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The Effect of *Talaromyces funiculosus* ST976 Isolated from *Pistacia vera* Rhizosphere on Phosphorus Solubility in Soil Samples with Different Physicochemical Properties

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ABSTRACT

In this study, a total of 78 *Talaromyces* isolates were isolated from the pistachio (*Pistacia vera* L.) rhizosphere heavily infested with *Neoscytalidium* spp. The identification studies of the four representative isolates based on morphological and molecular methods showed that all isolates were *T. funiculosus*. The 575 bp long sequence of the internal transcribed spacer region of *T. funiculosus* isolate ST976, selected as a representative of the isolates, was deposited in GenBank under accession no. MW130842. The Maximum Likelihood tree clustered the ST976 isolate with reference *T. funiculosus* isolates derived from the GenBank nucleotide database. The phosphorus dissolution ability of ST976 isolate was determined by an experiment using six soil samples collected from agricultural lands in various locations of Şanlıurfa province. The pH of the soil samples taken varied between 7.21 and 7.88. As a result of the analysis performed with the addition of the isolate ST976 applied to soil samples with different soil structures (Clay and Clay-Loam), it was determined that the isolate ST976 dissolved 109–311% more phosphorus than the control sample. The study is one of the first studies proving the ability of *T. funiculosus* isolate ST976 to dissolve phosphorus without any additives to soil solution was determined.

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Talaromyces funiculosus,
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Fıstık Ağaçları (*Pistacia vera*) Rizosferinden İzole Edilen *Talaromyces funiculosus* ST976'nın Farklı Fizikokimyasal Özelliklere Sahip Toprak Örneklerinde Fosfor Çözünürlüğüne Etkisi

ÖZET

Bu çalışmada *Neoscytalidium* spp. ile yoğun bir şekilde bulaşık antepfıstığı (*Pistacia vera* L.) rizosferinden toplam 78 *Talaromyces* izolatı izole edilmiştir. Dört temsilci izolatın morfolojik ve moleküler yöntemlere dayanarak tanımlama çalışmaları, tüm izolatların *T. funiculosus* olduğunu göstermiştir. İzolatları temsilen seçilen *T. funiculosus* ST976 izolatının internal transcribed spacer bölgesinin 575 bç büyüklüğündeki sekansı, GenBank tarafından sağlanan MW130842 erişim numarası ile kaydedilmiştir. Maximum Likelihood dendogramı, ST976 izolatını, GenBank nükleotid veri tabanında bulunan referans *T. funiculosus* izolatları ile birlikte kümelemiştir. ST976 izolatının fosfor çözünme kabiliyeti, Şanlıurfa ilinin farklı lokasyonlarında bulunan tarım arazilerinden alınan altı toprak numunesi ile yapılan deney ile belirlenmiştir. Alınan toprak örneklerinin pH'sı 7.21 ile 7.88 arasında değişmektedir. Farklı toprak yapılarına (Killi ve Killli-Tın) sahip toprak örneklerine uygulanan ST976 izolatının ilave edilmesi ile yapılan analiz sonucunda ST976 izolatının kontrol numunesine göre % 109-311 daha fazla fosfor çözündüğü tespit edilmiştir. Çalışma, *T. funiculosus* izolatı ST976'nın fosforu toprak çözeltisine herhangi bir katkı maddesi olmadan çözebildiğini kanıtlayan ilk çalışmalardan biridir.

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INTRODUCTION

Microorganisms consisting of root fungi and many bacterial species that coexist in the soil and living symbiotically in the roots of plants increase the solubility of inorganic phosphates that are insoluble in the soil due to their metabolic functions (Gupta et al., 2012; Özyılmaz and Benlioğlu, 2012; Mehta et al., 2013; Shi et al., 2017). Microorganisms, including *Aspergillus*, *Penicillium*, *Talaromyces*, *Pseudomonas*, *Bacillus*, *Rhizobium* and *Burkholderia* species, are capable of producing soluble-inorganic and organic phosphate with the help of organic acids and phosphatase enzymes that they excrete (Rodriguez and Fraga, 1999; Doilom et al., 2020).

Phosphorus (P) in the earth naturally occurs in mineral forms such as phosphate rocks and apatite, the most important feature of which is that they are insoluble (Whitelaw, 1999). The main source of P, which is useful for plants in the soil, is formed as a result of the breakdown of rocks and minerals. The soluble P concentration in soil is usually low, normally 1 mg kg^{-1} or less (Rodriguez and Fraga, 1999). In addition, since organic substances contain P, there are organic P compounds providing P in the soil (Bolat and Kara, 2017). Phosphorus is essential for agricultural production (Nawara et al., 2017), and plants can absorb P as a nutrient in the form of $HPO4^{2-}$ or $H2PO4^{-}$ from the soil to use in the structure of some very important organic compounds including nucleic acids (Çakmakçı, 2005; Pişkin and Turhan, 2017). In general, it is very effective in the initial development stages of the plant and plays an important role in flowering, root development, seed and fruit formation. Phosphorus is also needed for the formation of substances such as sugar and starch and energy metabolism, which is necessary for the plant to maintain its vital activities.

Another source of P in the soil for plants is fertilizers containing P in normal soluble form, especially used in agricultural areas. The use of commercial fertilizer in developing countries has increased significantly in recent years and although it is not high compared to developed countries in agriculture, there is a wrong use of fertilizers. A large part of this soluble inorganic phosphate applied in the form of iron (Fe), aluminum (Al) and calcium (Ca) compounds at the rate of 75-90% becomes insoluble in the soil in a short time and precipitates (Gyaneshwar et al., 2002; Musafa et al., 2017). Approximately 43% of the 1.319 billion hectares of arable land in the world is insufficient in P, which is among the important limiting factors in agricultural practices (Dumas et al., 2011).

Mobilization of soil P has been reported for numerous species of soil and rhizosphere fungi (Srinivasan et al., 2012; Sharma et al., 2013), particularly in genera *Aspergillus*, *Penicillium*, and *Trichoderma*, traversing long distances within the soil more easily than bacteria and may be more important to the solubilization of inorganic phosphate in soils (Kucey, 1983) as they produce and secrete more acids than bacteria (Sharma et al., 2013) and mediate the formation of new secondary minerals of P, reducing the P loss from soil because of surface run off (Burford et al., 2003; Fomina et al., 2005). The most powerful P mobilizers are mycorrhizal fungi being able to solubilize and mineralize soil P and also absorb and transport P into the host root as plant growth-promoting microorganisms (Owen et al., 2015).

The initial aim of this study was to screen rhizospheric fungi established in the zone of soil directly surrounding the pistachio root system in a representative location of the pistachio growing Southeastern Anatolia region to garner a broad perspective regarding their presence and functioning in combination. The study also aimed to identify *Talaromyces* isolates colonized the soil in great amounts together with the pathogenic *Neoscytalidium* spp. with morphological and molecular tools and to assess the ability of the *T. funiculosus* isolate ST976 to dissolve P in six soil samples taken from different parts of Şanlıurfa province. This research is among the originate studies evaluating the ability to dissolve P with only *T. funiculosus* ST976 without any additives to the soil solution is determined.

MATERIALS and METHODS

Collection of soil sample

In August 2019, soil samples were collected from rhizospheres of pistachio trees in an orchard of Bozova, Şanlıurfa, Turkey. Four samples, each consisting of 200 grams of soil, were collected from around symptomatic plants with a sterile container at a depth of 20 cm and at a distance of 25 cm from the roots, immediately sent to the laboratory, mixed thoroughly, shade-dried for 15-20 days, crushed, and sieved through a 2-mm sieve.

Isolation of rhizospheric fungi

A common protocol of soil dilution plate technique was applied to isolate fungi from the shade-dried and

sieved soil samples. At the beginning of the procedure, 10 g of each soil was suspended in 200 mL of sterile distilled water in a screw-capped bottle, and the mixture was stirred with a magnetic stirrer (250 rpm at room temperature) for 20 min. then one ml of aliquot was added to 9 ml of sterile distilled water up to 10^{-6} dilution. In this way, 0.5 ml aliquot of 10^{-6} dilution was transferred with a pipette onto the surface of 15 ml potato dextrose agar (PDA; Difco, Detroit, MI, USA) containing streptomycin sulphate at the concentration of 100 mg L^{-1} in a 100-mm diameter petri dish and spread evenly across the agar surface using a sterile glass spreader. Along these lines, each suspended soil sample was poured over 10 plates (in ten replicates). The plates were labeled and incubated at 25°C in the dark and observed for fungal growth. After 4-7 days of incubation, growing colonies were subcultured on PDA and malt extract agar (MEA; Difco, Detroit, MI, USA) media plates.

Cultural and morphological characteristics of the fungi

After 7 days of incubation at 25°C in the dark on MEA and PDA, colony characteristics were observed. The growth diameter and coloration of the colonies and the texture of the mycelia were noted. For further characterization of tentatively identified *Talaromyces* species (Samson et al., 2011; Yilmaz et al., 2014), isolates were also incubated at 37°C on MEA for 7 days. The microscopic features (spore formation, size and mycelium arrangement) were examined after three additional days of incubation. Microscopic observations were made using an Eclipse E200 model light microscope (Nikon Inc., Tokyo, Japan). The total number of colonies identified was counted for each soil sample. All isolates were deposited in the culture collection of the Plant Pathology Laboratory at the GAP Agricultural Research Institute, Şanlıurfa.

DNA extraction

To extract genomic DNA, a PDA plug (5 mm in diameter) from an actively growing each single-spore culture of Taloromyces spp. was transferred into a 50 ml centrifuge tube containing 20 ml of potato dextrose broth (Difco, Detroit, MI, USA) and incubated for 7 days at 25°C in a shaker (ThermoStable™ IS-30: Daihan Scientific Co., Seoul, Korea) at 100 rpm. Mycelial mass was harvested by filtration through a filter paper and rinsed three times with sterile distilled water. Following drying, the mycelia were ground to a homogenized powder with sterilized mortar and pestle under liquid nitrogen. Approximately 100 mg of powder was suspended in preheated 750 µl of extraction lysis buffer (2% CTAB, 2% PVP-40, 0.8 M NaCl, 125 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0, 0.5% sodium disulphide, and 0.5% sarcosyl) in a 2 mL Eppendorf tube, vortexed briefly and then incubated at 65°C for 1 h. After this incubation, 750 µL of chloroform/isoamyl alcohol (24:1 w/w) was added to the suspension, gently mixed for 5 min. The suspension was centrifuged at 13.000 g for 15 minutes to clarify and the supernatant was transferred to a new Eppendorf tube. This step was repeated twice. DNA was then precipitated with 0.6 volume of cold isopropanol and collected by centrifugation at 13.000 g for 10 min. The resulting pellet was rinsed with 70% ethanol and dried at room temperature in a laminar flow for 60 min. The final concentration and quality of the resulting DNA were estimated by a nano spectrophotometer (DS-11 FX+: Denovix Inc., Wilmington, DE, USA) and adjusted to 10 ng μL^{-1} with sterile distilled water for PCR assay. The DNA was stored at -20°C until used.

ITS rDNA sequencing and phylogenetic analysis

The internal transcribed spacer region (ITS) of ribosomal DNA of isolates was amplified with the primer pair of ITS1 (5′ - TCC GTA GGT GAA CCT GCG G - 3′) and ITS4 (5′ - TCC TCC GCT TAT TGA TAT GC - 3′) primer pair described by White et al (1990). Reaction mixtures contained 1× polymerase reaction buffer, 200 µM each dNTPs, 0.4 µM of each primer, 1.25-unit of Taq DNA polymerase (New England BioLabs, Ipswich, MA, USA), 10 ng of template DNA, and sterile distilled water up to 50 µL. Conditions for PCR of ITS constituted an initial denaturation step of 3 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 52°C and 1 min at 72°C, and a final denaturation step of 10 min at 72°C, which was carried out in a T100 thermal cycler (Bio-Rad, Hercules, CA, USA). PCR product was confirmed by visualizing on a 1.4% agarose gel containing 1× TAE buffer using a gel documentation system (G: Box F3: Syngene, Cambridge, UK) and bidirectional sequenced with the same primers by a commercial company (Macrogen Inc., Seoul, Korea).

The ITS sequence of isolates was compared to those of all known *Talaromyces* spp. deposited into the National Centre for Biotechnology Information (NCBI) nucleotide database using the Basic Local Alignment Search Tool (Anonymous, 2021). The ITS sequence of representative isolate ST976 was submitted to the NCBI GenBank and deposited under accession no. MW130842. For further analyses, a phylogenetic tree was produced with ST976 and eleven *Talaromyces* isolates belonging to six species. All sequences were aligned with Clustal W system (Thompson et al., 1994) using MEGAX software (Kumar et al., 2018). The Maximum Likelihood (ML) analysis was performed on a maximum parsimony (MP) analysis starting tree automatically generated by the software and the robustness of phylogeny was assessed by 1,000 bootstrap re-samplings (Felsenstein, 1985).

Soil material used in phosphorus solubility analysis

The pH of the soils taken into the trial from 6 different pistachio fields in Şanlıurfa province ranged between 7.21 and 7.88, according to the soil pH limit values reported by Kacar (1995). Haliliye soil sample was determined to be slightly alkaline, while samples taken from other regions were in the middle alkaline class (Table 1).

When the texture classes of the soils were examined according to the method reported by Eyüpoğlu (1999), the samples in Eyyübiye and Suruç regions had clayey structure and the samples in other regions had clayey-loam texture, and total P ratios were determined via Induced by Plasma-Optical Emission Spectrometry (ICP-OES). The concentrations of P were measured by the Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-OES Varian Liberty Series II). Some physical and chemical properties of the soils used in the research are given in Table 1.

Table 1. Some properties of soil materials experimented in this study.
Çizelge 1. *Çalışmada denemeye alınan toprak materyalinin bazı özellikleri.*

Sample	pH	Soil texture	EC (mS cm^{-1})	$CaCO_3$ (%)	Total P (mg kg^{-1})
Akçakale	7.70	Clay Loam	1.45	26.6	828
Bozova	7.68	Clay Loam	0.86	25.7	676
Eyyübiye	7.88	Clay	1.36	27.7	694
Haliliye	7.21	Clay Loam	4.02	31.1	2.075
Karaköprü	7.84	Clay Loam	0.74	27.3	2.094
Suruç	7.75	Clay	1.04	12.9	839

Determination of the solvent effect of the phosphorus in the soil solution of ST976

Soil samples were dried and sieved to determine the solvent effect of the P in the soil solution of ST976. After being sterilized, 5 g of soil for each sample was weighed and placed in a 150 ml sterile Erlenmeyer flask, 1 g of *T. funiculosus* ST976 (by weighing of 7 mm diameter mycelial agar plugs cut with a sterile cork borer from the centers of the colonies grown on PDA in aseptic conditions) and 100 mL of sterile distilled water were added into the flask and covered with parafilm. Only 5 g of soil and 100 ml of sterile distilled water were added to the control flasks and kept at 25°C in the dark for 14 days (in three replicates). After extraction of samples kept in the dark for 14 days, favorable P concentrations were determined on the ICP-OES device.

Statistical Analysis

The data were analyzed for significance by analysis of variance (ANOVA), followed by Fisher's least significant difference test (LSD) at $P < 0.01$ with Statistical Analysis System (SAS Version 9.0; SAS Institute Inc.; Cary, NC, USA).

RESULTS and DISCUSSION

Fungal species were easily isolated and purified because they formed surface colonies that were well dispersed at 10-6 dilution. Colonies were identified as *Neoscytalidium* spp. and *Talaromyces* spp. based on the description of their anamorphs (Crous et al., 2006; Samson et al., 2011; Yilmaz et al., 2014). Out of 726 colonies counted in all soil samples, the mean percentage of soil colonization by *Neoscytalidium* spp. (Figure 1) and *Talaromyces* spp. with similar colony type on both media and morphologically similar characteristics for each species were 77.7 and 10.7%, respectively (Table 2). The remaining colonies were minor in proportion (11.6%) and composed of *Penicillium* spp., *Fusarium* spp., a *Pythium* sp. and a *Rhizopus* sp. Among these genera, *Neoscytalidium* (*N. dimidiatum*) has been previously reported to cause canker, shoot blight, and root rot of pistachio in Turkey (Derviş et al., 2019).

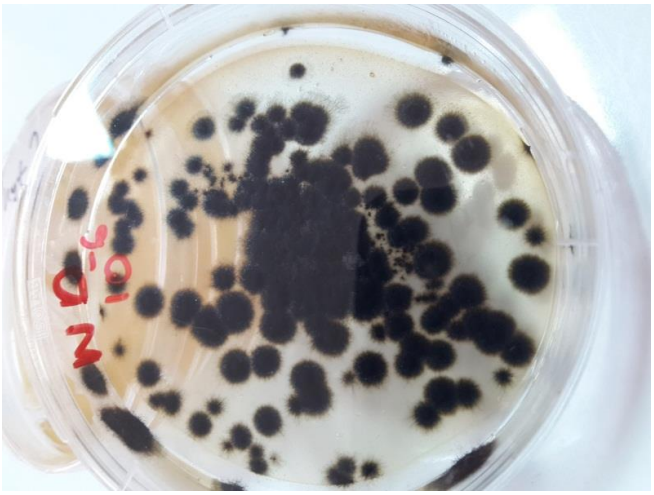

Figure 1. The colonies of *Neoscytalidium* spp. from pistachio rhizosphere.
Şekil 1. Antepfıstığı rizosferinden elde edilen Neoscytalidium spp. Kolonileri

Morphological and molecular studies were initiated by selecting 4 isolates, one isolate representing each sampling. Fungal isolates of *Talaromyces* spp. formed dull green and loosely and strongly funiculose (Figure 2. a) colonies on PDA and MEA of 35- and 30-mm diameter after 7 days incubation at 25°C, respectively.

The reverse of colonies was light green and pale brown on PDA and MEA, respectively. Conidiophores were biverticillate (Figure 2. b) with a few subterminal branches. Conidia were unicellular, gray-green in color, smooth-walled, ellipsoidal and 2 to 3 × 1 to 2 µm in size (Figure 2. c). The distinguishing featured colonies grew well at 37ºC in 50 mm-diameters on MEA after 7 days of incubation. These characteristics were consistent with those of *Talaromyces funiculosus* (Thom) described by Samson et al. (2011) and Yilmaz et al. (2014).

Table 2. Frequency of fungi in soil samples from pistachio rhizosphere on potato dextrose agar medium.
Çizelge 2. *Patates dekstroz agar ortamında antepfıstığı rizosferinden alınan toprak örneklerinde fungusların bulunma oranı*

Fungus	Number of colonies	Soil colonization (%)
Neoscytalidium spp.	564	77.7
Talaromyces spp.	78	10.7
Fusarium spp.	34	4.7
Penicillium spp.	30	4.1
Rhizopus sp.	16	2.2
Pythium sp.	4	0.6

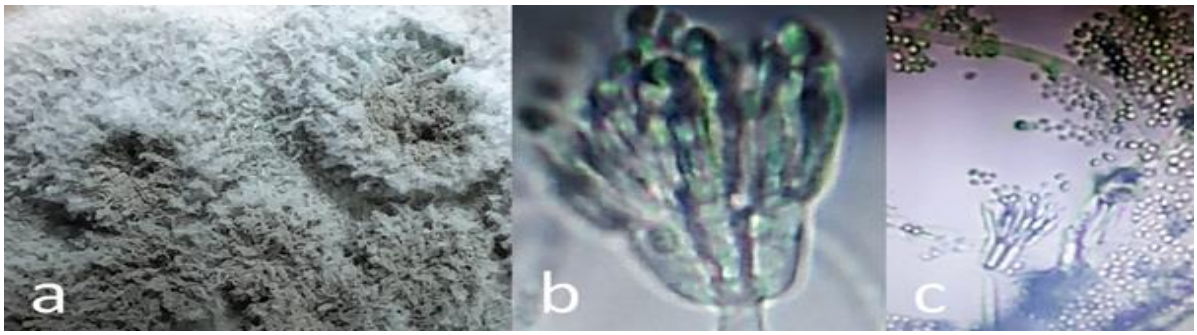

Figure 2. Morphological and distinguishing characters of *Talaromyces funiculosus* a. Strongly funiculose colony type on malt extract agar after 7 days incubation at 25°C in the dark b. Biverticillate conidiophore c. Ellipsoidal conidia

Şekil 2. Talaromyces funiculosus'un morfolojik ve ayırt edici karakterleri a. Karanlıkta 25 °C'de 7 gün inkübasyondan sonra malt ekstraktı agarında yoğun füniküloz koloni tipi b. Bivertisilat konidiyofor c. Elipsoidal konidiler

The PCR sequencing of the ITS locus of rDNA was successfully employed in this study to confirm the identification of isolates. The identification of four representative isolates was confirmed by the BLAST algorithm running on the NCBI based on the ITS sequences which were 575 bp long with identity matches of 100% with those of *T. funiculosus* isolates in the GenBank database. The ITS sequence of the isolate ST976 submitted to the NCBI GenBank was deposited under accession no. MW130842. *Talaromyces* isolates were differentiated from other *Talaromyces* species in the phylogenetic tree (Figure 3). The separation of *T. funiculosus* isolates into a clade was conducted by the Maximum Likelihood method with a high bootstrap value of 94%, which confirm that the sequence analysis of the ITS region is a reliable tool for the identification of *T. funiculosus* isolates.

The results obtained were determined to be statistically significant at the level of $P < 0.01$ in the soils to which the isolate ST976 was added (Table 3).

It was determined that *T. funiculosus* ST976 has the ability to dissolve P quite effectively in the experiment, which was established by bringing the soil solution and the fungus together, within 14 days compared to the control (in three replicates). As a result of the analyses made to determine the solvent effect of P in the soil solution of ST976, it was determined that ST976 (soil + ST976) increased the favourable P ratio in the soil by 109-311% compared to the control (soil) groups. (Table 3, Figure 4).

It was concluded that the contents of the soil samples did not affect the P dissolution rate in *T. funiculosus* ST976 due to the salt-free or less salty structure. It has been stated that the increase in salinity does not decrease phosphate solubility in *T. funiculosus* SLS8 and it may be beneficial in the protection of phosphate levels in salty soils with the use of fungi (Kanse et al., 2015).

In a study of the state of soil fractions and the ability of fungi to dissolve phosphates, fourteen of the thirty-three isolated fungi were considered to be high or very high P solvents based on a dissolving capacity of >1.000 µg $PO4^{3-}$ ml^{-1} (Barroso and Nahas, 2005).

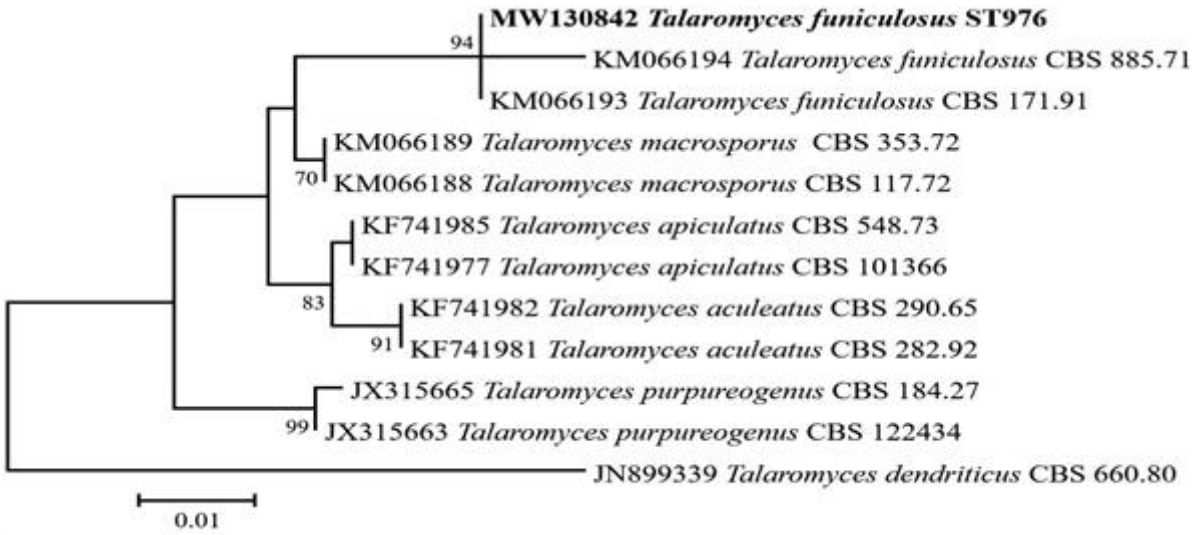

Figure 3. The Maximum Likelihood tree estimated using MEGAX based on ITS sequences showing the relationship among members of the *Talaromyces* genus. The bootstrap support percentages of the analysis are shown at the nodes. The isolate ST976 in bold is obtained in this study.

Şekil 3. Talaromyces cinsi üyeleri arasında ilişkiyi gösteren ve ITS dizileri tabanlı MEGAX kullanılarak tahmin edilen Maximum Likelihood dendogramı. Analizin bootstrap destek yüzdeleri nodlarda gösterilmektedir. Kalın harflerle gösterilen ST976 izolatı bu çalışmada elde edilmiştir.

Table 3. Effect on available P solubility by applying the isolate ST976 to soils.

Çizelge 3. ST976 izolatının topraklara uygulanmasıyla alınabilir P çözünürlüğü üzerindeki etkisi.

Sample	Control (soil, mg P L^{1})	Soil + ST976 (mg P l^{-1})*	Increase (%)
Akçakale	10.7	26.9	151
Bozova	9.50	19.9	109
Eyyübiye	7.52	25.9	244
Haliliye	40.2	119	196
Karaköprü	9.24	24.4	164
Suruç	4.94	20.3	311

**Statistically significant at the level of P < 0.01*

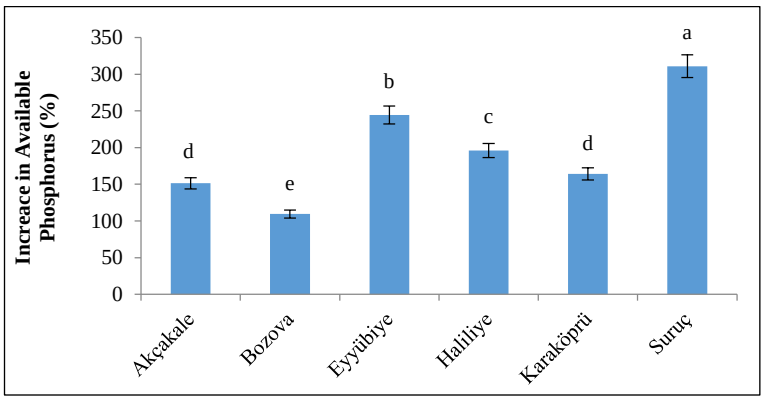

Figure 4. Effect on phosphate solubilization activity of the *Talaromyces funiculosus* isolate ST976. Means of the bars indicated with the same letter are not significantly different from each other according to Fisher's LSD test (P < 0.01).

Şekil 4. Talaromyces funiculosus izolatı ST976'nın fosfat çözündürme aktivitesi üzerindeki etkisi. Fisher'ın LSD testine (P <0.01) göre aynı harfin ardından gelen sütündaki ortalamalar önemli ölçüde farklı değildir.

Whitelaw et al. (1997), stated that increases in P uptake by plants may be due to phosphate dissolution in the soil as a result of the presence of *Penicillium radicum*. The inoculation of plant seeds with P

dissolving bacteria was reported to promote plant growth by increasing the availability of P fertilizers fixed in the soil or applied (Çakmakçı, 2005). In soil conditions, various phosphate dissolving microorganisms have features such as helping to dissolve insoluble phosphates and improving soil health and quality (Richardson, 2001; Vassilev et al., 2006; Naik et al., 2008; Dash and Gupta, 2011; Sun et al., 2017). Many researchers stated that depending on the soil structure and at low pH, P solubility is more active by fungi (Whitelaw et al., 1999; Çakmakçı, 2005; Song et al., 2008; Chai et al., 2011; Sharma et al., 2013). Seshadri et al. (2000) and Antoun (2005) reported that the soil pH is affected by the microorganisms that dissolve P in the soil, organic acids such as gluconic acid, citric acid and H+ (proton) pumping, and the P turns into a form that can be taken by plants.

CONCLUSIONS

In the present study, which was conducted to determine the solvent effect of the P in the soil solution of ST976 isolated from the roots of the pistachio tree, it was determined that ST976 can dissolve P at a rate of 109% to 311% compared to the control, depending on the P ratio in the soil.

Although it has been reported in the literature that organisms dissolving P at low pH are generally more active, the soil samples taken for the research had slightly alkaline and medium alkaline pH, and there were positive increases in P solubility in soils with ST976 added against control groups. Although the element P is found in high amounts in both organic and inorganic forms in the soil, it is still a limiting factor in terms of plant nutrition since it is in a form that cannot be taken by plants. Therefore, the presence of phosphate-dissolving microorganisms in the soil plays an important role in plant nutrition by increasing P uptake by the plant. To minimize chemical applications in terms of increased efficiency and quality in production, it is known that the role of P-dissolving microorganisms in soils with different P content is substantially important. Considering the small amount of research on their use as biological fertilizers and their encouragement of plant growth, more and broader studies should be conducted.

The findings demonstrate the ability of this beneficial rhizospheric fungus to solubilize P as a potential growth promoter under high pathogen pressure (*Neoscytalidium* spp. in this study). In light of other studies, the ability of *T.* f*uniculosus* ST976 to dissolve phosphorus directly in the soil solution was demonstrated in this study. It is thought that the data obtained in soil conditions will be included in scientific studies in a way that will form the basis for the studies on the application of *T. funiculosus.*

Author's Contributions

The contribution of the authors is equal

Statement of Conflict of Interest

Authors have declared no conflict of interest.

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