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In vitro and *in vivo* antagonism of pathogenic turfgrass fungi by *Streptomyces hygroscopicus* strains YCED9 and WYE53

K Chamberlain and DL Crawford

Department of Microbiology, Molecular Biology and Biochemistry, University of Idaho, Moscow, ID 83844–3052, USA

Disease prevention is a current practice used to minimize fungal diseases of turfgrasses in lawns and golf greens. Prevention is accomplished through fungicide applications, and by periodic thatch removal. During the development of a microbial biodethatch product utilizing the lignocellulose-degrading *Streptomyces hygroscopicus* strains YCED9 and WYE53, we demonstrated using *in vitro* plate antagonism bioassays that both strains are antagonists of various turfgrass fungal pathogens. These activities were present when the cultures were growing on thatch, as demonstrated by antifungal antagonism bioassays with culture filtrates. Experiments conducted using a growth chamber demonstrated that a bio-dethatch formulation containing spores of strains YCED9 and WYE53 in a zeolite carrier, provided protection for Kentucky bluegrass seedlings against turfgrass pathogens, including *Pythium ultimum*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Sclerotinia homeocarpa*, *Gaeumannomyces graminis* and *Microdochium nivale*. Results showed that by integrating the use of the *S. hygroscopicus* YCED9/WYE53 bio-dethatch formulation into routine turf management practices, it should be possible to both minimize thatch build-up while also controlling fungal turfgrass diseases by way of the antifungal biocontrol activity of these strains. This in turn would help control fungal pathogens in turfgrass while minimizing the need for routine chemical fungicide applications.

Keywords: *Streptomyces hygroscopicus*; biocontrol; fungi; turf; thatch

Introduction

Pathogenic fungi survive in turfgrass, in part, due to its perennial nature. Depending on environmental conditions and management practices, the turfgrass may experience fungal diseases, either seasonally or many times during a single season. Therefore, prevention of fungal disease is usually achieved through coordinated management and cultural practices, which include thatch removal and preventative fungicide applications [31]. In intensely managed turf, ie on golf courses, fungicides are heavily used. However, there are disadvantages to use of fungicides in a preventative manner, including chemical pollution, increased incidence of secondary diseases, selection for fungicide-resistant fungal strains and increased thatch accumulation [26]. While the use of some fungicides has been correlated with increased thatch accumulation [26], the presence of a thatch layer is in turn correlated with increased incidences of fungal diseases [8,9,26,27,33].

Streptomyces species are soil actinomycetes that are often rhizosphere-associated [6,16]. Some strains produce antibiotics in both soil [7,14,23] and in thatch [5,29,30]. The antibiotics are often antagonistic to fungal pathogens [11,15,16,35]. Antibiotic production may confer a selective advantage over other resident microbes [14,20,32]. Certain *Streptomyces hygroscopicus* (syn: *violaceusniger*) strains produce antibiotics with antifungal properties [15,22,30]. Compared to chemical fungicide treatments, natural antibiotics produced within the microhabitat of the rhizosphere can be considered less polluting and less stressful on the turf microorganisms [28].

Melvin *et al* [19] found that fungal disease development in turf can be reduced by keeping the thatch and upper soil layers moist in order to provide optimal conditions for the development of antagonistic microbial populations. One problem specific to golf courses is the higher sand content of typical golf course soil matrices, combined with the use of sand as a topdressing to maintain a smooth firm surface. These management practices are correlated with lower microbial populations [2,17] including fewer thatch decomposers and fungal antagonists. The combination of sand, thatch and favorable environmental conditions can lead to severe outbreaks of diseases caused by *Pythium* [1,31] and *Rhizoctonia* species [4,18,31]. These same conditions favor the growth of *Streptomyces hygroscopicus* strains YCED9 and WYE53 in thatch [5]. Their growth in turn might enhance thatch turnover/degradation, as well as the accumulation of effective antifungal metabolites in the turf. Strain YCED9 has been shown to produce three distinctly different antifungal antibiotics in laboratory culture [29]. We have not yet identified those produced by strain WYE53.

Our research presented here focused on determining the simultaneous effectiveness of *S. hygroscopicus* YCED9 and WYE53 as thatch colonizers, thatch degraders, and biocontrol agents against turf pathogenic fungi. In the study, we examined biocontrol of several turfgrass fungal pathogens which are problematic at golf courses (warm- and cool-season regions). These pathogenic fungi included *Pythium ultimum*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Sclerotinia homeocarpa*, *Gaeumannomyces graminis* and *Microdochium nivale*. Antagonism tests were based on *in vitro* plate bioassays, and on thatch-culture filtrate plate

Correspondence: Dr DL Crawford, Department of Microbiology, Molecular Biology and Biochemistry, University of Idaho, Moscow, ID 83844-3052, USA
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bioassays. These assays showed that strains YCED9 and WYE53 successfully antagonized all of the fungal pathogens in *in vitro* plate bioassays and, over the short-term, so did the thatch-culture filtrates. An experiment conducted in a growth chamber with Kentucky bluegrass plots treated with the bio-dethatch spore formulation showed that the two *Streptomyces* strains also antagonized most of the fungi, though with different degrees of success. Additionally, we found that these rhizosphere-colonizing strains may also enhance turf growth, independent of the other activities, as shown by experiments where we compared growth of uninoculated and streptomycete-treated turf in the absence of fungal pathogens in growth chambers.

Material and methods

Cultures and inocula

Streptomyces hygroscopicus strains YCED9 and WYE53 were originally isolated from an English soil, and they were maintained on yeast extract-malt extract-dextrose agar (YEMED) [6]. Strain YCED9 has also been called *Streptomyces violaceusniger*, though the species are considered synonymous [30]. All inoculations consisted of aseptic spore transfers of spores from well-sporulated agar plates or slants. Fungal strains included *Pythium ultimum* strain P8, *Fusarium oxysporum* and *Rhizoctonia solani* [35], *Gaeumannomyces graminis* strains FL-199 and 179, *Microdochium nivale* and *Sclerotinia homeocarpa* [29]. All fungi were maintained on parafilm-covered Difco brand potato dextrose agar (PDA), or cornmeal agar plates (CMA; Difco, Detroit, MI, USA) at room temperature.

In vitro antagonism bioassays

Antifungal antagonism was determined using a modified agar plate antagonism bioassay [6,24,25]. All streptomycete cultures were spot-inoculated onto agar plates from a well-sporulated stock culture using an inoculating loop. Plates were then inocubated at 30°C for 3 days prior to fungal inoculation. the pathogenic fungi were inoculated by transferring agar plugs from fresh, 2-week-old stock cultures grown on PDA or CMA agar. Once the fungi were inoculated onto plates containing the streptomycete colonies, they were incubated at 28°C. All controls were run in duplicate, while test plates were run in triplicate. Antagonism was determined by measuring radial growth from the transferred fungal colonies measured from the edge of the agar plug perpendicularly to the streptomycete colonies (see Equation 1). Growth was measured at 2, 3, 5 and 7 days. Each plate was marked at the first measurement, and all measurements for each time interval were measured from that location. All fungal control measurements were ranked as 'optimum'. Radial growth from the streptomycete-inoculated fungal plates were based on the perpendicular measurement from the edge of the fungal plug towards the streptomycete colony and recorded as percent antagonism (= percent of growth inhibition) in comparison to the fungal controls (see Equation 2).

$$\text{Total growth diameter} - \text{fungal plug inoculum diameter}/2 = \text{radial growth} \quad (1)$$

$$1 - (\text{Radial growth with streptomycete}/\text{radial growth fungal control})\,(100\%) = \%\ \text{antagonism} \quad (2)$$

These antagonism assays were determined on a variety of agar media, including potato dextrose agar (PDA, Difco), cornmeal agar (CMA, Difco), and P/YE agars with and without carbohydrate polymer supplements at 0.10% (w/v): medium viscosity carboxymethylcellulose (P/YE-cel), (Difco) soluble starch (P/YE-s) and (Sigma) oat spelt xylan (P/YE-xyl). P/YE agar consisted of 30 mM potassium phosphate monobasic, 0.02% (w/v) yeast extract with 15 g L^{-1} agar adjusted to pH 6.5.

Thatch culture filtrate antagonism plate bioassays

Experiments using culture filtrate were based on other antagonism bioassays [11,24,25,34]. Thatch cultures were prepared as described by Chamberlain [5] and included non-inoculated thatch culture controls, single culture inoculated, and co-inoculated (WYE53/YCED9) cultures. Thatch cultures consisted of 10.0 g thatch per flask inoculated with 70.0 ml spore suspension. Replicates of four streptomycete thatch-cultures were prepared per inoculum treatment, two of which were harvested at 5 days and two of which were harvested after 20 days. Culture filtrates were harvested by vacuum filtration (using No. 413 filters; VWR) and then filter-sterilized by passage through a sterile 0.5-μm filter (Gelman, Ann Arbor, MI, USA). Each filtrate was used to amend PDA and CMA to a 5.0% (v/v) concentration. The filtrates were added to sterile molten agar (45°C) prior to pouring the plates.

Duplicate fungal controls included fungi inoculated on non-supplemented PDA and CMA and triplicate non-inoculated (streptomycete-free) thatch-culture filtrate-supplemented plates. Triplicate test cultures were streptomycete-inoculated filtrate-amended agars. Antagonism was measured as the difference in radial growth from a centrally located fungal plug, comparing growth on the corresponding non-amended agar and filtrate-supplemented agar. Measurements used the following equations to determine radial growth (see Equation 3) and percent antagonism (see Equation 4) [11,24]:

$$\text{Total growth diameter} - \text{fungal plug inoculum diameter})/2 = \text{radial growth} \quad (3)$$

$$1 - (\text{ave. radius with streptomycete}/\text{ave. radius fungal control})\,(100\%) = \%\ \text{antagonism} \quad (4)$$

In vivo antagonism assay in growth chambers

The effectiveness of strains YCED9 and WYE53 as antagonists toward pathogenic fungal infections of Kentucky bluegrass was tested in a growth chamber. Plant culture flasks containing 20.0–20.5 g sand and 0.50 g thatch were steam-sterilized [5]. Then, 0.50 g each of strains YCED9 and WYE53 formulation (as spores in sterile powdered zeolite carrier) [5] were added to each of 20 flasks, along with 2.0 ml sterile distilled water. (The formulation spore densities of both strains was *ca* 1×10^7 colony forming units g^{-1}, as determined by viable plate counting [5].) All culture flasks including controls were placed in the growth

chamber (12 h lighting cycles at 25°C) for 3 days to initiate streptomycete growth on the thatch. Then, 0.25 g of Kentucky bluegrass seed was added to each culture flask, and the flask was gently agitated after addition of 3.0 ml of sterile distilled water. Flasks were then returned to the growth chamber. When cultures began to dry, 2.0-ml aliquots of sterile distilled water were added as needed to keep them moist.

Grass seedling emergence was monitored, and when all flasks had 10 or more seedlings, the fungal pathogens were added to the culture flasks by transfer of mycelia directly from well-grown PDA agar plates. Each of the seven pathogenic fungi were tested in duplicate against strains YCED9 and WYE53. Five replicates for each assay were run, along with controls, which included pathogen only, untreated seedlings only, strain YCED9 alone, and strain WYE53 alone. Results were based on grass yield after 10 days; yield was ranked according to observable density (approximate number of leaf blades) and grass height. A ranking of between 0 and 5.0 was assigned to each treatment. The untreated control (no pathogen or biocontrol agent) was ranked 3, and all other microbe-amended cultures were compared to this control.

Results

In vitro antagonism plate bioassays

Table 1 shows the antifungal antagonism expressed as the percentage of pathogenic fungal growth inhibited by strains YCED9 and WYE53 on agar plates after 5 days incubation. Antagonism was examined on six different media. Strain YCED9 showed similar antagonism against *Pythium ultimum* strain P8 on all agars. With the other five pathogens the amount of antagonism varied more between media, though growth of all of the test fungi was inhibited by the presence of extracellular metabolites produced by colonies of strain YCED9. The minimal medium (P/YE), regardless of the carbohydrate supplement used (xylan, starch, or cellulose), showed less antagonism compared with those on the richer media (PDA and CMA). Strain WYE53 demonstrated similar inhibition patterns as strain YCED9.

Thatch culture filtrate antagonism plate bioassays

Filtrate was prepared at two culture ages (5 and 20 days) and tested for antagonism as shown by reduced fungal growth on CMA and PDA when filter-sterilized filtrate was added to the media. The co-inoculated, 'Both', thatch culture filtrate (5%, v/v addition to the medium), tested after 5 days growth, inhibited *S. homeocarpa* growth by 20% on PDA, and all the *Streptomyces* culture filtrates inhibited its growth approximately 10% on CMA (data not shown). None of the other fungi were inhibited by 5.0% (v/v) filtrate additions to either agar type. Table 2 shows antagonism by 20-day thatch-culture filtrate-amended CMA or PDA agars as a percentage of growth inhibition compared with fungal growth on unsupplemented (filtrate-free) PDA or CMA. The addition of thatch-culture filtrate to PDA and CMA had a variety of effects on fungal growth, depending on the streptomycete used and the specific agar medium. Since these pathogenic fungi can grow as saprophytes, the thatch culture filtrates also potentially contained water-soluble substances and leachates which enhanced the growth of different fungi under the experimental conditions. These are reported as negative inhibition percentages. There were different antagonism patterns between the two media as demonstrated by all the fungi except for *P. ultimum* strain P8 and *M. nivale*. *R. solani* was significantly inhibited at 3 days but not after 5 days incubation. *F. oxysporum* and *S. homeocarpa* had similar growth inhibition patterns after both incubation periods. *G. graminis* strain 179 showed increased inhibition at 5 days *vs* 3 days on PDA filtrate-amended agars but not on CMA filtrate-amended agars.

Table 1 Percent of fungal pathogen growth inhibited by *Streptomyces hygroscopicus* strains WYEC108 and YCED9 in *in vitro* plate bioassays

Medium	Per cent inhibition of:					
	Pythium ultimum strain P8	*Fusarium oxysporum*	*Rhizoctonia solani*	*G. graminis* strain 179	*M. nivale*	*Sclerotinia homeocarpa*
Strain WYE53						
P/YE	19	42	25	2	37	49
P/YE-cellulose	16	45	9	13	44	50
P/YE-starch	22	54	41	47	59	59
P/YE-xylan	17	31	26	60	24	52
PDA	39	60	53	78	42	63
CMA	37	64	49	64	24	63
Strain YCED9						
P/YE	18	40	14	8	20	54
P/YE-cellulose	21	39	19	11	32	55
P/YE-starch	16	31	42	44	48	55
P/YE-xylan	10	49	25	43	22	46
PDA	23	59	56	75	53	74
CMA	14	67	63	53	42	75

Assays were performed on each of six media, including phosphate-yeast extract agar (P/YE), P/YE agar supplemented with 0.1% (w/v) carboxymethylcellulose (P/YE-cellulose), P/YE agar supplemented with 0.1% (w/v) soluble starch (P/YE starch), P/YE agar supplemented with 0.1% (w/v) xylan (P/YE-xyl), potato dextrose agar (PDA), and cornmeal agar (CMA). Fungal growth inhibition of each fungus was calculated as a percentage of the radial growth observed on the same medium in the absence of streptomycete.

Table 2 Percentage of fungal pathogen growth inhibited on potato dextrose agar (PDA) or cornmeal agar (CMA) containing filter-sterilized 20-day-old thatch culture supernatants that were added to the agar (5%, v/v) prior to its solidification

Fungal pathogen	Per cent inhibition on:							
	Potato dextrose agar				Cornmeal agar			
	Control	Strain WYE53	Strain YCED9	Both	Control	Strain WYE53	Strain YCED9	Both
T = 3 days								
Pythium ultimum P8	0	21	0	0	0	0	0	0
Fusarium oxysporum	13	12	17	14	9	7	20	20
Rhizoctonia solani	−1	13	15	12	−6	28	28	36
Sclerotinia homeocarpa	−9	2	2	2	−49	38	23	24
G. graminis strain 179	−5	11	3	13	6	39	20	33
M. nivale	−2	56	34	37	−72	55	30	42
T = 5 days								
Pythium ultimum P8	0	0	0	0	0	0	0	0
Fusarium oxysporum	8	8	14	13	8	2	19	13
Rhizoctonia solani	1	0	0	0	0	12	20	29
Sclerotinia homeocarpa	0	0	0	0	−24	42	28	32
G. graminis strain 179	−1	22	10	23	7	34	23	28
M. nivale	52	81	69	74	−39	59	48	60

The control medium was PDA or CMA agar supplemented with filter-sterilized thatch extract from non-inoculated, sterile thatch cultures. The control values were calculated as the relative amount of fungal growth on the control medium as compared to the equivalent non-amended medium (filtrate-free PDA or CMA). Fungal growth inhibition values for media amended with thatch culture supernatants were calculated as a percentage of the radial growth observed on unsupplemented (filtrate-free) medium.

M. nivale showed greater growth inhibition in both filtrate-amended agars at 5 days as compared to 3 days. The principal finding of these experiments was, however, that growth of the *Streptomyces* on thatch led to their production of antifungal metabolites that could be detected in the thatch culture filtrates via the bioassays.

In vivo antagonism experiment conducted in a growth chamber

Experiments in growth chambers were done to determine the *in vivo* effectiveness of strains YCED9 and WYE53 as biocontrol agents to control pathogenic fungal agents of grass. The data are presented in Figure 1. Most pathogenic turfgrass fungi require humid and moist conditions for disease development. Under these conditions, the Kentucky bluegrass treated with formulations of strains YCED9 and WYE53 grew better than the untreated controls, judged by differences in length and number of leaf blades. Controls with fungi only showed that all of the fungi tested significantly affected turfgrass growth and caused disease, though disease severity differed between fungi, as measured by biomass yields.

With each of the streptomycete treatments, it was seen that strain WYE53 protected Kentucky bluegrass against *F. oxysporum*, *R. solani*, *P. ultimum* and *G. graminis*. Growth was comparable to the untreated controls. Strain WYE53 also protected the turf from *M. nivale*, but to a lesser degree. It failed to protect the turf from *S. homeocarpa*. Strain YCED9 significantly protected the Kentucky bluegrass cultures from *R. solani*, *M. nivale*, *P. ultimum* and *G. graminis* strain 179. Some protection was seen against *F. oxysporum* and *G. graminis* strain Fl-199, but none was observed against *S. homeocarpa*.

Discussion

In this study, we screened two lignocellulolytic *Streptomyces hygroscopicus* strains, YCED9 and WYE53, which have been developed to control thatch accumulation in turf while possibly also acting as antifungal biocontrol agents [5], for their abilities to antagonize six turfgrass pathogens *in vitro* and *in vivo*. Results from the *in vitro* bioassays showed that both strains, YCED9 and WYE53, clearly antagonized all six fungal turf pathogens on nutrient-rich agar media (PDA and CMA), and to a lesser extent on minimal (P/YE) agar. Any antagonism observed *in vitro* is good, but not clearly indicative of the potential antagonism in actual field situations [3]. The use of the more nutrient-rich media probably promoted better streptomycete growth and, therefore, antifungal metabolite production [3]. We also included minimal P/YE medium amended (0.10% w/v) with starch, cellulose and xylan in the *in vitro* plate bioassays because thatch typically contains carbohydrate polymers that would be the major carbon sources for the *Streptomyces*. In general these media resulted in stronger fungal inhibition by the *Streptomyces* colonies. For instance, *R. solani* was inhibited by strains YCED9 $>55\%$ on PDA, $>40\%$ on P/YE with starch, but only $\approx 14\%$ on P/YE alone, and antagonism with P/YE-cellulose and P/YE-xylan showed inhibition values between P/YE-starch and P/YE alone (Table 1). These data suggest that, because these streptomycetes utilize the xylan in thatch, they should also be antagonistic against the fungal pathogens during growth on thatch. However, thatch contains a heterogeneous mixture of polysaccharides, soluble sugars, lignin, and other nutrients. Therefore, while the levels of antagonism observed on the different P/YE media were reproducible

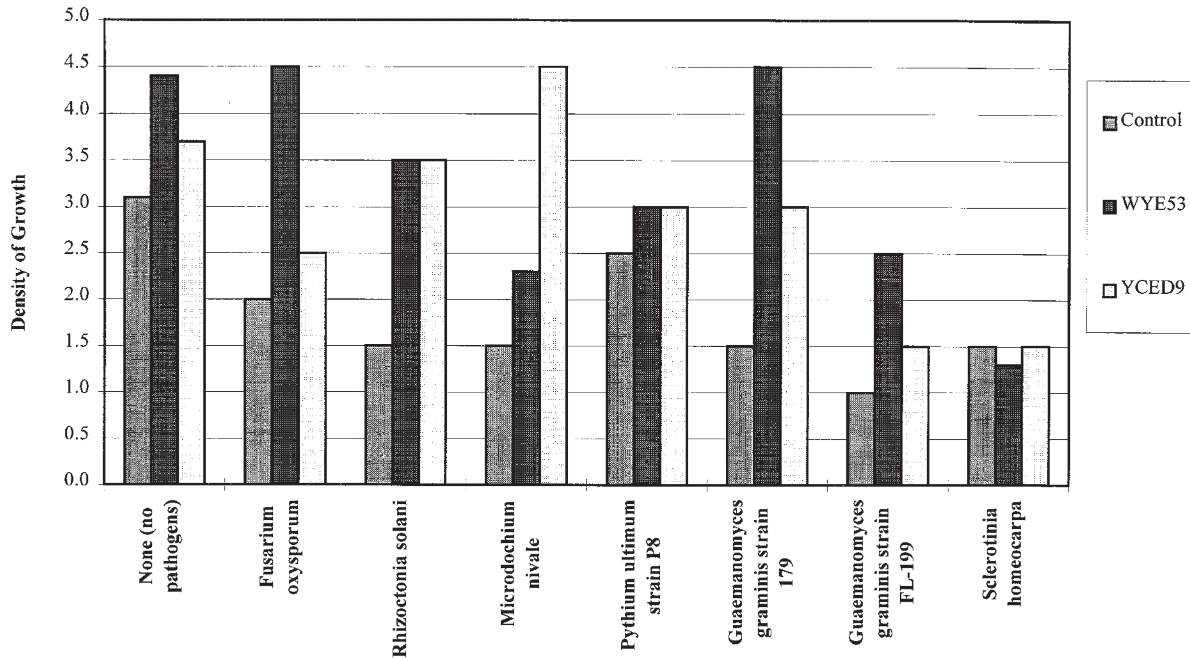

Figure 1 Pathogenicity of turfgrass fungal pathogens to Kentucky bluegrass as influenced by treatments with biocontrol formulations containing the biocontrol agents *Streptomyces hygroscopicus* strains YCED9 and WYE53. (1) No pathogen; (2) treated with *Fusarium oxysporum*; (3) treated with *Rhizoctonia solani*; (4) treated with *Microdochium nivale*; (5) treated with *Pythium ultimum* P8; (6) treated with *Gaeumannomyces graminis* 179; (7) treated with *Gaeumannomyces graminis* FL-199; (8) treated with *Schlerotinia homeocarpa*. Control, treated with strain WYE53, and treated with strain YCED9, respectively left to right.

and nutritionally-dependent, depending on the carbohydrate supplement, these effects could be more or less dramatic *in vivo*. They might depend on the specific mix of available nutrients in the thatch. Also, secondary metabolite (antibiotic) production might be subject to other forms of regulation, such as catabolite repression.

For this reason we also determined if strains YCED9 and WYE53 excreted active antifungal metabolites during growth on thatch (Table 2). Bioassays of sterile filtrates from streptomycete-inoculated thatch cultures and sterile control filtrates (uninoculated thatch cultures) were used to determine if the streptomycetes specifically produced the antifungal metabolites during growth on this lignocellulolytic substrate. Thatch-culture filtrates are really a better measure of potential *in situ* antagonistic activity, since chemically defined media often result in significantly different inhibition effects [10]. The sterile filtrates from thatch cultures were added to PDA and CMA to ensure that each fungus was not nutritionally deprived. Control plates supported excellent growth of the fungi, and the growth diameters of the fungal colonies were easily measured over time. Differences in growth could be attributed only to extracellular metabolites produced by the streptomycetes in thatch-cultures and/or to solubilized compounds leached from the thatch. Filtrates from the streptomycete-amended thatch cultures antagonized all of the fungal pathogens except *P. ultimum*. This suggests that an antagonistic substance required for inhibition of oomycetes, such as *Pythium* or *Aphanomyces* species, which is known to be geldanomycin for strain YCED9 [29], was not produced in the thatch cultures, or its concentration was below our detection limit. The activity might also be due to enzymes not produced during growth on thatch, and/or the medium provided enough other nutrients for *P. ultimum* to overcome the antagonism. Still, metabolite(s) active against the other fungi were definitely present in the thatch culture filtrates. Control filtrates, with a few exceptions, did not possess antifungal activity. In contrast, in several instances, growth stimulation was observed. Thus, it is clear that these actinomycetes were antagonists *in situ*. Our results are generally in agreement with another filtrate antagonism assay done by El-Abyad *et al* [11]. They found that streptomycete-filtrates antagonized three fungi on plates, and subsequent experiments found that the filtrates provided short-term protection when used as a seed coating in a growth chamber.

Applying spore formulations of strains YCED9 and WYE53 in growth chamber experiments involving Kentucky bluegrass led to two significant observations (Figure 1). First, the strains YCED9 and WYE53 formulation-treated controls demonstrated enhanced growth compared to the untreated controls. This result is consistent with other experiments wherein other *Streptomyces* species can enhance the growth of plants [12,13,21]. Second, for all but one of the six pathogens tested, the formulation-treated cultures demonstrated less disease incidence in comparison to the pathogen controls.

In total, the data show that there is good potential for using spore-containing formulations of these thatch-degrading *Streptomyces hygroscopicus* strains, not only to control thatch build-up, but also for the biocontrol of turfgrass fungal diseases. However, research still needs to be done on a field scale, under *in situ* environmental conditions favoring fungal disease development, in order to establish the true

effectiveness of strains YCED9 and WYE53 as biodethatch and antifungal biocontrol agents. To our knowledge this is a first report showing the possibility of using the combined abilities of one organism to control both the build-up of thatch and to control fungal diseases in turf.

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