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ORIGINAL ARTICLE

Antagonist activities and phylogenetic relationships of actinomycetes isolated from an *Artemisia* habitat

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Abstract Diverse habitats have been screened for novel antimicrobial actinomycetes, while others remain unexplored. In this study, we analyzed the bioactivities of actinomycetes cultured from rhizosphere soils of the desert plant *Artemisia tridentata* and the nearby bulk soils. Actinomycetes were screened for antifungal and antibacterial activities toward a panel of plant pathogens; all comparisons were between activities of rhizosphere soil isolates toward those of its counterpart bulk soil. A selected group of the strongest antifungal isolates were also tested against two antifungal-drug resistant strains of *Candida albicans*. 16S rDNA partial sequences and phylogenetic analysis of isolates that showed broad-spectrum antifungal activities were performed. Forty-two out of 200 and two soil isolated actinomycetes were selected for their strong antifungal activities. The highest proportion of isolates ($p < 0.05$) from rhizosphere soil of an old plant showed antagonism against gram-positive bacteria (0.483 and 0.224 proportions against *Bacillus subtilis* and *Rathayibacter tritici*, respectively), and phytopathogenic fungi (0.259, 0.431, and 0.345 proportions against *Fusarium oxysporum*, *Rhizoctonia solani* and *Pythium ultimum*, respectively), while the highest antagonism against the gram-negative bacteria predominated in isolates from the bulk soils. Isolates from a rhizosphere soil of a young plant were characterized for strong antagonist activities against *Fusarium oxysporum* (0.333 proportion, $p < 0.05$). Phylogenetic analysis of 16S rDNA sequences showed that isolates that exhibited strong antifungal activity were genetically similar. We conclude that the rhizosphere soil of *A. tridentata* is an excellent source for discovery of actinomycetes with potentially novel antifungal compounds.

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PALABRAS CLAVE
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Suelo rizosférico;
Actividad anti-Candida;
Streptomyces

Actividades antagónicas y relaciones filogenéticas de actinomicetos aislados de un hábitat de *Artemisia*

Resumen En la búsqueda de actinomicetos antimicrobianos se han estudiado diversos hábitats, pero muchos permanecen aún sin explorar. En este estudio analizamos las actividades biológicas de cultivos de actinomicetos provenientes de suelos rizosféricos de la planta desértica *Artemisia tridentata* y de suelos no asociados a sus raíces. Los actinomicetos fueron seleccionados por sus actividades antifúngicas y antibacterianas contra un panel de patógenos de plantas. Todas las comparaciones fueron entre las actividades de los aislados rizosféricos y aquellas de los aislados no asociados a las raíces. Un grupo selecto de los aislados con las mayores actividades antifúngicas fueron también evaluados contra 2 cepas de *Candida albicans* resistentes a antifúngicos. Se realizó la secuenciación parcial del ARNr 16S y el análisis filogenético de los aislados que mostraron actividades antifúngicas de amplio espectro. Se seleccionaron 42 de 202 actinomicetos aislados por sus fuertes actividades antifúngicas. La mayor proporción de aislados de suelo rizosférico de plantas viejas mostraron antagonismo contra bacterias gram positivas y hongos fitopatógenos (proporciones de 0,259; 0,431 y 0,345 contra *Fusarium oxysporum*, *Rhizoctonia solani* y *Pythium ultimum*, respectivamente), mientras que la mayor actividad antagónica contra las bacterias gram negativas predominaron en aislados de suelo no asociado a raíces. Los aislados de suelo rizosférico de plantas jóvenes se caracterizaron por una fuerte actividad antagónica contra *F. oxysporum* (proporción de 0,333, $p < 0,05$). El análisis filogenético de secuencias del ADNr 16S mostró que los aislados que presentaron fuerte actividad antifúngica fueron genéticamente similares. Concluimos que el suelo rizosférico de *A. tridentata* es una fuente excelente para el descubrimiento de actinomicetos productores de compuestos antifúngicos potencialmente novedosos.

Introduction

Actinomycetes comprise an extensive and diverse group of gram-positive, aerobic, mycelial bacteria that play an important physiological and ecological role in soil.[29,31] They can degrade a wide diversity of recalcitrant compounds such as lignocelluloses and many other polymers occurring in soil and litter, as well as a range of xenobiotic compounds.[4,7,19] Because of their metabolic diversity, actinomycetes are a great source of lytic enzymes, antibiotics and a great deal of other bioactive metabolites.[5,10,28] This group of organisms produce more than half of the naturally occurring antibiotics discovered to date and continue to be screened for useful compounds.[5,8]

Because actinomycetes are largely spread in nature, they have been isolated from many environments including soils, composts, plant materials and waters.[5,10,23,26] Actinomycetes, especially *Streptomyces* spp. isolated from rhizosphere soils, sometimes represent novel species.[16,20] They are great root colonizers, where they protect the plants against phytopathogens and promote plant growth.[1,11,24,33]

Desert plants such as *Artemisia* spp. are known to produce a great diversity of phenols and terpenoids with antimicrobial activities.[30] Thus, rhizosphere actinomycetes from *Artemisia*, which can endure unfavorable growth conditions, are worthy of examination for antimicrobial activities.

The present study involved the screening for antimicrobial activities of actinomycetes from rhizosphere soils of *Artemisia tridentata* and their counterpart bulk soils. Their antagonistic activity was characterized based on *in vitro* bioassays against a broad panel of bacteria and fungal plant pathogens. A select group of the actinomycetes strongly antagonistic to all filamentous fungi tested was further characterized for antagonism toward two drug-resistant strains of *Candida albicans*: ATCC MY-204276 (fluconazole-resistant) and ATCC 44373 (5-fluorocytosine-resistant). In addition, a selection of three strongly antifungal groups of isolates were further characterized by partial 16s rRNA gene amplification, and phylogenetic trees were constructed to determine if there was a relationship between antimicrobial activity and genetic relatedness.

Material and methods

Source of actinomycetes

Two hundred and two actinomycete strains were previously isolated from two sagebrush rhizosphere soils [one from a young plant (RSYP) and one from an old growth plant (RSOP)], and two non-rhizosphere bulk soils near the sagebrush (B1Y and B1O, for the young and old plant system, respectively).[9]

Evaluation of antimicrobial activities

Antimicrobial susceptibility tests were performed *in vitro* by using a panel of plant pathogen strains including: *Fusarium oxysporum* ATCC 070233 as well as *Rhizoctonia solani* and *Pythium ultimum*; two gram-positive bacteria (*Bacillus subtilis* and *Rathayibacter tritici*) and two gram-negative bacteria (*Xanthomonas campestris* pv. *campestris* and *Burkholderia cepacea*). All fungal strains were from Dr. Don Crawford's laboratory stock, Department of Microbiology, Molecular Biology and Biochemistry, while the bacterial strains were from the Bacteriology laboratory, Department of Entomology, Plant Pathology and Nematology.

The antimicrobial susceptibility for filamentous fungi were assessed following the *in vitro* plate bioassay. Actinomycete isolates were streak-inoculated to one side of PDA and YDA plates and incubated at 30 °C for eight days to allow the production and diffusion of metabolites and extracellular hydrolytic enzymes. An agar plug containing actively growing fungus was then placed onto the opposite side of the inoculated plates and incubated at 30 °C. Fungal mycelial plugs were placed on noninoculated plates as controls. Growth inhibition was recorded at different time intervals, depending on the fungus, for 7 days.

The antimicrobial bioassay plates for bacteria were performed by streak-inoculation of the actinomycete to one side of multiple PDA plates and incubated at 30 °C for 10 days to allow the production and diffusion of metabolites and extracellular hydrolytic enzymes. Forty-eight-hour bacterial growth from NBY (ATCC Medium 763) plates was then inoculated as lines perpendicular to the actinomycete growth and incubated at 30 °C. Bacteria were also streaked on non-inoculated plates to serve as controls.[2] Bacterial growth inhibition was recorded at different time intervals for 5 days.

A selected group of actinomycetes strongly antagonistic to all filamentous fungi tested were further characterized for antagonism toward two drug-resistant strains of *Candida albicans*: ATCC MY-204276 (fluconazole resistant) and ATCC 44373 (5-fluorocytosine-resistant). Anti-*Candida* activity was tested as described for bacteria except for PDA and YDA were used instead.

PCR amplification of partial 16s rRNA gene sequences

Genomic DNA from the isolates with potent antifungal activity were extracted using the UltraClean™ Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, CA) according to the manufacturer's instructions. 16S rDNA partial sequences of cultured isolates were amplified using the primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 907R (5′-CCGTCAATTCMTTTRAGTT-3′). Each PCR mixture contained 20 μmol/l (each) primer, 0.2 mmol/l (each) dNTPs, 25 mmol/l $MgCl_2$, 5 μl of 10x PCR universal buffer (Invitrogen TECH-LINE™, USA) 1 μl of the DNA template, and 1.25 U of Taq DNA polymerase (Invitrogen TECH-LINE™, USA) to a final volume of 50 μl.

Thermocycling conditions were as follows: one cycle at 95 °C for 5 min; followed by 30 cycles (each) at 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min; and finally, one cycle at 72 °C for 7 min in a Gene Amp PCR System 2400 thermocycler (Applied Biosystems). The positive control consisted of reaction mixtures containing 8 μg of DNA of *Streptomyces lydicus* WYEC-108. The negative control lacked the DNA template but contained all other reactants. A 1-kb plus ladder (GIBCO BRL Life Technologies) was used as a DNA size marker.

The purified PCR amplicons were sequenced by the Laboratory of Biotechnology and Bioanalysis at Washington State University (Pullman, WA). Sequences were compared against known sequences using the NCBI BLAST database.

Phylogenetic analysis

The BioEdit program was used as an editing tool to facilitate sequence analysis. Multiple alignments were obtained using the Clustal W program. Phylogenetic trees were inferred by three algorithms, the maximum-parsimony, neighbor-joining, and maximum-likelihood methods using the PAUP* package. In addition, the 16S rDNA sequence of *Haemophilus paragallinarum* was added to the analysis as an outgroup. Bootstrap analyses for the neighbor-joining and the maximum-likelihood results were generated based on 200 re-samplings.

Evolutionary distance matrices for the neighbor-joining method were generated. The TreeView (WIN 32) program was used for viewing the trees generated by the three algorithms.

Statistical analysis

The generalized linear model was used, assuming a binomial distribution to test for significant activity effect across soils. Pair-wise comparisons among soils were done using contrasts and chi-square tests. All computations were carried out using SAS 8.2 Copyright (c) 1999–2001 by SAS Institute Inc., Cary, NC, USA.

Results

Two hundred and two actinomycetes were isolated from the four mentioned soils and their antifungal activities showed that isolates from both rhizosphere soils, RSYP and RSOP, had the highest activity against *F. oxysporum*; however, they were not significantly different from those of their bulk soils (Table 1). The highest antagonistic activities against *R. solani* were detected in RSOP; this was significantly different from the rest of the soils, followed by the activities of bulk soils B1Y and B1O. The highest anti-*Pythium* activities were detected in organisms found in three soil samples: bulk soil B1Y and rhizosphere soils, RSOP and RSYP. Bulk soil B1O activity was significantly lower than that in the other three soils (Table 1).The antibacterial activities of isolates from bulk soils B1Y and B1O had higher activities against Gram-negative bacteria, *X. campestris* pv. *campestris* and *B. cepacea* than their counterparts RSYP and RSOP; however, no statistical differences were detected among soils (Table 2). On the other hand, the highest activities against Gram-positive bacteria, such as *B. subtilis*, were detected in the isolates of bulk soil B1Y and rhizosphere soil RSOP, with

Table 1 Distribution of antifungal activities in four soils of a sagebrush habitat.

Soil samples	Active isolates against		
	F. oxysporum	*R. solani*	*P. ultimum*
RSYP	0.333[a]	0.074[b]	0.333[a]
B1Y	0.203[a]	0.257[b]	0.487[a]
RSOP	0.259[a]	0.431[a]	0.345[a]
B1O	0.209[a]	0.256[b]	0.140[b]

The data presented are proportions of the active isolates with respect to the total population in each soil (RSYP, n = 27; B1Y, n = 74; RSOP, n = 58; B1O, n = 43). RSYP and RSOP are rhizosphere soils from a young and old sagebrush plants, respectively; B1Y and B1O are their counterpart bulk soils, respectively. Different letters within a column indicate significant difference at $p < 0.05$. All comparisons are based on logit transformations [$(P/1-P)$, where P is the transformation values of active isolates].

no statistical difference between the two, followed by the antagonistic activity of bulk soil B1O (Table 3). Finally, isolates from soil B1Y showed the highest antagonistic activity against *Rathayibacter*, followed by both rhizosphere soils, RSYP and RSOP; isolates from bulk soil B1O had the lowest activity against *Rathayibacter* (Table 2).

Selected isolates

Forty-two isolates out of two hundred and two actinomycetes were selected for their strong antifungal activity. They were divided into three groups: Group 1 was comprised of antagonistic isolates with broad-spectrum antifungal activity against lower (*P. ultimum*) and higher filamentous fungi (*F. oxysporum* and *R. solani*); Group 2 contained isolates with only anti-*Pythium* activity, and Group 3 was comprised of those isolates with both anti-*Rhizoctonia* and anti-*Fusarium* activities (Table 3). Of the selected strains, 17 were from RSOP (overall 29.3% of isolates with strong antifungal activity); bulk soil B1O accounted for six isolates (overall 13.95% of isolates with strong antifungal activity); none of the isolates of this soil showed strong inhibition against *P. ultimum*. On the other hand, only four isolates (overall 14.8% of isolates with strong antifungal activity) from RSYP were selected; none of the isolates were classified into Group 3. Its counterpart bulk soil BYO accounted for 15 strong antifungal isolates (20.3% of isolates with strong antifungal activity).

Anti-*Candida* activities

Twelve isolates that showed antagonism against all three phytopathogenic filamentous fungi tested were also screened for anti-*Candida* activities (Fig. 1). Antagonistic activity was detected toward both strains of *C. albicans* (ATCC MY-204276, fluconazole-resistant and ATCC 44373, 5-fluorocytosine-resistant) in both media (PDA and YDA), with different levels of activities observed between both media. *C. albicans* ATCC 44373 was inhibited to the greatest extent on PDA by strain R1O41, followed by B1O9, R1Y9 and R1Y10 while isolates R1O44 and B1O10 only showed antagonistic activity on YDA, but to a great extent, after R1O41. Isolates R1O7-2, R1O3-2, R1O4-2 and B1Y54, all showed lesser antagonistic activity in both media (Fig. 1A). Inhibition toward *C. albicans* ATCC MY-204276 was higher on PDA in most isolates (Fig. 1). On PDA, strain R1O41 was the best inhibitor, followed by B1Y14, B1Y54 and R1O7-2 while isolates R1O41, B1Y14 and R1O7-2 showed the highest activity on YDA; isolate R1Y9 only showed antagonism on PDA. The comparison of anti-Candida activity on PDA and YDA by strain is shown in Figure 1.

16S rDNA sequence analysis

Analysis of the partial 16S rRNA gene sequences of 42 selected strong antifungal isolates by BLAST revealed that 31 belonged to the genus *Streptomyces* while the remaining 11 strains were only identified as actinobacteria (Table 3). Analysis of the 16S rRNA gene of the isolates B1Y37 and R1O49 showed 100% sequence identity with *Streptomyces ciscaucasicus* strain DSM 40275 (accession AY508512), and *Streptomyces africanus* (accession AY208912), respectively. Isolates B1Y3, R1054 and R1O59 had 100% sequence identity with the undefined *actinobacterium* 17a-5 (accession AY561563). Isolates B1Y54, R2O4, R1Y25, B1Y64, and R1O31 had 98% sequence identity to *S. violaceusniger* (accession AJ391823), *Streptomyces* sp. LK4-2 (AY277376), *S. griseocarneus* (X99943), *Streptomyces* sp. (accession AY167807, AJ621613 and AJ621604), and *Streptomyces* sp. (Y15499), respectively. The remaining isolates had 99% sequence identity with the best match(es) in the blast search (Table 3).

Table 2 Distribution of antibacterial activities in four soils of a sagebrush habitat.

Soil samples	Active isolates against			
	B. subtilis	*R. tritici*	*X. campestris* pv. *campestris*	*Burkholderia cepacea*
RSYP	0.185[c]	0.260[b]	0.222 [a]	0.037[a]
B1Y	0.527[a]	0.500[a]	0.338 [a]	0.243[a]
RSOP	0.483[a]	0.224[b]	0.103[a]	0.052[a]
B1O	0.209[b]	0.023[c]	0.163[a]	0.116[a]

The data presented are proportions of the active isolates with respect to the total population in each soil (RSYP, n = 27; B1Y, n = 74; RSOP, n = 58; B1O, n = 43). RSYP and RSOP are rhizosphere soils from a young and an old sagebrush plants respectively; B1Y and B1O are the counterpart bulk soils respectively. Different letters within a column indicate a significant difference at $p < 0.05$. All comparisons are based on logit transformations.

Table 3 Blast search of partial sequences of the 16s RNA gene of selected isolates.

Isolates[a]	Match	Identity[b] (%)	GenBank accession number
Group 1			
R1Y9	*Streptomyces kasugaensis*	721/725 (99%)	DQ629032
R1Y10	*Streptomyces kasugaensis*	786/791 (99%)	DQ629033
B1Y14	*Streptomyces* cf. *griseus*	645/648 (99%)	DQ629050
	Streptomyces argenteolus		
B1Y54	*Streptomyces violaceusniger*	756/764 (98%)	DQ629051
B1Y71	*Streptomyces* sp. IM-8062	710/715 (99%)	DQ629052
R1O41	*Streptomyces* sp. KACC 91020	776/791 (98%)	DQ629031
R1O44	Actinobacterium 17a-5	684/688 (99%)	DQ642601
R1O3-2	*Streptomyces* sp. LK4-2	706/713 (99%)	DQ629028
R1O4-2	*Streptomyces* sp. LK4-2	680/687 (98%)	DQ629029
R1O7-2	*Streptomyces* sp. LK4-2	709/716 (99%)	DQ629030
B1O9	*Streptomyces erumpens*	771/773(99%)	DQ629048
B1O10	*Streptomyces luteogriseus*	768/772 (99%)	DQ629049
	Actinobacterium 17a-5	765/768 (99%)	
Group 2			
R1Y11	Actinobacterium 17a-5	740/742 (99%)	DQ629038
R1Y25	*Streptomyces griseocarneus*	771/782 (98%)	DQ629039
B1Y4	Actinobacterium 17a-5	767/771 (99%)	DQ629053
B1Y37	*Streptomyces ciscaucasicus*	732/732 (100%)	DQ629054
B1Y40	Actinobacterium 17a-5	740/742 (99%)	DQ629055
B1Y42	*Stretomyces* sp. KN-0647	724/730 (99%)	DQ629056
B1Y43	*Stretomyces* sp. KN-0647	726/732 (99%)	DQ629057
R1O4	Actinobacterium 17a-5	767/770 (99%)	DQ629034
R1O23	Actinobacterium 17a-5	737/739 (99%)	DQ629035
R1O24	*Streptomyces cyaneus*	703/704 (99%)	DQ629036
R1O46	*Streptomyces turgidiscabies*	681/686 (99%)	DQ629037
Group 3			
B1Y2	*Streptomyces* sp. IM-8062	763/769 (99%)	DQ629062
B1Y3	Actinobacterium 17a-5	733/733 (100%)	DQ629063
B1Y16	*Streptomyces* sp. KN-0647	730/736 (99%)	DQ629064
B1Y21	*Streptomyces* sp. IM-6899	729/740 (98%)	DQ629065
B1Y33	*Streptomyces* sp. KN-0647	746/752 (99%)	DQ629066
B1Y48	*Streptomyces* sp. IM-8062	683/688 (99%)	DQ629067
B1Y64	*Streptomyces platensis*	765/778 (98%)	DQ629068
	S. catenulae	765/778 (98%)	
	S. tubercidicus	765/778 (98%)	
R1O3	*Streptomyces* sp. Sm22	657/661 (99%)	DQ629040
	Streptomyces sp. IM-6784		
R1O31	*Streptomyces* sp.	709/718 (98%)	DQ629041
R1O195	Actinobacterium 17a-5	737/739 (99%)	DQ629047
R1O37	*Streptomyces lincolnensis*	721/723 (99%)	DQ629042
	Streptomyces ciscaucasicus		
R1O45	*S. peruviensis*	706/713 (99%)	DQ629043
	S. ciscaucasicus		
R1O49	*Streptomyces africanus*	655/655 (100%)	DQ629044
R1O54	Actinobacterium 17a-5	661/661 (100%)	DQ629045
R1O59	Actinobacterium 17a-5	722/722 (100%)	DQ629046
B1O22	*Streptomyces coerulescens*	749/750 (99%)	DQ629058
	Streptomyces bellus		
B1O26	*Streptomyces* sp.	745/750 (99%)	DQ629059
B1O27	*Streptomyces* sp.	693/697 (99%)	DQ629060
B1O32	Actinobacterium 17a-5	734/741 (99%)	DQ629061

[a] The isolates were the strongest fungal inhibitors from 202 isolates of a sagebrush habitat. They were divided into three groups: antagonistic isolates toward *Pythium*, *Rhizoctonia* and *Fusarium* (Group 1), isolates with anti-*Pythium* activities (Group 2), and those with anti-*Rhizoctonia* and anti-*Fusarium* activities (Group 3).

[b] Nucleotides identical to nearest GenBank relative/total nucleotides of the isolate sequenced.

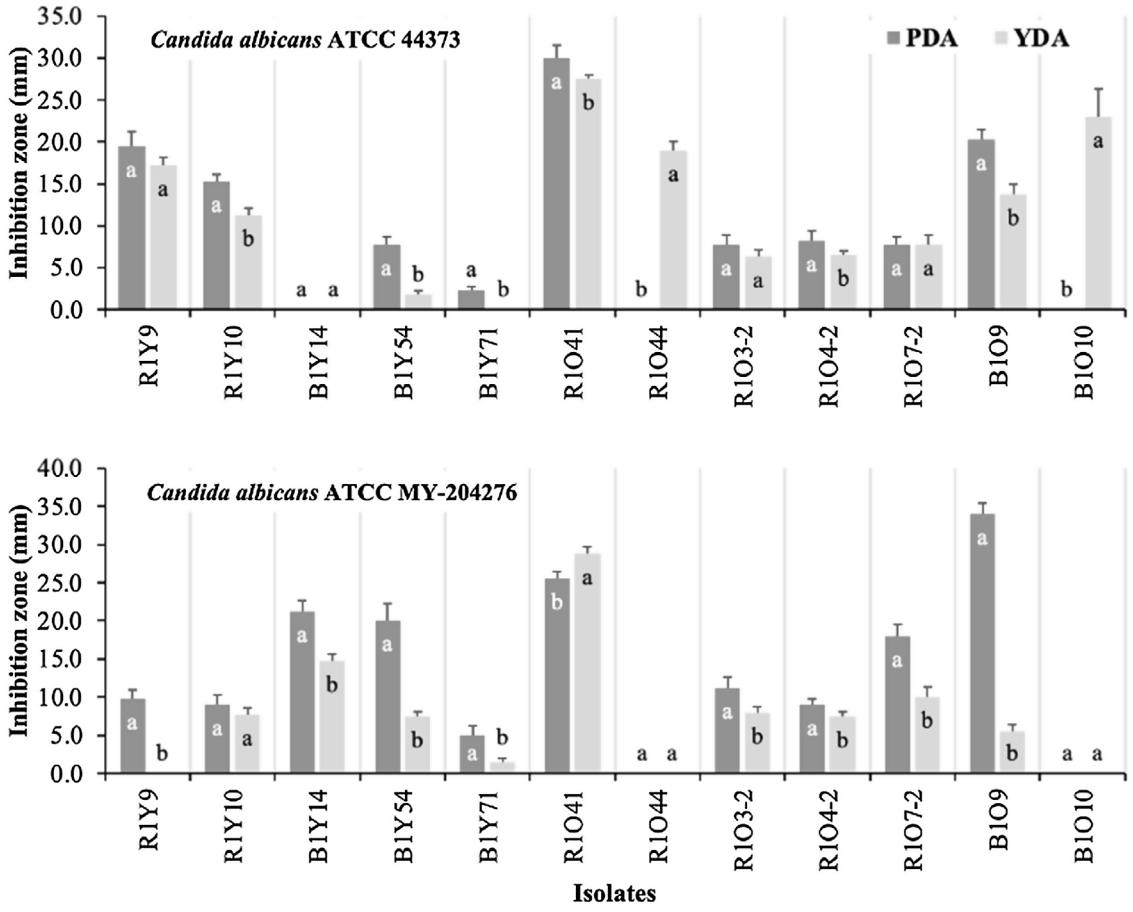

Figure 1 Anti-*Candida* activity of selected actinomycetes from four soils of a sagebrush habitat in two different media. The data presented are the means of quadruplicate measurements of inhibition zones. Bars represent their standard deviations. Mean values with equal letters are not statistically different (Tukey, $p < 0.05$) within each isolate.

Fragment 27F-907R of the forty-two selected isolates was also used to construct a multiple alignment and a phylogenetic tree, which is shown in Figure 2. Bootstrap analysis of the tree revealed that all of the phylogenetic relationships were not resolved by using only partial 16S rDNA sequences. Additional studies will be needed to resolve all the intraspecies relationships among various *Streptomyces* strains; however, very defined branches and some clades were generated with the three different algorithms used. One of the defined branches grouped all the isolates identified in the blast search as actinobacteria, while those isolates with 98% identity to *Streptomyces* sp. were in one of the major clusters of the neighbor-joining tree (Fig. 2). All the antagonists against filamentous fungi of group one that also showed anti-yeast activity were located on one major cluster by the analysis. Those isolates within group two (strong anti-*Pythium* activity) and group three (strong anti-*Rhizoctonia* and Anti-*Fusarium* activity), were found all over the tree, and did not fall into a specific branch or clade on the tree (Fig. 2).

Discussion

Within the rhizosphere, plant roots have a direct effect on composition and density of the soil microbial populations. Root exudates selectively influence the growth of bacterial and fungal populations by altering the presence of substrates in soil in the vicinity of roots.[27] The varieties of organic compounds released by plants have been postulated to be a key factor influencing the diversity of microorganisms in the rhizosphere of different plant species.[3,32] Sagebrush roots are known for their wide production of phenols, and other aromatic compounds as well as terpenoids with antimicrobial activities.[13,17,30] Those antimicrobial compounds can be used as a carbon source by some microorganisms, including actinomycetes.[12,19] Therefore, it is not surprising that actinomycetes actively grow and colonize root systems like those of desert sagebrush plants.[9,15]

In the present work, the sagebrush rhizosphere soil of the old plant (RSOP) appears to be enriched in highly active antifungal and antibacterial Gram-positive compound-producing actinomycetes; while the rhizosphere soil of the young plant (RSYP) enriched for anti-*Fusarium* actinomycetes with lower antibacterial actinomycetes compared to its counterpart bulk soil showing a lower rhizosphere effect than that in the old plant. These differences can be explained by the rhizosphere effect, and by the qualitative and quantitative differences in root exudates due to the different plant ages.[21,27] In this context, it was demonstrated that the roots of *Artemisia tridentata* produce different types of antimicrobial and phytotoxic secondary metabolites depending on

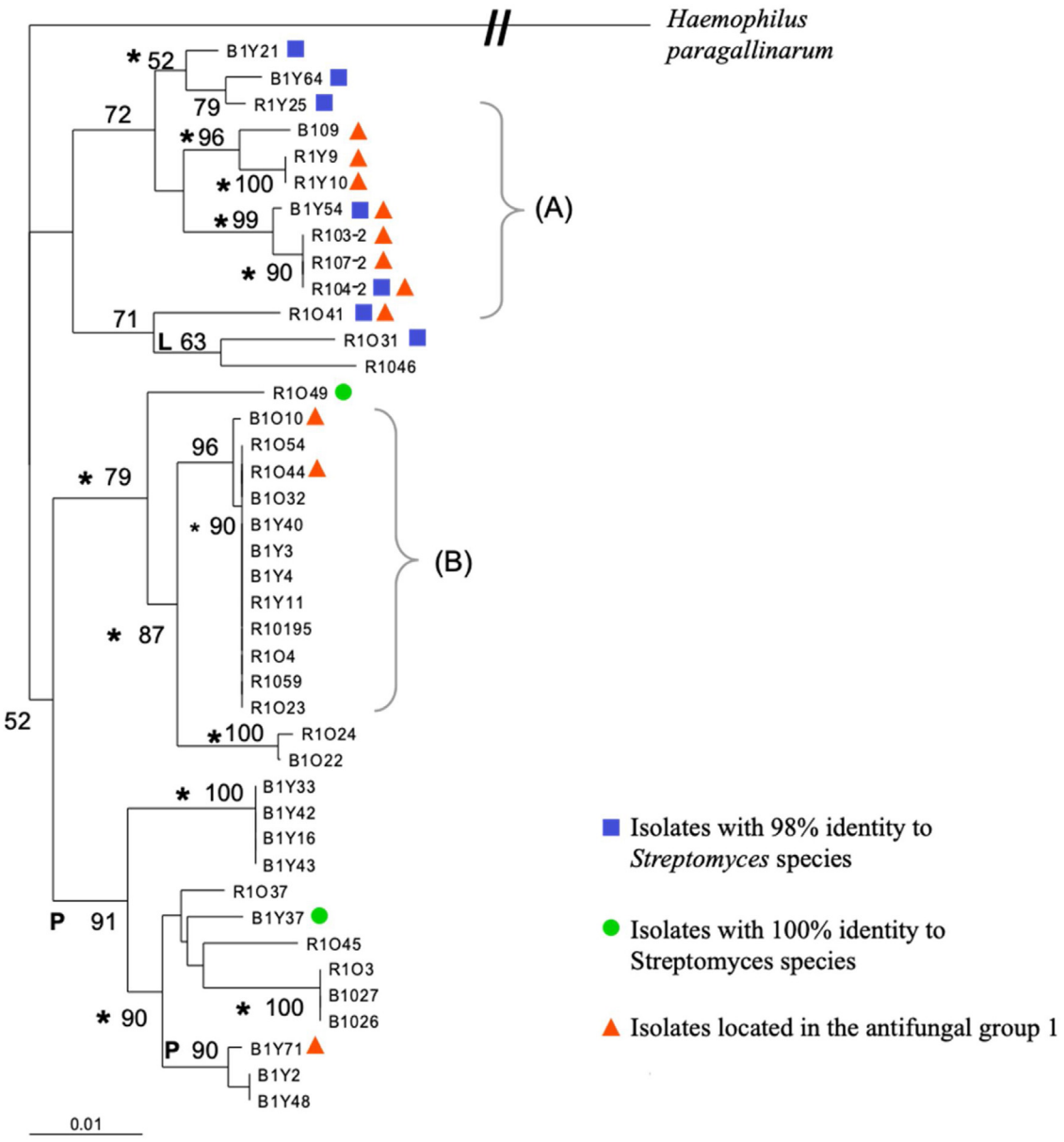

Figure 2 Phylogenetic relationship of partial 16S rRNA gene sequences of 41 streptomycetes in a neighbor-joining tree. L and P indicate branches that were also found when we used the maximum-likelihood and maximum-parsimony methods, respectively; the asterisks indicate branches recovered with all three methods. The numbers at the nodes indicate the level of bootstrap support based on a neighbor-joining analysis; only values that were >50% are given. The scale bar indicates 0.01 substitutions per nucleotide position. The bracket (A) indicates the isolates with antimicrobial activity against filamentous fungi and *C. albicans* and (B) indicates the clade of isolates identified with actinobacterium 17a-5.

the age of the plant.[13] On the other hand, in our study, the antimicrobial activities of each rhizosphere soil were compared to its counterpart bulk soil to avoid soil effect. It is well known that the soil type qualitatively and quantitatively affects microbial communities in the rhizosphere.[3]

Forty-two actinomycetes (21% of the total number of isolates) were selected for their strong antifungal properties and classified into three groups based on their activities toward *F. oxysporium* and *R. solani* (higher fungi rich in chitin in their cell wall), and *P. ultimum* (a lower fungus rich in cellulose in its cell wall).

Isolates from the four soils were present in each of the three antifungal groups, except for isolates from RSYP and B1O. RSYP isolates showed strong antagonism against *R. solani* or *F. oxysporum*, but none of them showed antagonism against both fungi while B1O isolates did not show strong antagonism, only against *P. ultimum*.

In our study of anti-yeast activity, diverse results were observed. Isolate R1O41 showed the strongest antagonism in both media compared to the remaining antagonistic isolates evidencing the potent broad-spectrum antifungal activities and their complex strategies to control the two antifungal

drug-resistant *Candida albicans* strains. A significant difference ($p < 0.05$) in anti-yeast activities between media within the same antagonist was observed in some of them, which may be influenced by diverse factors that involved the synthesis of antibiotics and other secondary metabolites in culture media. Data previously published showed that medium composition and their concentrations are strongly related to antibiotic production.[22] Simply metabolizable carbon sources such as glucose generally repress production of many antibiotics, particularly when they are used as the sole carbon source.[25] Studies on fermentation media show that polysaccharides are generally the best carbon sources for antibiotic production as they support a slow growth rate which is desirable for antibiotic production.[6,22,25] Moreover, there are also cases where glucose is an excellent carbon source for antibiotic production. It has been reported that dextrose was a great carbon source for the antibiotic production of *Streptomyces kanamyceticus* M27.[18]

Nitrogen is another component strongly related to antibiotic synthesis. As with the carbon component, simply metabolizable nitrogen sources usually decrease antibiotic production while complex nitrogen sources such as yeast extract, malt extract and soybean meal can increase the production of antibiotics produced by streptomycetes, which can be attributed to the slow decomposition of these compounds in the medium.[18,22]

In addition to nutrients, microbial interactions can regulate the production of antibiotics and other secondary metabolites. In such interactions, production of secondary metabolites can facilitate communication, but can also act as defensive molecules which help microorganisms to defend themselves against competitors.[31] In our study of the anti-yeast bioassay, the signaling between the yeast and each antagonist must be unique in each case and may be reflected in some antifungal activities or the lack of them; for example, B1Y14 showed antifungal activity against *C. albicans* ATCC MY-204276 in both media; however, no activity was detected against *C. albicans* ATCC 44373. Similarly, other isolates, R1O44 and B1010, showed activity against *C. albicans* ATCC 44373 in YDA; however, no activity was observed against *C. albicans* ATCC MY-204276.

In some studies, mainly members of the genus *Streptomyces* were detected by screening for antifungal actinomycetes from the rhizosphere soil of different plants including medicinal and forage plants.[14,33] Similarly, our results of the 16S rDNA analyses revealed that most of the selected antifungal isolates belong to the genus *Streptomyces*, except for eleven isolates that showed a best identity match with actinobacterium 17a-5. After actinobacterium 17a-5, *Streptomyces luteogriseus* and *S. tuirus* followed in the search, each with lower scores and percent sequence identity (data not shown), suggesting that those eleven isolates may be members of a new *Streptomyces* species not yet identified. Furthermore, the phylogenetic analysis showed that all the isolates identified with actinobacterium 17a-5 were in a very defined terminal clade. On the other hand, all the antifungal isolates with 98% identity with *Streptomyces* sp. were in one of the major clusters of the neighbor-joining tree; they are potentially novel species; however, further analysis are required to determine that. In addition, almost all the strains of antifungal group one, which also exhibited anti-*Candida* activity (Fig. 1), were in close proximity on the phylogenetic tree (R1O41, R1O4-2, R1O7-2, R1O4-3-2 B1Y54, R1Y10, R1Y9 and B1O9), suggesting the presence of a potential clade of strong antifungal *Streptomyces* with potential novel bioactive metabolites. The isolate denominated R1O41, isolated from RSOP, seems to be a novel actinomycete isolate with strong broad antifungal activity against the three phytopathogens and the two drug-resistant *Candida* strains, showing a great potential that can be exploited for use in agriculture and medicine.

Conclusions

Our study showed that the rhizosphere soil of sagebrush plants may preferentially favor antifungal actinomycete colonizers, particularly plants with high rhizosphere effect. The isolates with strong antifungal activities were identified as members of the genus *Streptomyces*. A relationship was detected by phylogenetic analysis between the genetic relatedness and the antifungal activities of those actinomycetes exhibiting strong antifungal activity against all the filamentous fungi tested along with anti-*Candida* activity. Finally, the rhizosphere soil of *A. tridentata* seems to be a great source of novel actinomycetes with strong antifungal activity that can be exploited for use in agriculture and medicine.

Conflict of interest

The authors declare that they have no conflicts of interest.

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