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SHORT COMMUNICATION

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Antifungal activities of actinomycete strains associated with high-altitude sagebrush rhizosphere

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Abstract The antifungal-producing potential of actinomycete populations from the rhizosphere of low-altitude sagebrush, *Artemisia tridentata*, has been examined. In a continued investigation of new sources of antifungal-producing microorganisms, this study examined the antifungal-producing potential of actinomycetes from the rhizosphere of high-altitude *A. tridentata*. With high-altitude sagebrush, rhizosphere soil actinomycete numbers were one to four orders of magnitude higher than those found in nonrhizosphere bulk soils and different from those found with the low-altitude plants. A total of 122 actinomycete isolates was screened against nine fungal species and six bacterial species for the production of antimicrobial compounds. Four rhizosphere isolates, *Streptomyces amakusaensis*, *S. coeruleorubidus*, *S. hawaiiensis* and *S. scabies*, showed broad-spectrum antifungal activity against three or more fungal species in plate assays. In liquid antagonism assays, mycelium production by *Aspergillus niger* was reduced by up to 50% by two of the actinomycete isolates. These results demonstrate the potential of rhizosphere microbiology in the search for new antimicrobials.

Keywords Actinomycete · Rhizosphere · Sagebrush · Antifungal · *Streptomyces*

Introduction

In the search for new antifungals to inhibit material degradation and in the protection of animal and plant health, Streptomycetes (Gram-positive, mycelia-forming soil bacteria) are known to produce a variety of antimicrobial compounds. The plant rhizosphere represents an uncharacterized source of microorganisms producing novel antimicrobial metabolites [11]. The sagebrush *Artemisia tridentata* provides an ideal habitat for novel Streptomycetes isolation, as actinomycetes may be among the most dominant rhizosphere colonizers of plants in harsh, arid environments. As an unexplored habitat for antimicrobial-producing microorganisms, the actinomycete community of the rhizosphere of *A. tridentata* was recently examined in low-altitude (295, 305 and 975 m elevations) plant populations in a study by Basil et al. [2]. As a follow-up, the current study examined high-altitude sagebrush populations (2,273 and 2,364 m elevations). While both studies found potential novel antimicrobial-producing actinomycetes using similar growth media and conditions, differences in the actinomycete populations and associated antimicrobial activities were evident and are reported here.

The aim of this study was to isolate novel plant growth-promoting actinomycetes with antifungal activity from the rhizosphere of high-altitude sagebrush *Artemisia tridentata* Nutt. (= *Seriphidium tridentatum* (Nutt.) W.A. Weber). Our study found several previously unreported and uncharacterized actinomycete species with antifungal activity associated with the rhizosphere of *A. tridentata*.

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Materials and methods

Sample collection

The sampling sites were sagebrush communities located approximately 5 miles outside of Wolcott, CO (elevation 2,273 m) and in the town of Silverthorne, CO, USA (elevation 2,364 m). Intact sagebrush root systems were stored at 4°C until analysis. Rhizosphere soil was collected by gently scraping and rinsing the roots with sterile distilled water. Bulk soils were collected to a depth of 10 cm at a minimum distance of 2 m from vegetation. Particle size analysis, pH and soil water content were determined for each soil.

Actinomycete isolation

Actinomycetes were cultured on yeast casamino acids extract and dextrose agar (YCED) and water yeast extract agar (WYE) [3]. Cyclohexamide (100 μg/ml) was added to reduce fungal contamination. Dilution and plating of soil slurries (5 g dry wt soil in 50 ml 0.1% sterile glycerophosphate, homogenized at 40 rpm for 30 min) assessed culturable actinomycete and eubacterial counts. Plates were incubated at 25°C for 14 days until sporulated actinomycete colonies were visible. Isolates were purified and stored at 4°C.

Actinomycete spores were collected using glass beads rolled over sporulated colonies [5]. The beads were then washed in sterile 0.9% NaCl:0.85% Tween 80. The suspension was filtered through Whatman no. 2 filter paper to remove hyphae. Spore suspensions were enumerated via dilution and plating prior to storage at −20°C.

Fungal cultures

Aspergillus niger (ATCC 16404), *Alternaria alternata* (Ward's Natural Science, Rochester, NY, USA) and *Armillariella mellea* (Ward's) fungal isolates were grown on corn meal agar, pH 6.0 (Difco, Inc., Detroit, MI, USA), while *Candida albicans* (ATCC 90027), *Saccharomyces cerevisiae* (laboratory strain), *S. pastorianus* (ATCC 10575), *Phytium ultimum* P8 (J. Kraft, Irrigated Agricultural Research and Extension Center, Prosser, WA, USA), *Rhizoctonia solani* (D. Crawford, University of Idaho, Moscow, ID, USA) and *Fusarium oxysporum* (ATCC 070233) were grown on yeast potato and potato dextrose agars (Difco, Inc.).

Microbial antagonism plate assays

Using a modified method from Crawford et al. [3], each actinomycete isolate was streaked onto one half of a corn meal agar plate and incubated at 25°C until sporulation. A 0.5-cm^2 agar plug with actively growing fungal mycelia was then placed near the front of the actinomycete growth on the plate. Following a 48-h incubation, antagonism was determined as the distance between actinomycete growth and fungal growth. Growth of fungi at a distance greater than 7.5 mm from the actinomycete colonies was considered strong antagonism. Bacterial antagonism was similarly tested for using *Bacillus subtilis*, *Enterobacter faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus agalactiae*.

Liquid fungal antagonism assays

Isolates showing antagonism against *A. niger*, a known degrader of plastics, paints and polycarbonates, were chosen for examination for *A. niger* inhibition in a liquid medium [10]. Agar plugs (0.5-cm^2) of actively growing fungal mycelia were placed in petri dishes with 15-ml of YCED broth enriched with 20 mM glucose. Following incubation at 25°C for 48 h, 1-ml of an actinomycete spore suspension (10^5 spores/ml) was added to the fungal cultures and incubated for an additional 48–120 h. Cultures were filtered through preweighed Whatman no. 2 filter paper every 24 h. Retained mycelia on the filter paper was dried overnight at 60°C and reweighed.

Identification of antagonistic actinomycetes

16S rRNA gene sequencing on isolates was performed according to Heuer et al. [6]. Isolates were also sent to Microbial ID Systems Inc. (MIDI; Newark, DE, USA) for identification using fatty acid methyl ester (FAME) analysis.

Statistics

All assays were performed in triplicate and subjected to statistical analysis. Cell enumerations were analyzed using a *t* test comparison. Fungal antagonism assays were analyzed using the Wilcoxon Rank Sum test due to the nonnormality of the data. Analyses were performed using SAS Statistical Software (SAS Institute, Cary, NC, USA).

Results

While actinomycete and eubacterial numbers were consistently higher in the two rhizosphere soil samples as compared to the nonrhizosphere soil samples, ranging from one to four log units higher (Table 1), rhizosphere actinomycete numbers were significantly higher than the bulk soil actinomycete numbers at the Wolcott site ($P < 0.05$) but were not at the Silverthorne site. Three percent of the actinomycetes in this study showed strong broad-spectrum antifungal activity.

The 16S rRNA gene sequence analysis of four isolates with broad-spectrum antifungal activity showed all to be members of the genus *Streptomyces* (99% similarity). Since the 16S rRNA gene sequencing did not identify to the species level, FAME analyses were performed and used to identify the species as *S. amakusaensis* (SIM = 0.641), *S. coeruleorubidus* (SIM = 0.788), *S. hawaiiensis* (SIM = 0.729) and *S. scabies* (SIM = 0.636), where an SIM > 0.6 indicates a positive identification (Microbial ID Systems, Inc.). Each isolate showed strong antagonism against three or more fungi and one or more bacteria (Table 2).

Based on the initial antifungal screening, quantitative fungal inhibition was monitored in a liquid medium. Within 48 h, *S. amakusaensis* and *S. hawaiiensis* significantly ($P < 0.05$) reduced the growth of *A. niger* by 49%

Table 1 Sagebrush-associated soil characteristics at the Wolcott and Silverthorne sites

Soil	pH	Sand (%)	Total eubacteria (CFU/g dry wt soil)[a]	Actinomycetes (CFU/g dry wt soil)	Streptomycete isolates used in this study
Wolcott, CO bulk	8.0	97	$1\times10^5 \pm 0$ (YCED) $1\times10^5 \pm 1\times10^2$ (WYE)	$3.5\times10^3 \pm 1.3\times10^3$ (YCED) $5.6\times10^2 \pm 1.7\times10^2$ (WYE)	
Rhizosphere	7.2	97	Plant A	Plant A	*S. amakusaensis* (YCED)
			$1.6\times10^7 \pm 2.3\times10^6$ (YCED) $1.1\times10^7 \pm 7.1\times10^5$ (WYE)	$1.2\times10^6 \pm 5.6\times10^5$ (YCED) $1.3\times10^6 \pm 1.1\times10^6$ (WYE)	*S. coeruleorubidus* (YCED), *S. scabies* (YCED)
			Plant B	Plant B	
			$1.1\times10^7 \pm 1.4\times10^6$ (YCED) $9.4\times10^6 \pm 2.0\times10^6$ (WYE)	$7.5\times10^5 \pm 3.1\times10^5$ (YCED) $3.5\times10^5 \pm 3.1\times10^5$ (WYE)	
			Plant C	Plant C	
			$1.3\times10^6 \pm 2.6\times10^5$ (YCED) $1.2\times10^6 \pm 2.5\times10^5$ (WYE)	$1.7\times10^6 \pm 2.8\times10^5$ (YCED) $1.3\times10^6 \pm 3.5\times10^5$ (WYE)	
Silverthorne, CO bulk	8.1	95	$5.7\times10^5 \pm 7.1\times10^4$ (YCED) $9.5\times10^5 \pm 1.2\times10^5$ (WYE)	$< 10^2$ (YCED) $< 10^2$ (WYE)	
Rhizosphere	7.8	95	Plant A	Plant A	*S. hawaiiensis* (YCED)
			$3.6\times10^6 \pm 5.6\times10^4$ (YCED)	$7.0\times10^2 \pm 1.4\times10^2$ (YCED)	
			Plant B	Plant B	
			$3.0\times10^8 \pm 7.1\times10^6$ (YCED)	$6.5\times10^2 \pm 2.1\times10^2$ (YCED)	

[a]Mean ± standard deviation based on triplicate analyses

Table 2 Antagonism of rhizosphere *Streptomyces* spp. against various fungi and bacteria measured as the distance (mm) between actinomycete growth and fungal/bacterial growth

	Actinomycete isolate			
	S. amakusaensis	*S. coeruleorubidus*	*S. hawaiiensis*	*S. scabies*
Fungal isolate				
Alternaria alternata	20	ND	ND	7.5
Armillariella mellea	20	ND	ND	10
Aspergillus niger	30	20	10	7.5
Candida albicans	0	0	9	0
Fusarium oxysporium	0	5	9	0
Phytium ultimum P8	0	1	25	0
Rhizoctonia solani	11	0	18	19
Saccharomyces cerevisiae	60	35	7	25
S. pastorianus	60	32	11	31
Bacterial isolate				
Bacillus subtilis	0	0	33	0
Enterobacter faecalis	0	0	0	0
Escherichia coli	0	0	0	0
Pseudomonas aeruginosa	35	60	60	60
Staphylococcus aureus	0	0	21	0
Streptococcus agalactiae	0	0	26	0

Antagonism was defined as distances greater than 7.5 mm
ND not determined

(from 18.3 ± 1.1 to 9.3 ± 1.5 mg) and 50% (20.8 ± 7.8 to 10.4 ± 3.3 mg), respectively. The reduced fungal growth (15.7 ± 3.5 to 13.0 ± 2.5 mg) by *S. coeruleorubidus* was not statistically significant. Fungal inhibition in the liquid medium was maintained until approximately 96–120 h of coincubation, after which the difference in mycelium production in tests with the actinomycete and in actinomycete absent controls was no longer statistically significant (data not shown).

Discussion

The Basil et al. [2] study of low-altitude sagebrush found eleven out of 153 isolates tested (7%) showed broad-

spectrum antifungal activity. Our study found 4 out of 122 actinomycetes (3%) recovered from high-altitude sagebrush rhizosphere samples showed strong broad-spectrum antifungal activity and varying antibacterial activities. Tested against known fungal biodeterioration agents and plant or animal fungal pathogens, the antifungal activities of the actinomycete isolates were evident in both plate and liquid assays. In the liquid assays, fungal inhibition did decrease with time, possibly due to nutrient limitations or negative feedback by the antimicrobial [9]. While having known antibacterial activities, *S. amakusaensis* produces cytosaminomycins [4] and nagastatin [1], *S. coeruleorubidus* produces anthracyclines [8], and *S. hawaiiensis* produces thiostrepton [7], the antifungal activities of these actinomycetes and *S. scabies* have not been well characterized.

Basil et al. [2] concluded that microbial differences between rhizosphere and bulk soils were more a reflection of the sampled site rather than the rhizosphere association. In the two sites sampled in this study, differences were observed both between sites and between rhizosphere and bulk soils, although both studies observed greater actinomycete diversity in rhizosphere versus bulk soils. These studies imply the presence of novel rhizosphere antifungal-producing actinomycetes. In our need for new antimicrobial compounds, further investigation of actinomycete habitats will prove a fruitful enterprise.

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