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Screening of Acidophilic Actinobacteria That Show Activity against Paddy Pest Fungi

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Abstract

This study aimed to isolate and identify acidophilic actinobacteria. Acidophilic actinobacteria isolates were had from a paddy field soil in Osmancık placed near Çorum province in Turkey. The dilution plate technique on seven selective media with pH 5.5 was used for isolation. 16S rRNA gene PCR amplification of acidophilic actinobacteria was performed. Three different algorithms were used in the phylogenetic analyzes made with MEGA 7.0 software. Twenty-two isolates were obtained from seven selective media, and according to 16S rRNA gene sequence analysis of 22 isolates, twenty-one *Streptomyces* isolates and one *Rhodococcus* isolate were identified. The antifungal activities of isolated acidophilic actinobacteria against *Fusarium moniliforme* and *Rhizoctonia solani*, the rice pathogenic fungi were evaluated. The isolates with antifungal activity have the potential to be used as biological control agents against rice pathogens.

Keywords: Genomic DNA isolation, 16S rRNA gene, Acidophilic actinobacteria, Paddy field

Introduction

Actinobacteria, which are in the group of gram positive bacteria, have high GC content. They are widely distributed in the soil and other different environments such as marine sediments (Veyisoglu and Sahin, 2015; Veyisoglu et al., 2016; Veyisoglu et al., 2020). It is the most economical and biologically valuable group of bacteria among prokaryotes and actinobacteria synthesize different biologically active compounds such as antitumor agents, enzymes and, antibiotics (Sanglier et al., 1996; Lazzarini et al., 2000; Procópio et al., 2012).

Acidophilic actinobacteria are divided into two main groups as neutrotolerant acidophils and strict acidophils. Typical neutrotolerant acidophils (optimum growth between pH 5.0 and 5.5) grow in environments between pH 4.5-7.5. Members of the strictly acidophilic group typically grow in environments between pH 3.5 and 6.5 and provide optimum growth at pH 4.5 (Williams et al., 1971; Xu et al., 2006; Poomthongdee et al., 2015).

Rice is a staple product that meets the needs of about half of the people living in the world. However, in rice cultivation, fungal rice diseases create significant problems (Ou, 1987). The use of chemical synthetic substances for prevention and treatment is considered an effective method, but these chemicals have harmful effects on the environment and human health (Tsukano et al., 1986; Pingali et al., 1995).

Actinobacteria are recognized as potential biocontrol agents against different phytopathogenic fungi due to the bioactive metabolites synthesis or production of enzymes that hydrolyze fungal cell walls (Basilio et al., 2003; Li et al., 2011; Patil et al., 2011; Yuan and Crawford, 1995; Gomes et al., 2000; El-Tarabily and Sivasithamparam, 2006; Xue, 2013). Numerous studies have focused on the capability of actinobacteria isolated from isolation media with neutral pH. Nevertheless, It has been reported that acidophilic actinobacteria inhibit fungi under acidic conditions more than neutrophilic actinobacteria

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(Zakalyukina and Zenova, 2007).

According to our screenings, there is no study on acidophilic actinobacteria isolation, molecular analysis based on 16S rRNA gene region and antifungal activity from paddy fields in Osmancık district of Çorum province in Turkey. The aim of this study is to isolate acidophilic actinobacteria from paddy fields in Osmancık district of Çorum province in Turkey, to determine the species to which the isolates belong by performing 16S rRNA sequence analysis, to make phylogenetic dendograms of the isolates according to the result of 16S rRNA sequence analysis, to identify candidate isolates to become new species and to determine antifungal activities of isolates.

Materials and Methods

Soil Sample Collection

Acidophilic actinobacteria isolates were isolated from soil samples from a paddy field (head of the field) (40°58'42.4"N 34°47'16.2"E), (middle of the field) (40°58'42.4"N 34°47'15.5"E) and (the field end) (40°58'40.1"N 34°47'14.4"E) in Osmancık located near Çorum province using a dilution plate on seven selective media with pH 5.5 (Table 1). Media used were Humic acid Vitamin Agar (Hayakawa and Nonomura., 1987), Starch-Casein Agar (Küster and Williams, 1964), Gause no. 1 Agar (Gauze et al., 1957), NZ-Amine agar-DSMZ medium 554 (Atlas, 2010), SM3 Agar (Tan et al., 2006), Nocardia Agar (Sanglier et al., 1992) and M1 Agar (Mincer et al., 2002). Soil samples were taken from a depth of 20–30 cm.

Table 1. List of selective media used.

	Name of media	Antibiotics	References
1	Humic Acid-Vitamin Agar (pH 5.5)	Nystatin (50 µg/ml) Nalidixic acid (25 µg/ml)	(Hayakawa and Nonomura, 1987)
2	Starch-Casein Agar (pH 5.5)	Nystatin (50 µg/ml) Nalidixic acid (25 µg/ml)	(Küster and Williams, 1964)
3	Gause no. 1 Agar (pH 5.5)	Nystatin (50 µg/ml) Nalidixic acid (25 µg/ml)	(Gauze et al., 1957)
4	NZ-Amine Agar- DSMZ medium 554 (pH 5.5)	Nystatin (50 µg/ml) Nalidixic acid (25 µg/ml)	(Atlas, 2010)
5	SM3 Agar	Nystatin (50 µg/ml) Rifampicin (5 µg/ml)	(Tan et al., 2006)
6	Nocardia Agar	Cycloheximide (50 µg/ml) Nystatin (50 µg/ml)	(Sanglier et al., 1992)
7	M1 Agar	Cycloheximide (50 µg/ml) Nystatin (50 µg/ml), Rifampicin (5 µg/ml)	(Mincer et al., 2002)

Acidophilic actinobacteria were isolated from four selective media with pH 5.5. The organisms were maintained on related agar slopes added cycloheximide (50 µg mL^{-1}), and stocked in glycerol (25%, v/v) at -20 °C.

Isolation of Actinobacteria

After adding 1 g of soil sample to 9 ml of Ringer's solution, it was mixed at room temperature for homogenization. Then this 10^{-1} dilution was kept for 30 min at 55 °C in a preheated water bath. Serial dilutions (10^{-1}, 10^{-2} and 10^{-3}) were spread over the surface of related agar plates, and the plates were incubated at 28 °C for 10–14 days. Actinobacteria were subcultured onto related media and incubated for up to 4 weeks at 28 °C. Suspensions of spores and mycelia were maintained in 25% glycerol (w/v) at −20 °C.

Genomic DNA Extraction

For molecular identification and phylogenetic analysis, the genomic DNA of test organisms was isolated by using Purelink Invitrogen genomic DNA isolation kit.

Amplification and Detection of 16S rRNA Gene Sequence

PCR mixture (50 µl) included chromosomal DNA (50–300 ng), primers (20 µM), Taq polymerase (2.5 U, HotStarTaq®), Taq polymerase buffer (HotStarTaq®) and deoxynucleoside triphosphates mixture (Promega) (25 µM). The 16S rRNA genes were amplified by using specific primers 27F and 1525R. The PCR conditions were initial denaturation at 95 °C (5 min), 35 cycles at 95 °C (1 min), 55 °C (2 min), and 72 °C (3 min), and a final extension at 72 °C (10 min). Then the PCR products were separated using electrophoresis in 1% agarose gel (Merck) and were imaged with the Gene Genius Bioimaging system.

Sequencing of PCR Products

The PCR products of the 22 isolates were purified with

QIAquick purification kit (Qiagen). According to Chun and Goodfellow (1995) PCR-mediated amplification and sequencing of the 16S rRNA gene were performed as described by using an ABI PRISM 3730 XL automatic sequencer with previously mentioned oligonucleotide primers (Table 2). Chromatogram files in ABI format are turned to FASTA format using Chromas 1.7.5. An almost complete 16S rRNA gene sequences of the 48 isolates were compared to sequences of type strains in GenBank (Boratyn et al., 2013) and EzBioCloud (Yoon et al., 2017) databases.

Table 2. List of oligonucleotide primers used for 16S rRNA PCR amplification and sequencing.

Primer Code	Sequences (5'-3')	Base Length	References
27F	AGAGTTTGATC**M**TGGCTCAG	20	(Lane, 1991)
518F	CCAGCAGCCGCGGTAAT	17	(Buchholz-Cleven et al., 1997)
800R	TACCAGGGTATCTAATCC	18	(Chun and Goodfellow, 1995)
MG5F	AAACTCAAAGGAATTGACGG	20	(Chun and Goodfellow, 1995)
MG6F	GACGTCAAGTCATCATGCC	19	(Chun and Goodfellow, 1995)
1525R	AAGGAGGTG**W**TCCA**R**CC	17	(Lane, 1991)

Degeneracies according to Lane (1991) M = A:C; R = A:G; W = A:T.

Phylogenetic Analysis

The determination of phylogenetic neighbors and computation of pairwise 16S rRNA gene sequence similarity were obtained using the Ezbiocloud server (https://www.ezbiocloud.net)(Yoon et al., 2017). Multiple alignments with sequences from closely related species were applied with the program CLUSTAL W in MEGA 7.0 (Kumar et al., 2016). Phylogenetic trees were formed with the neighbor-joining (Saitou and Nei, 1987) maximum likelihood (Felsenstein, 1981) and maximum parsimony (Kluge and Farris, 1969) algorithms in MEGA 7.0 (Kumar et al., 2016). Evolutionary distances were calculated using the Kimura two-parameter (Kimura, 1980) and topologies of the resultant trees evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. The 16S rRNA gene sequences obtained in this study were deposited in GenBank (Table 3).

In Vitro Antifungal Activity

Twenty-two acidophilic actinobacteria isolates were examined for their ability to inhibit the growth of two pathogens rice pests, including *Fusarium moniliforme* and *Rhizoctonia solani.*

The ability to inhibit the growth of twenty-four acidophilic actinobacteria isolates was observed using an overlay technique (Williams et al., 1983). For each isolate, 2 ml Ringer's solution was added to small bottles with lids and sterilized by autoclaving at 121 ºC for 15 minutes. The spores and substrate micelles of the isolates grown at 28 ºC in ISP 2 agar medium were transferred to small glass bottles containing Ringer's solution in aseptic conditions. Spot-inoculated colonies on modified Bennett's Agar (Jones, 1949) surface were inverted over 1-5 ml chloroform for 40 min. Killed colonies were then overlaid with 5 ml sloppy agar (0.7 %, w/v, nutrient agar) inoculated with the pathogen test organisms. Zones of inhibition were measured after 48 h at 30 ºC.

Results and Discussion

A total of 22 morphologically distinct actinobacterial isolates were obtained from a paddy field soil in Osmancık. Seven different selective isolation media were used. Seven strains were isolated on Starch-Casein agar, seven strains from Gause no. 1 agar, three strains from M1 agar, two strains from Humic Acid-vitamin (HV) agar, two strains from SM3 agar, one strain from Nocardia agar and incubated at 28°C for about 10-14 days (Fig 1 and Table 3).

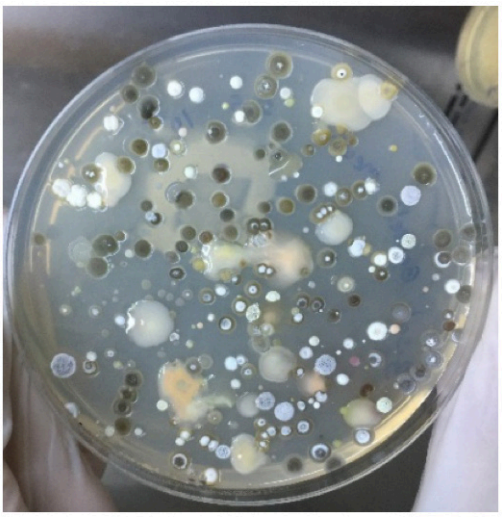

Figure 1. Isolation petri sample - Gause no. 1 agar.

16S rRNA gene sequences of all 22 isolates were amplified using universal primers (Table 2). Most of the strains belonged to the genus *Streptomyce*s (21 isolates). Other one strain belonged to the genus *Rhodococcus* (1 isolate) (Table 3).

Table 3. Nucleotide similarity of *Actinobacteria* isolates according to 16S rRNA sequence analysis.

Number	Strain	Isolation medium	Genbank Number	Highest match	Similarity (%)-Nucleotide difference
1	PT503	SM3 Agar	MZ025943	*Streptomyces bobili* NRRL B-1338^T	99.72 % - 4/1448
2	PT510	Gause no. 1 Agar	MZ026800	*Streptomyces scabiei* NRRL B-16523^T	99.86 % - 2/1448
3	PT511	Starch-Casein Agar	MZ026801	*Streptomyces bobili* NRRL B-1338^T	99.72 % - 4/1448
4	PT513	Starch-Casein Agar	MZ026802	*Streptomyces abietis* A191^T	98.34 % - 24/1444
5	PT517	Nocardia Agar	MZ026846	*Rhodococcus wratislaviensis* NBRC 100605^T	99.79 % - 3/1441
6	PT539	Humic Acid-Vitamin Agar	MZ027070	*Streptomyces bobili* NRRL B-1338^T	99.38 % - 9/1448
7	PT542	Humic Acid-Vitamin Agar	MZ027080	*Streptomyces clavifer* NRRL B-2557^T	100 % - 0/1448
8	PT557	Gause no. 1 Agar	MZ027591	*Streptomyces clavifer* NRRL B-2557^T	100 % - 0/1448
9	PT559	Gause no. 1 Agar	MZ027347	*Streptomyces fulvissimus* DSM 40593^T	99.93 % - 1/1448
10	PT564	Gause no. 1 Agar	MZ027400	*Streptomyces cyslabdanicu*s K04-0144^T	99.24 % - 11/1448
11	PT566	Gause no. 1 Agar	MZ027592	*Streptomyces fulvissimus* DSM 40593^T	99.93 % - 1/1448
12	PT572	SM3 Agar	MZ027483	*Streptomyces bobili* NRRL B-1338^T	99.72 % - 4/1448
13	PT573	M1 Agar	MZ027489	*Streptomyces bobili* NRRL B-1338^T	99.79 % - 3/1448
14	PT575	M1 Agar	MZ027494	*Streptomyces rochei* NRRL B-2410^T	100 % - 0/1448
15	PT579	M1 Agar	MZ027496	*Streptomyces caeruleatus* NRRL B-24802^T	97.51 % - 36/1448
16	PT597	Starch-Casein Agar	MZ031923	*Streptomyces aurantiogriseus* NBRC 12842^T	98.55 % - 21/1447
17	PT598	Gause no. 1 Agar	MZ031924	*Streptomyces virginiae* NRRL ISP-5094^T	100 % - 0/1446
18	PT599	Starch-Casein Agar	MZ061921	*Streptomyces fulvissimus* DSM 40593^T	99.93 % - 1/1448
19	PT600	Gause no. 1 Agar	MZ040132	*Streptomyces bobili* NRRL B-1338^T	99.72 % - 4/1448
20	PT605	Starch-Casein Agar	MZ040598	*Streptomyces bobili* NRRL B-1338^T	99.72 % - 4/1448
21	PT609	Starch-Casein Agar	MZ040488	*Streptomyces paradoxus* NBRC 14887^T	99.38 % - 9/1447
22	PT613	Starch-Casein Agar	MZ040755	*Streptomyces rochei* NRRL B-2410^T	100 % - 0/1448

In a study conducted in Thailand, acidophilic actinobacteria were isolated from the rhizosphere soil of rice plant, and the antifungal activity of these isolates was examined (Poomthongdee et al., 2015).

In another study conducted in Turkey, the effect of light intensity on the nitrogenase activities of cyanobacteria was investigated after the isolation of cyanobacteria was carried out by taking samples of irrigated soil from the regions where paddy cultivation was carried out in Osmancık district of Çorum province (Ökmen and Dönmez, 2007**)**.

Based on 16S rRNA gene sequence analysis, 21 of 22 isolates are members of the genus *Streptomyces*. Members of the genus *Streptomyces* are dominant in the paddy field located in Osmancık (Fig. 2). Based on 16S rRNA gene sequence analysis, 21 *Streptomyces* isolates were determined. Actinobacteria commonly found in acidic habitats belong to the genus *Streptomyces* (Zenova et al., 2011).

According to the neighbor-joining algorithm, the phylogenetic tree indicated that twenty-one strains were members of the genus *Streptomyces* (Fig. 2; Supp. Figs. S1 and S2). Based on the 16S rRNA gene sequence analysis, 21 *Streptomyces* isolates showed that close 16S rRNA gene sequence similarity with the type strain of *Streptomyces* which are 100% and 97.51%. Strains PT513, PT579 and PT597 may be new species belong to the genus *Streptomyces*. Strain PT513 had the closest 16S rRNA gene sequence similarity with *Streptomyces abietis* A191^T (98.34%). PT579 indicated the closest 16S rRNA gene sequence similarity with *Streptomyces caeruleatus* NRRL B-24802^T (97.51%), and Strain PT597 had the closest 16S rRNA gene sequence similarity with *Streptomyces aurantiogriseus* NBRC 12842^T (98.55%) (Table 3). Isolates with a 16S rRNA similarity rate below 98.65% have the possibility of being a new species (Stackebrandt and Ebers, 2006; Kim et al., 2014; Chun et al., 2018).

Based on the neighbor-joining algorithm, the phylogenetic tree indicated that one strain was member of the genus *Rhodococcus* (Fig. 2; Supp. Figs. S1 and S2). PT517 indicated the closest 16S rRNA gene sequence similarity with *Rhodococcus wratislaviensis* NBRC 100605^T (99.79%) (Table 3).

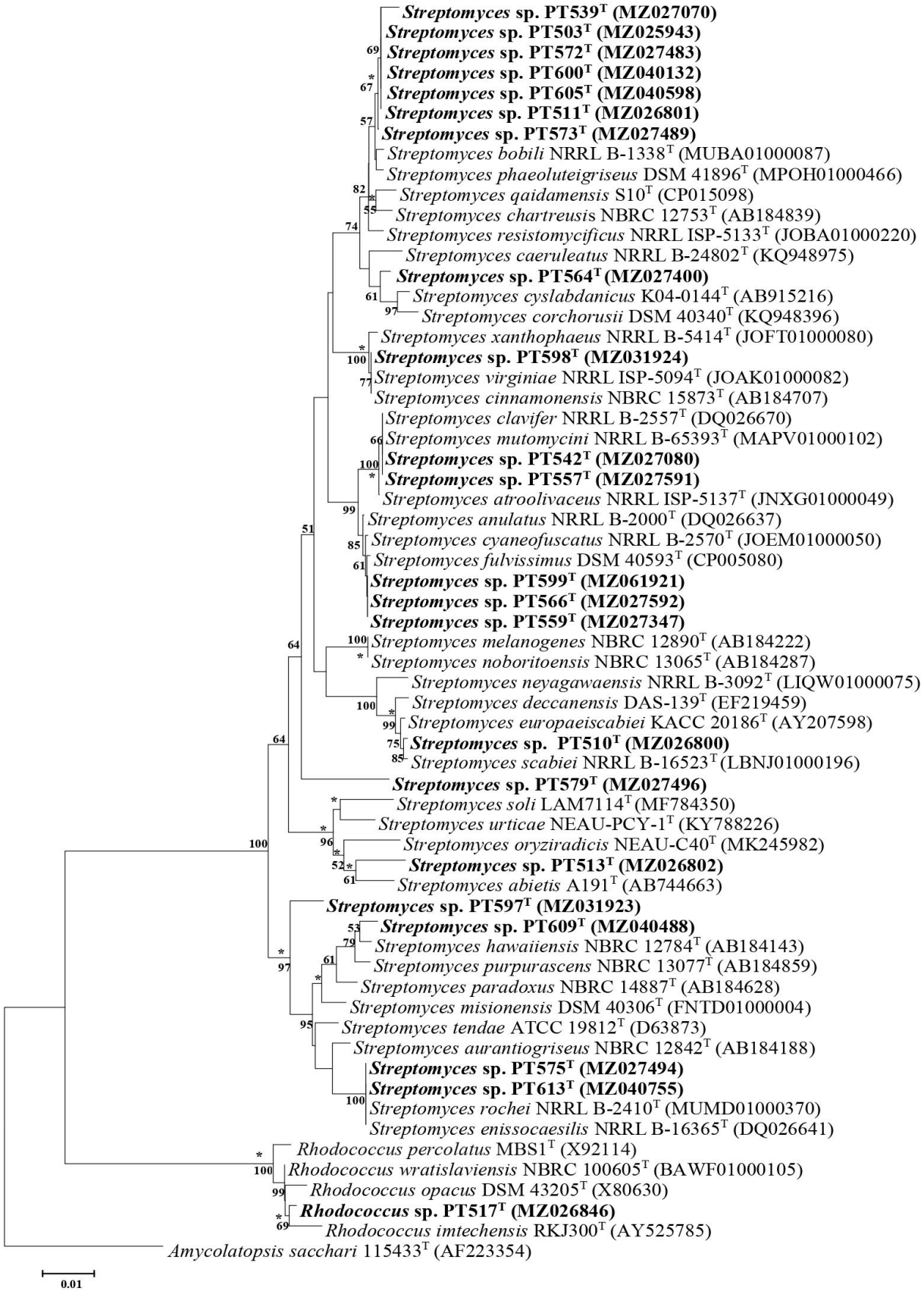

Figure 2. Neighbor-joining tree (Saitou and Nei, 1987) according to 16S rRNA gene sequences of test isolates

The antifungal activity of twenty-two acidophilic actinobacteria isolates was determined against rice pest two fungi, and their inhibition zone diameters were measured. While of the isolates 27.27 % showed antifungal activity against the rice pathogen *F. moniliforme*, 100% did not show antifungal activity against *Rhizoctonia solani*. Six acidophilic actinobacteria test isolates were found to show good antifungal activity against *F. moniliforme* fungus. PT510 coded isolate formed 38 mm, PT559 coded isolate 24 mm, PT566 coded isolate 40 mm, PT575 coded isolate 28 mm, PT599 coded isolate 14 mm and PT613 coded isolate 20 mm inhibition zone diameter. Measured zone diameters are given in Figure 3.

Poomthongdee et al. (2015) 351 acidophilic actinobacteria were isolated from 21 rhizospheric soils, and 57.8% of these actinobacteria showed antifungal effect against *Fusarium moliniforme*, 32.5% *Helminthosporium oryzae* and 50% *Rhizoctonia solani*. While 25.9% of the isolates showed activity against all pathogenic fungi tested, more than 68.1% showed activity against at least one pathogenic fungus.

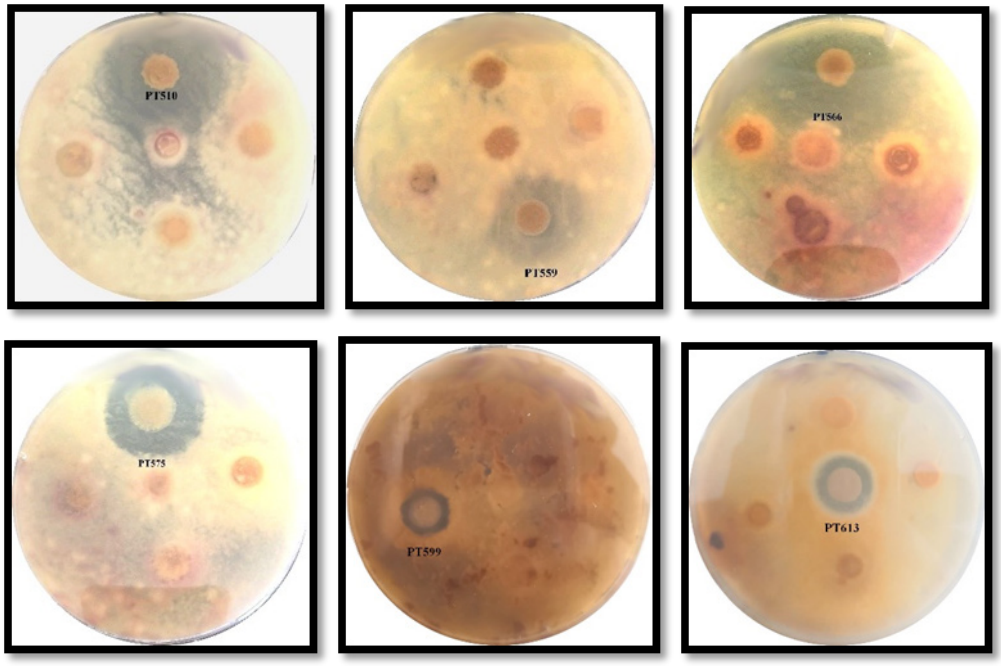

Figure 3. Zones of acidophilic actinobacteria isolates formed by against the rice pathogen *Fusarium moniliforme*

Conclusion

Consequently, isolation and phylogenetic analysis of acidophilic actinobacteria living in a paddy field soil in Osmancık were performed.

In future studies, it is possible to introduce *Streptomyces* sp. PT513, *Streptomyces* sp. PT579 and *Streptomyces* sp. PT597 isolates obtained in this study as a new species in the literature by making necessary analyzes.

Fungi cause important problems in rice cultivation. Although the use of chemical substances against fungi seems to be effective, it can be harmful to human and environmental health. Actinobacteria have the potential to be used as biocontrol agents against a variety of phytopathogenic fungi. In this study, six acidophilic actinobacteria test isolates were found to show good antifungal activity against *F. moniliforme* fungus. Isolates showing activity in this study can be used in biological control. It is more beneficial for the environment and people than chemicals.

Compliance with Ethical Standards

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal.

Ethical approval

Not applicable.

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Data availability

Not applicable.

Consent for publication

Not applicable.

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