grows only in hyper-arid areas in the Southern and Southeastern coast of Iran, which are located on the Persian Gulf (26–29 °N and 52–61 °E). The soil analysis showed that soils are loamy, alkaline, calcareous, low in organic matter, and non-saline. Altitude was low in the growing areas of *T. pinoyi*. It has been previously reported that *T. pinoyi* widespread in Saharan areas and harvested near *H. lippii* and *H. hirtum* on calcareous alkaline sandy soil with low organic matter (Alsheikh and Trappe 1983; Ravolanirina 1986; Diez et *al.*2002; Slama et *al*. 2004, 2006; Jamali and Banihashemi 2012). Jamali and Banihashemi (2012) show that, the distribution pattern of the desert truffles species correlated with soil texture, soil pH, EC and host, and *T. pinoyi* live in siliceous sands and gypsum soils. In the case of *Tirmania* spp., *T. nivea* was found in basic soils associated with the basophilous plant *H. salicifolium*. Also *T. pinoyi* was collected under the basophilous plant *H. salicifolium*. It has been reported previously that *T. pinoyi* were collected under the acidophilous plant *H. guttatum* (Diez *et al*. 2002). In the study areas, the predominant plant species that associated with *T. pinoyi* were *H. salicifolium* and *H. lippii*, and *Cistus* and *H. almeriense* are not found in these areas.

Today, a lot of attention has been paid to cultivate *T. pinoyi* like the other successfully cultivated truffles. In Iran, cultivation of this species can be very important in desertification and can also a source of income to local people. Transplantation and utilization of mycorrhizal plants in desertification could reducing the effects of climatic change (Slama et al. 2021). In this study, mycorrhizal association *T. pinoyi* with four plant species; *H lippii*, *H. almeriense*, *C. laurifolius*, and *C. ladanifer* in a mixture of sterilized peat-vermiculate-sand (2:1:1 v/v) that containing a low phosphorus concentration under greenhouse conditions were investigated which could be the first step towards the successful cultivation of this truffle. Our results showed that *T. pinoyi* could form ectendomycorrhizae with all four species. Mycorrhizae of *H. ledifolium* and *H. salicifolium* with different *Tirmania* species (*T. nivea* and *T. pinoyi*) were described by Awameh and Alsheikh (1980). In all these syntheses, an endomycorrhiza was observed. Leduc et al. (1986) and Fortas and Chevalier (1992) demonstrated the existence of mycorrhizae formed by *T. pinoyi* with *Helianthemum* and *Cistus*. These fungi can form an ectendomycorrhiza lacking a mantle and, depending on the phosphorus content of the soil, with or without a Hartig net. Although the genus *Tirmania* is primarily ectomycorrhizal and forming a sheath around the roots of their host plant, it is highly adaptable. Some species, like *T. pinoyi*, form endomycorrhizal associations in phosphate-poor soils and ectomycorrhizal associations in phosphate-rich soils (Fortas and Chevalier, 1992). In this study, the percentage of mycorrhizae and relative mycorrhizal dependency was higher in *H. lippii* than other plants. In the study areas, the predominant plant species that associated with *T. pinoyi* was *H. lippii*, and *H. almeriense*, *C. laurifolius*, and *C. ladanifer* are not found in these areas. Relative mycorrhizal dependency was defined as the degree on which a plant species depends on the mycorrhizal association to reach a maximal growth at a given environmental condition (Plenchette et al., 1983). This index allows assessing the dependency of a plant species on the mycorrhizal symbiosis to achieve its maximum growth and development (Torres et al. 2021). The remarkable rates of mycorrhizal colonization and relative mycorrhizal dependency observed in *T. pinoyi-H. lippii* combination is quite logical since the *H. lippii* is natural host

plant of *T. pinoyi* in the arid and semi-arid regions of Iran. Adequately colonized plants under controlled conditions are essential for the commercial cultivation and production of truffles. In this study, although all four plant species formed mycorrhizae with *T. pinoyi*, but our results indicated that *H. lippii* is a better option than other as potential host for the production of *T. pinoyi* inoculated plants, and its subsequent application on desert truffle cultivation. Also, this species found in the vicinity where the fruiting bodies were collected and therefore had the potential to be host. Any attempt to cultivate this economically important desert truffle demands a useful tool for tracking dynamics it in cultivated soils. The presence of sheath is not sufficient evidence to confirm a relationship between plant and truffle. Nested PCR using species-specific primers have greatly improved the detection of fungal hyphae present in the root of host plants. PCR based assays are accurate, sensitive, rapid, and specific and have been often implemented for the routine diagnostics of mycorrhiza. In this study, we have used nested PCR as a rapid approach for the detection of *T. pinoyi*. Analysis of ITS sequences of rDNA of *T. pinoyi* and other desert truffles was performed to design primary PCR primer pair. The developed protocol was successfully used for the exclusive amplification of the 300 bp fragment from *T. pinoyi* genomic DNA. Thus, this method can discriminate *T. pinoyi* from all the other desert truffle tested. Detection of *T. pinoyi* in the root of the *H. lippii* in arid and semi-arid regions, using a single round of PCR with the primer pair ITS1/ITS4, was not consistent, probably because of the low concentration of the target DNA. The genomic DNA was extracted from the artificially inoculated, and control healthy plants followed by detection of the *T. pinoyi* by nested PCR approach. The results showed that the developed protocol successfully detected the *T. pinoyi* in the artificially infected plants. No PCR products were obtained in healthy plants. Many other researchers have used nested PCR to increase the sensitivity of the reaction for the detection of fungal hyphae in the root of host plants (Turnau et al. 2001; Zampieri et al., 2009). In this work, nested PCR increased the sensitivity of *T. pinoyi* specific primers by 100 fold to 50 pg. The nested PCR assay has potential as a diagnostic tool for detecting *T. pinoyi* in the host plant roots. Detection of *T. pinoyi* using the described PCR assay was positive for inoculated seedlings in the greenhouse. The Nested PCR could be useful for monitoring the establishment of mycorrhizal colonization by the inoculated fungal species in controlled experimental conditions.

Declarations

Author Contributions

Conceived the idea of the research, designed the scientific experiments and methodology, writing-original draft preparation, writing-review and editing, phylogenetic investigation, formal analyses, phylogenetic visualization/data presentation, GenBank sequences submission, S. Jamali.; Field surveys, sample preparation, laboratory works, sequencing of fungal isolates, M. Sheibani.

Ethical Approval and Consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

All authors concur with the submission.

Availability of supporting data

Not applicable

Competing interests

The authors declare no competing financial interests.

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Tables

Table 1. Origins of species studied

Species	Location	Latitude	Longitude	Elevation(m)	Host plant	pH	EC	Silt%	Sand%	Clay%	CaCO3%	OM%	Na
Tirmania pinoyi	Beyram	27^0 28´ 64" N	53^0 35´ 29" E	989	*H. lippii/H. salicifolium*	7.78	0.446	31	68	1	54	1.78	166.2
Tirmania pinoyi	Beyram	27^0 28´ 45" N	53^0 35´ 27" E	1084	*H. lippii/H. salicifolium*	7.8	0.451	27.23	65.31	7.46	48	2.35	113.8
Tirmania pinoyi	Beyram	27^0 28´ 17" N	53^0 35´ 30" E	1091	*H. lippii/H. salicifolium*	7.85	0.4	29	63.2	7.8	63	1.31	172.8
Tirmania pinoyi	Evaz	27^0 47´ 90" N	530 49´ 06" E	943	*H. lippii/H. salicifolium*	8.1	0.53	32.14	67.72	0.14	44	1.9	163.1
Tirmania pinoyi	Evaz	27^0 46´ 43" N	530 56´ 95" E	1033	*H. lippii/H. salicifolium*	7.83	0.47	30.14	69.72	0.14	45	2.35	152.4
Tirmania pinoyi	Kahnuyeh	27^0 55´ 97" N	530 20´ 79" E	706	*H. lippii/H. salicifolium*	7.69	0.40	49.4	26.6	24	58	0.44	156.7
Tirmania pinoyi	lozuyeh	27^0 55´ 95" N	530 20´ 78" E	709	*H. lippii/H. salicifolium*	8.2	0.43	50	29	21	47	2.3	125.4
Tirmania pinoyi	Lamerd	27^0 51´ 95" N	530 21´ 78" E	701	*H. lippii/H. salicifolium*	7.9	0.42	37.5	54	8.5	48	1.9	164.5
Tirmania pinoyi	Mohr	27^0 52´ 95" N	530 20´ 78" E	638	*H. lippii/H. salicifolium*	8.1	043	38.5	52	9.5	46	1.8	169.4
Tirmania pinoyi	Beyram	27^0 27´ 82" N	53^0 35´ 71" E	1169	*H. lippii/H. salicifolium*	7.75	0.42	35	59.6	5.4	49	1.85	-
Tirmania pinoyi	Beyram	27^0 27´ 72" N	53^0 35´ 60" E	1165	*H. lippii/H. salicifolium*	7.89	0.51	37.5	49	13.5	49	1.84	-
Tirmania pinoyi	Ehsham	27^0 27´ 31" N	53^0 19´ 84" E	537	*H. lippii/H. salicifolium*	7.95	0.43	31	52.4	16.6	52	2.21	-
Tirmania pinoyi	Evaz	27^0 46´ 37" N	53^0 48´ 26" E	944	*H. lippii/H. salicifolium*	8.1	0.42	28.4	62.74	8.86	57	0.79	-
Tirmania pinoyi	Evaz	27^0 46´ 64" N	53^0 47´ 85" E	937	*H. lippii/H. salicifolium*	7.98	0.47	29.14	68.72	2.14	49	2.21	-
Tirmania pinoyi	Mohr	27^0 28´ 13" N	53^0 35´ 41" E	1136	*H. lippii/H. salicifolium*	7.68	0.46	28.1	67.2	4.7	51	1.60	-
Tirmania pinoyi	Chahvarz	27^0 28´ 03" N	53^0 31´ 70" E	968	*H. lippii/H. salicifolium*	7.75	0.41	34	62.6	3.4	62	0.54	-
Tirmania pinoyi	Chahvarz	27^0 28´ 02" N	53^0 34´ 69" E	1142	*H. lippii/H. salicifolium*	7.93	0.50	39.5	52	8.5	46	2.11	-
Tirmania pinoyi	Agoseh	27° 49´ 34" N	53° 25´ 34" E	642	*H. lippii/H. salicifolium*	7.6	0.48	38.5	51	10.5	49	1.35	-
Tirmania pinoyi	Arad	28^0 43´ 00" N	53^0 58´ 00" E	630	*H. lippii/H. salicifolium*	7.69	0.41	31	63.2	5.8	50	1.46	-

Table 2. Mean plant fresh weight, plant dry weight, root fresh weight, root dry weight, shoot fresh weight, shoot dry weight, plant height, root height, shoot height and chlorophyll content of mycorrhizal and non-mycorrhizal plants

Combination	Treatment	Fresh weight of entire plant (g)	Dry weight of entire plant (g)	Root fresh weight	Root dry weight	Shoot fresh weight	Shoot dry weight	Plant height	Root height	Sh he
HL/TP	M	28.41 ± 1.49*	10.47±1.19*	10.57±0.52*	2.07±0.16 NS	17.84±1.33*	8.40±1.14*	57.30±2.49*	24.00±1.26*	33
	NM	19.90±1.49*	5.13±1.19*	7.37±0.52*	1.59±0.16 NS	12.53±1.33*	3.54±1.14*	47.80±2.49*	18.40±1.26*	29
HA/TP	M	21.66±1.39 NS	7.18±0.48 NS	10.48±0.89 NS	2.71±0.26 NS	11.00±0.66 NS	4.47±0.34 NS	44.00±2.27*	21.50±1.19 NS	21
	NM	18.19±1.39 NS	5.86±0.48 NS	8.76±0.89 NS	2.16±0.26 NS	9.65±0.66 NS	3.70±0.34 NS	35.40±2.27*	18.30±1.19 NS	17
CLad/TP	M	22.46±0.84*	8.11±0.42*	5.48±0.22 NS	1.56±0.09*	16.98±0.66*	6.55±0.36*	35.70±1.50*	17.80±0.89*	17 NS
	NM	18.95±0.88*	6.28±0.44*	5.47±0.23 NS	1.02±0.10*	13.08±0.7* b	5.26±0.37*	28.88±1.58*	13.77±0.93*	15 NS
CLau/TP	M	31.45±1.68 NS	8.83±0.46 NS	10.26±0.79 NS	2.00±0.23 NS	21.52±1.11*	6.89±0.35*	35.90±0.99*	15.3±0.69 NS	20
	NM	27.73±1.68 NS	7.83±0.46 NS	9.93±0.75 NS	1.94±0.23 NS	17.37±1.11*	5.83±0.35*	32.60±0.99*	15.2±0.69 NS	17

One-way ANOVA analysis. Values are means±standard error

NS: absence of significance, M: mycorrhizal plants, NM: non-mycorrhizal plants

HL (*Helianthemum lippii*), HA (*Helianthemum almeriens*), CLad (*Cistus ladanifer*), CLau (*Cistus laurifolius*), TP (*Tirmania pinoyi*)

*P<0.05, level of significance

Figures

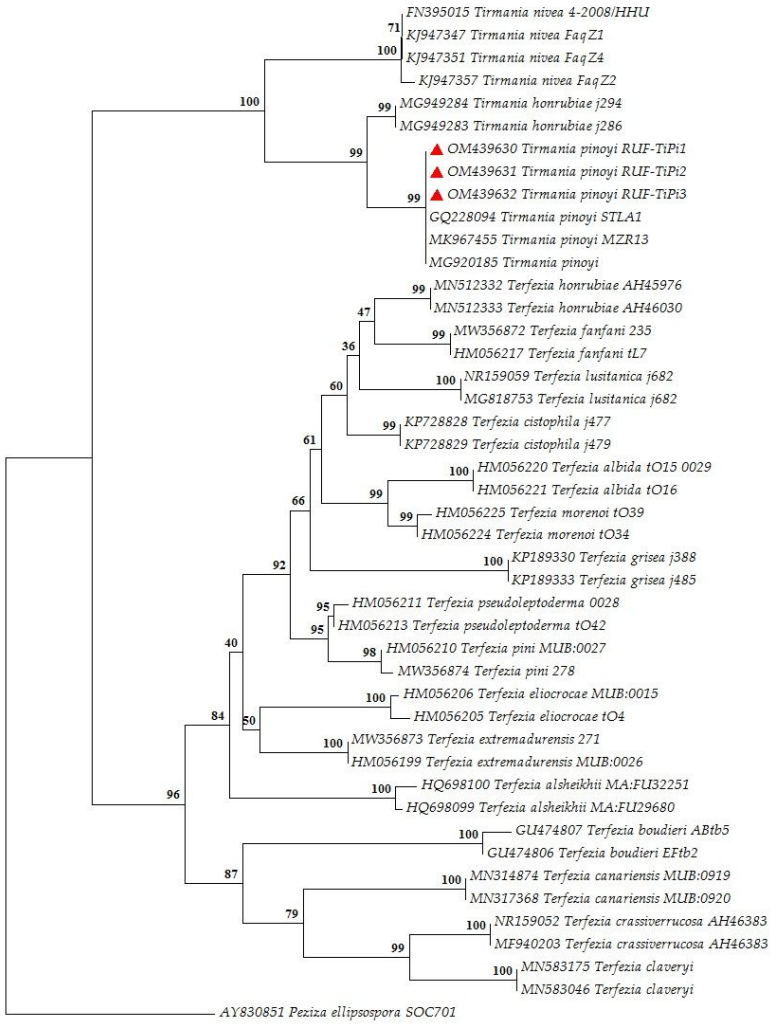

Figure 1

Maximum likelihood phylogram generated in Mega X program from the alignment of 25 combined internal transcribed spacer (ITS) and β-tubulin gene sequences of desert truffle species using Kimura 2-parameter model with complete deletion gap handling and 1,000-replication bootstrapping. *Thermoascus crustaceus* is used as outgroup. The values associated with each horizontal the line denote bootstrap support for the node.

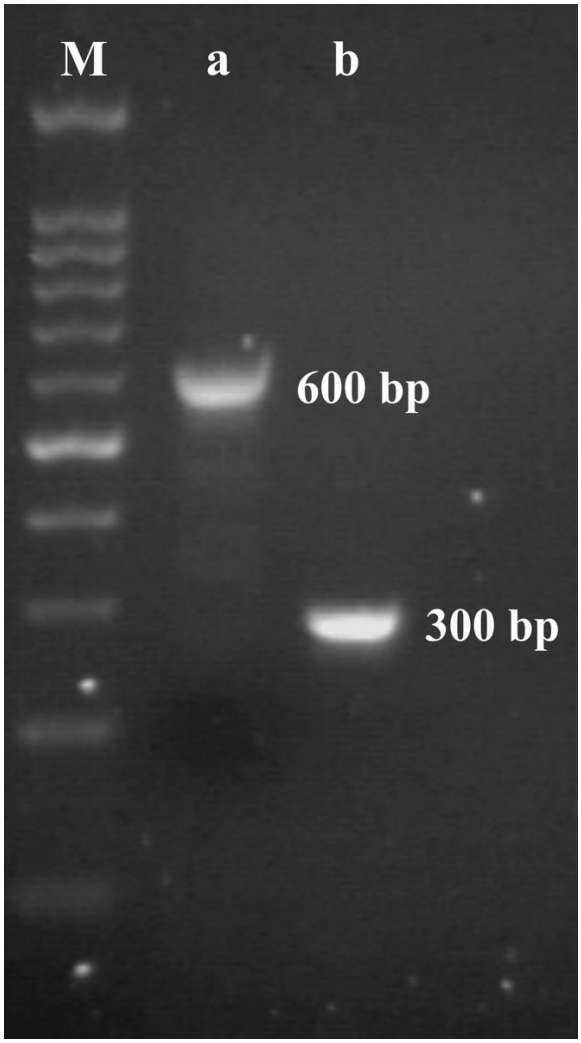

Figure 2

Gel electrophoresis of PCR-amplified product using *Tirmania pinoyi*-specific primers FTiPi/RTiPi. Lane M: 100 bp DNA ladder; Lane a: ITS1/ITS4 primer; Lane b: *Tirmania pinoyi*-specific primers FTiPi/RTiPi.

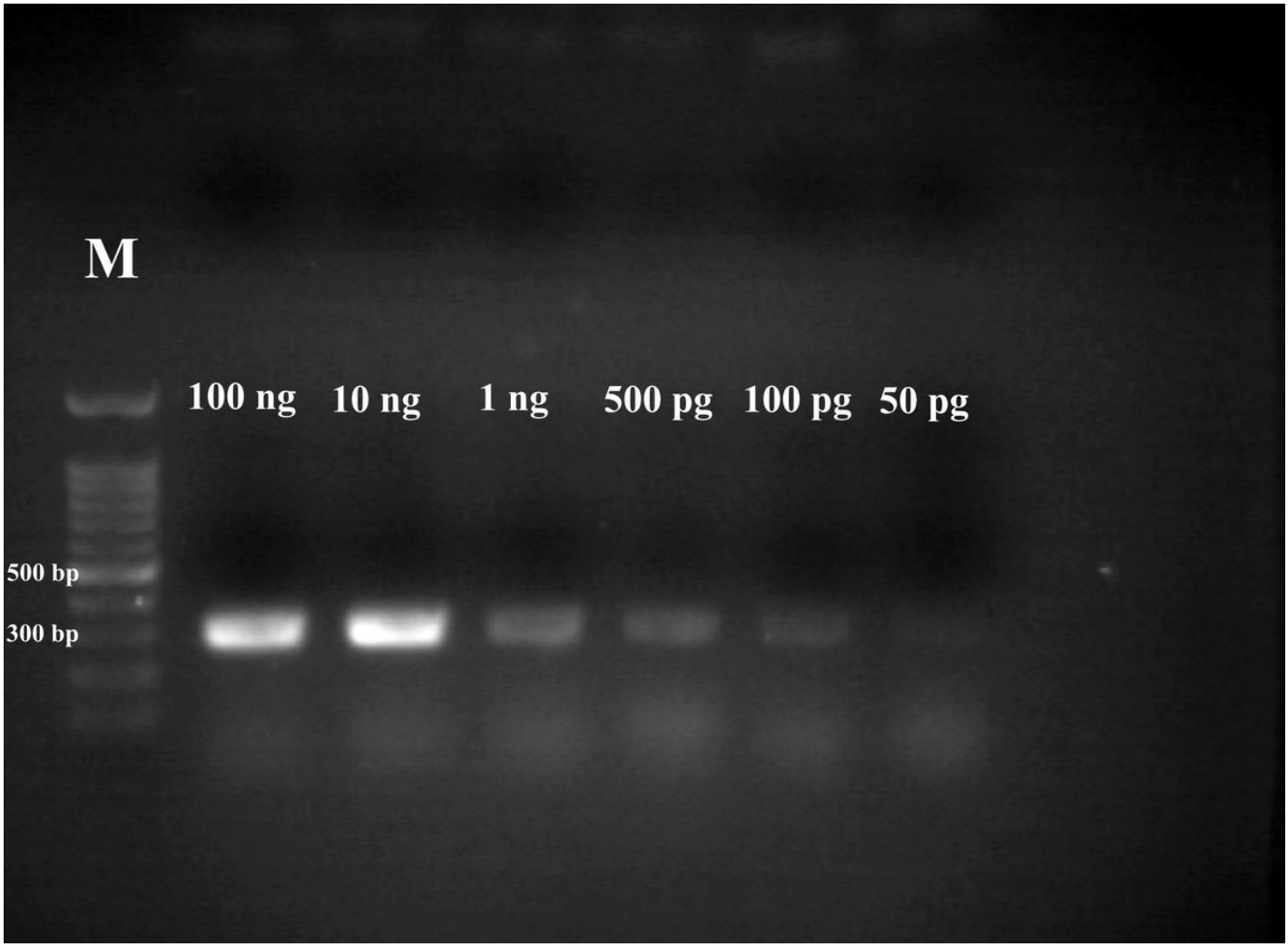

Figure 3

Sensitivity of PCR with primers FTiPi/RTiPi using different concentrations of fruit body of *Tirmania pinoyi* DNA. Lane M: 100 bp DNA ladder.

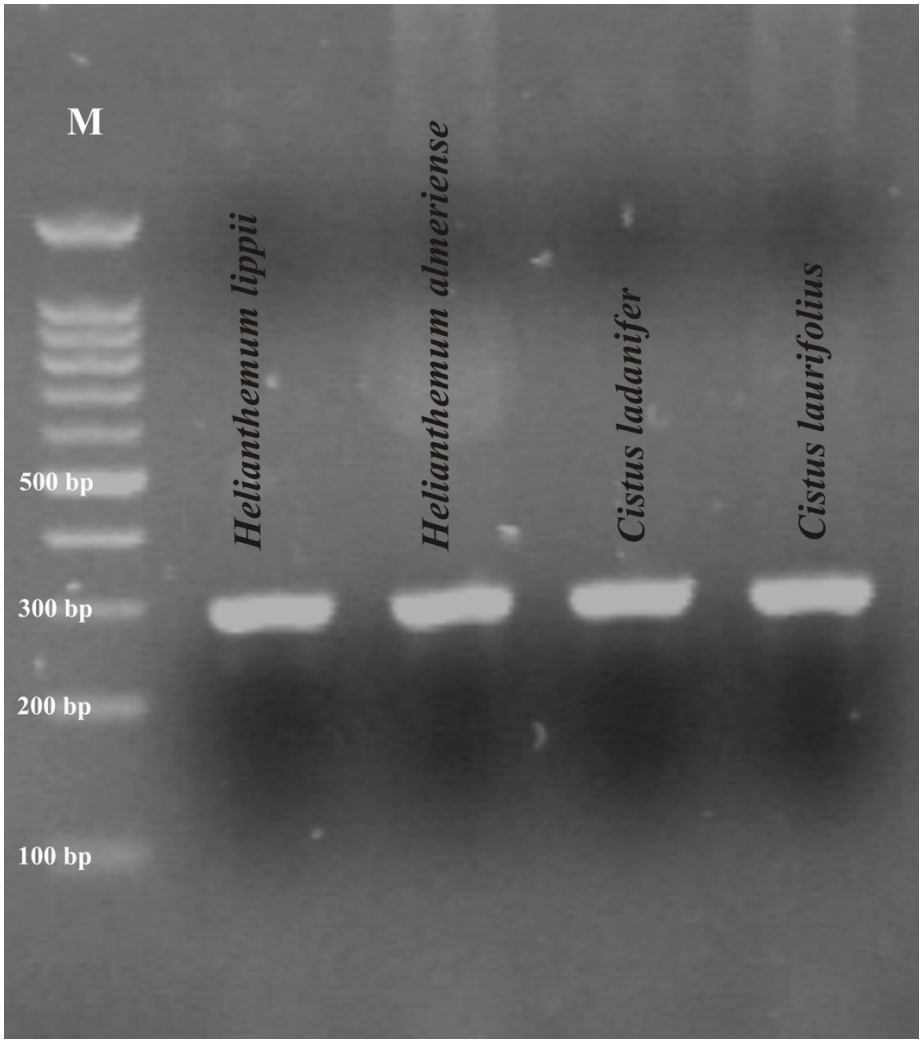

Figure 4

Specific primer FTiPi/RTiPi amplified DNA of artificially inoculated plants. Lane M: 100 bp DNA ladder.

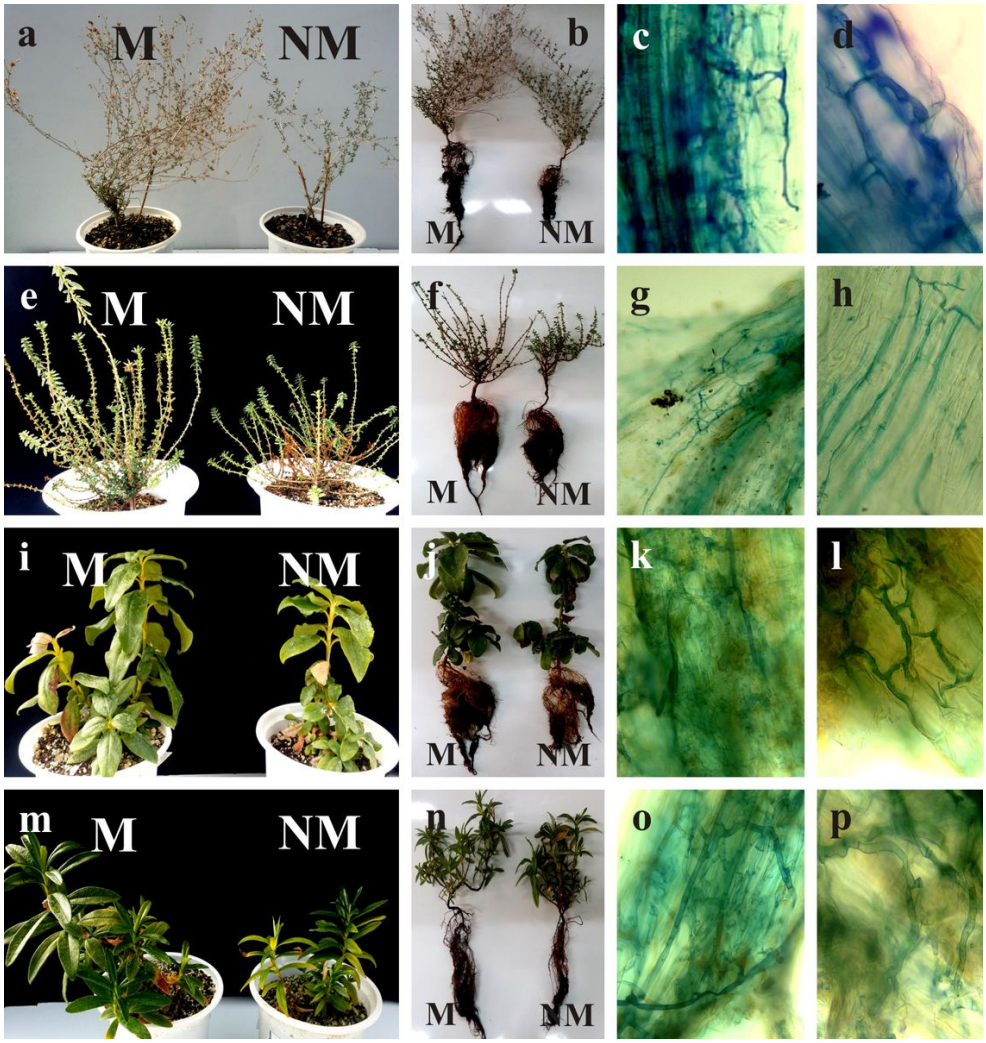

Figure 5

Light microphotographs of *Tirmania pinoyi*-plant mycorrhizal roots, (400×): (a, b) mycorrhizal and non mycorrhizal *H. lippii*, (c, d) *T. pinoyi* × *H. lippii* root showing well-developed sheath, (e, f) mycorrhizal and non mycorrhizal *H. almeriense*, (g, h) *T. pinoyi* × *H. almeriense* root showing rudimentary sheath, (i, j) mycorrhizal and non mycorrhizal *T. pinoyi* × *C. laurifolius*, (k, l) *T. pinoyi* × *C. laurifolius* root showing less-developed sheath, (m, n) mycorrhizal and non mycorrhizal *T. pinoyi* × *C. ladanifer*, (o, p) *T. pinoyi* × *C. ladanifer* root showing developed sheath.

Supplementary Files

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