Population Dynamics of *Laetisaria arvalis* and *Burkholderia cepacia*, Potential Biocontrol Agents in Soil Cores and Thatch of Creeping Bentgrass (*Agrostis palustris*)

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The fungus, *Laetisaria arvalis* (LA) and the bacterium *Burkholderia cepacia* (BC), were added separately to builder's sand at 10^4 sclerotia/ g and 10^7 colony forming units (cfu)/g sand, respectively. Forty ml of amended sand mixture was inserted into the center of an 11 cm diam- bentgrass cylinder, and 100 ml of water was applied evenly over the surface. Movement of each organism was monitored during a 5 wk period for LA and for 5 d for BC using selective media. LA moved horizontally in the thatch layer and vertically only within the upper 1 to 2 cm of soil, but was never recovered below 2 cm depth. Populations of LA were recovered from the outer edge of thatch within the first week but only moved radially through soil a total of 3 cm during the 5 wk period. BC moved horizontally in soil and on roots from the central plug within one d to establish populations of 10^5, 10^4, and 10^3 cfu/g at 1.0, 2.5, and 4 cm, respectively and was almost evenly distributed vertically throughout the cylinder. Lowest densities occurred at the bottom and outer edges of the grass cylinders. During days three to five, populations of BC within the 2.5 cm radius stabilized at 10^5 to 10^6 cfu/g soil, but dropped below detectable levels beyond this area. Knowledge of population dynamics in thatch and the rhizosphere of bentgrass will aid in deploying these biocontrol agents against soilborne diseases.

INTRODUCTION

Although the economic importance of turfgrass may not be appreciated by the general public, the turfgrass industry represents a multimillion dollar business (*1*). Pest control on highly visible or highly used turf areas also generates considerable dollar amounts to the economy.

Increasing concerns about pesticide use (*2*) by the general public and governmental agencies are severely limiting the availability and use of many important pesticides. Fungicides that have been used as standards in many disease control programs have been increasingly regulated. Biological control techniques offer alternative means of control. Although the concept of biological control is simple, in reality it is a complex system that requires well-developed strategies to optimize control potential.

There have been several reports concerning biological control of turfgrass pathogens (*3-7*). In general, modes of action for biological agents are attributed to nutrient competition (*4*), host-induced resistance (*3*), hyperparasitism (*4,8,9*) and antibiotic production (*3,10-13*). Research efforts with turf pathogens have indicated that most of the biocontrol organisms either did not control the pathogen (*14*) or they did not perform as well as selected fungicides (*11,14)*. However, our research indicated that biocontrol agents could be applied to turfgrass and maintain populations in excess of natural populations (*14,15*). Also, some biocontrols

were effective under field conditions (*3,13*), indicating that these systems could become more effective as techniques and formulations are developed and refined. For instance, *Fusarium heterosporum* and another antagonist isolated from turfgrass foliage reduced the incidence of dollar spot on creeping bentgrass when applied in a cornmeal sand top dressing (*5*).We have been interested in using fungi and bacteria to control various soilborne pathogens (*16-19*). Inherent in developing systems for biological control is research involved with delivery of these organisms into the field. Many novel systems have been developed including gels (*17*), alginate pellets (*10, 20,21*) and other carriers and seed treatments (*5, 10,15,22*). Antagonistic fungi have been used in many systems, but many have specificities to forms or species of pathogens (*20,21*). In other words, fungi used as biocontrol agents may not be effective against a disease complex, which is often the case in turf ecosystems. The expectation that one biological control agent will control one pathogen or a complex of pathogens was termed the "silver bullet" approach (*22*) that is often not successful under field conditions. On the other hand, bacterial systems can produce compounds with a broad host spectrum of activity and appear to colonize roots better than fungi (*16*). Conversely, fungi may be better able to survive in soil by producing resistant structures, such as sclerotia or chlamydospores, and provide long-term protection. Some fungal species, when used as seed or root treatments, also have the ability to enhance the growth of plants (*23,25*). Recently, a rhizosphere competent strain of *Trichoderma harzianum* was developed (*7*) and is available as a commercial product: Bio-Trek T- 22G (Willbur-Ellis Co., Fresno, CA). It was effective in controlling dollarspot, brown patch, and *Pythium* root rot on creeping bentgrass (*5*).

Creeping bentgrass (*Agrostis palustris* Huds.) is commonly used for golf course greens because of its low-growth and fine textured qualities (*26*). It is susceptible to many diseases including those caused by *Pythium* spp., and *Sclerotinia homeocarpa* (now considered species of *Lanzi*a and *Moellerodiscus*). In addition, a number of leafspots and root rots can be incited by other pathogens. Bentgrass is best adapted to moderately fertile, acidic soils that are well drained (*26*). Fungal diseases normally occur in a succession throughout the year, which necessitates long-term disease control over a variety of environmental conditions (*26*). For instance, pathogens in the Rhizoctonia complex are active depending on temperature (seasonal variation), with *R. cerealis* being favored by cooler temperatures (15° C to 25° C), and *R. solani* (8° to 40° C) with an optimum of 28° C and *R. zeae* by temperatures above 32° C. Evaluating potential biocontrol agents would have to be conducted under the appropriate conditions that favor development of the pathogen.

Our primary objective was to evaluate the ability of *Laetisaaria arvalis* Burdsall (LA) and *Burkholderia cepacia* (BC)to colonize the rhizosphere and thatch of creeping bentgrass cylinders. Thatch refers to the layer of tightly intermingled living and dead stems, leaves and roots that develops between the soil surface and the layer of green vegetation We think that applying biocontrol agents could be timed during green renovation, especially following aeration because sand is usually spread on the greens to fill the holes produced by the aerator. If biocontrol agents were formulated as a sand mixture, they could be applied at this time to allow for spread to roots and thatch of the existing grass to suppress the activity of soilborne pathogens.

MATERIALS and METHODS

Turf: Cylinders of 2 yr-old Creeping bentgrass (cv Pennlinks) (11 cm diam X 9 cm depth) were taken from a standard sand-based, 2-yr old putting green and placed into 15 cm clay pots. Each cylinder consisted of sand and an extensive root system of bentgrass. Pots were transferred to a growth room, (28° C/18° C and 14 h/10 h day/night, 700 micro-Einsteins light) several days prior to each experiment. Fertilization (Peters soluble plant food, 20 20 20) and watering were performed 3 times/wk, with each pot receiving 100 ml of either tap water or fertilizer as required.

Organisms: The fungus *LA* was obtained from the Forest Products Laboratory, Madison, WI (*2,6*) and is a basidiomycete producing only mycelia and sclerotia in culture. It has been used as a biological control agent against Pythium spp. (*11*), *Rhizoctonia solani*, and *Sclerotium rolfsii* (*14*). BC strain OK-1 was isolated from soil in Oklahoma and identified as *Burkholderia cepacia* (ex Burk.) Yabuuchi (*27*) (formerly *Pseudomonas cepcia* Burk) by fatty acid profile analysis. When used as a seed treatment for cotton, the bacterium colonized and multiplied on the root system (*16*). The strain, OK-2 used in this study was a selection from the original isolate and carried resistance to nalidixic acid (100 µg/mL), chloramphenicol (70 µg/mL), and had a natural resistance to copper (*17*).

Production of organisms. Sclerotia of LA were prepared by growing the fungus on potato-dextrose broth contained in Roux bottles. Cultures were maintained at 25 C in an incubator with 12/12 h light/dark cycle. Mycelial mats were harvested after three weeks of growth and strained through nested sieves (500 , 250 , and 180 µm aperture). Sclerotia were collected from each sieve and dried overnight in a laminar-flow hood. Sclerotia were either used directly prior to drying or mixed dry with sterile builder's sand at 10^4 sclerotia/ g for addition to the middle of the turf cylinders.

BC was grown on Kings medium B agar (KMB) (*28*) for 48 hr at 25° C. An initial suspension was prepared equivalent to 10^8 cfu / ml using a Spectronic 20 at an absorbance value of 0.1 at 660 nm. We made appropriate dilutions from this concentration. Sterile builder's sand was infested with the bacterium to provide a final concentration of 10^7 cfu/g.

Background population tests: In order to determine the native background populations of the biological control agents in the soil cylinders, soil samples with roots were collected from several pots of turfgrass, bulked and samples plated either directly using a multiple pellet soil sampler (*1*) (for LA) or (for *BC*) through a dilution series onto selective media for enumeration .

Test for Pathogenicity: Because red thread disease of turfgrasses is caused by the pathogen *Laetisaria fuciformis*, we thought that possible pathogenicity of bentgrass by LA should be evaluated. In the pathogenicity tests, 1 g of dried sclerotia of LA (10^4 sclerotia/g) was inserted into five inoculation sites /pot by cutting the turf surface along radii with a sterile razor blade to provide an opening 3 to 4 cm long and 3 cm deep. Inoculated turfgrass cylinders were observed for a five week period for discoloration or lesion development on the grass.

BC was formulated as a suspension (10^7 cfu/ ml) in 0.85% NaCl and 100 ml was applied evenly to the surface of 3 grass cylinders contained in pots. Three pots were selected at random from the total number prepared for each organism (15 pots for LA, 12 for BC) and sampled each week (5 wk) for LA and daily (5 d) for BC by taking soil cores between inoculation slits for LA and at random for BC. A total of three plugs were removed along a radial path, at the center, at the middle of the radius, and at the edge of the pot. A soil core device was constructed from a brass tube (1 cm inside diam.); a section of the tube was cut away from one side of the bottom half exposing a 15 cm length of the core in the tube to facilitate removal of the soil plug. The thatch was separated from each soil plug and the remaining plug below the thatch was divided into three 1 cm lengths. Ten to fifteen leaf blades from the thatch and forty roots sieved from the soil sample were plated directly onto each selective medium to determine the presence of either BC or LA on the thatch or root segments. For isolation of LA, each length of the core was separated, mixed, and 0.25 g spread over the surface of selective agar (*29*) contained in petri dishes. Dilutions of soil from *BC* treatments were incorporated into a modified (with the addition of nalidixic acid, 100 ppm and chloramphenicol, 70 ppm) molten selective agar (*30*) and poured into petri dishes to confirm the presence of the bacterium in the soil sample.

Population dynamics. These tests were conducted to determine the horizontal and vertical movement of the biological control agents. Both organisms were formulated into a sterile builder's sand to provide final concentrations of 10^4 sclerotia /g and 10^7

cfu/g for LA and BC, respectively. A central plug (1 cm diam X 9 cm deep) was removed from each turfgrass cylinder and 40 g of infested sand was added into the central core.. Fifteen turfgrass cylinders were treated with LA and 12 were treated with BC. Movement of each organism was monitored for 5 wk for *LA* (Table 1) and for 5 d for *BC (*Table 2). During the experimental period, 100 ml of tap water was added evenly over the surface of only the LA turfgrass, as needed. Watering for BC treated cylinders was from the bottom. Soil plugs were removed from the turf as previously described. Wet-weights of individual plugs were recorded to determine densities of each organism. Dry weights of each soil sample were determined gravimetrically, and densities are reported as cfu/g dry soil.

Data Analysis: Measurements of population densities for each organism were replicated three times at four different time periods. Data were subjected to an analysis of variance using the SAS system (SAS Institute Inc., Cary, NC) and when the F-test was significant, means were separated with a mean separation test. We conducted each test twice.

RESULTS and DISCUSSION

Background Populations. Isolations from soil of the cylinders prior to experimentation indicated that if *LA* and *BC* were present, their populations were below detectable levels using our methodology.

Pathogenicity Test. There were no discolorations or growth abnormalities associated with inoculation of either biological control agent on turf.

Population Dynamics. *LA* was isolated primarily from the thatch and in the upper 1 cm of soil within 1 wk of inoculation. Horizontal movement was most rapid in the thatch layer, spreading 4 cm from the inoculum plug. Greatest recovery occurred within 1 cm of the inoculum plug. Colony counts, as cfu/0.25 g, were averaged from 3 pots and were relatively low compared to the original inoculum density. Densities of LA dropped from 10^4 sclerotia/g sand to less than 5 cfu/ 0.25 g sand or thatch. Because LA produces no conidia, colony counts probably represented mycelial fragments and would raise the question whether *LA* would be able to colonize thatch in densities sufficient to control pathogens such as *Rhizoctonia solani* or *Sclerotinia homeocarpa*. The different sampling periods used for each organism, 5 wk for LA and only 5 d for BC, were related to the speed of movement for each organism. We conducted each experiments twice. There were no difference(*P*-0.05) between the two experiments therefore, data are presented from only one experiment.

After 4 d (Table 2), horizontal movement of *BC* was limited to only 2.5 cm from the inoculum plug, but was almost evenly distributed vertically within that cylinder. Densities of BC dropped from 10^7 cfu/g sand to 10^3 to 10^6 cfu/g and were never isolated from the thatch layer. Obviously, watering assisted in his distribution, because irrigation of turf is a common management technique, downward percolation with irrigation would not be unexpected in the field. Colonization of the rhizosphere by *BC* would make this organism a better choice in control of root diseases caused by *Pythium* spp. There were no significant differences (*P*-0.05) among populations of each organism either vertically or horizontally.

Recently, serious safety concerns about the agricultural use of *BC* have been issued by the U. S. Environmental Protection Agency and the Center for Disease Control (*31*). While we agree with the restriction on field release of this organism, we also believe that laboratory research should be continued to understand its modes of action and spectrum of activity against soilborne pathogens and to compare its efficacy to other bacterial biocontrol candidates that pose less risk to humans.

Both organisms were capable of growth and survival away from points of inoculation. In previous experimentation (*5*) a layer of amended sand cornmeal mix was top-dressed ($400 cm^3/m^2$) over bentgrass plots to control dollarspot. Our system required less inoculum thus our next objectives will be to evaluate LA in the field against selected soilborne pathogens and to develop better formulations and delivery

TABLE 1. Horizontal and vertical distribution of *Laetisaria arvalis*[a] inoculated in a central core[b] in a cylinder of bentgrass[c].

Week[d]	Depth[e](cm)	Distance[f] (cm)		
		1.0	2.5	4.0
1	thatch[g]	1.7	0.7	0.3
	1.0 [h]	0.0	0.0	0.0
	2.0	0.0	0.0	0.0
	3.0	0.0	0.0	0.0
2	thatch	3.7	1.0	0.3
	1.0	0.3 [h]	0.0	0.0
	2.0	0.0	0.0	0.0
	3.0	0.0	0.0	0.0
3	thatch	0.3	0.0	0.0
	1.0	0.0	0.0	0.0
	2.0	0.0	0.0	0.0
	3.0	0.0	0.0	0.0
4	thatch	2.3	0.3	0.0
	1.0	0.0	0.0	0.0
	2.0	0.0	0.0	0.0
	3.0	0.0	0.0	0.0
5	thatch	7.0	2.3	0.0
	1.0	1.7	0.0	0.0
	2.0	0.0	0.0	0.0
	3.0	0.0	0.0	0.0

a *L. arvalis* was formulated as sclerotia produced in potato dextrose broth and mixed (10^4 sclerotia/g white builder's sand.

b A central plug(2.0 cm diam X 9 cm deep) was removed from the center of bentgrass cylinders and filled with 40 g of sclerotia - sand mixture.

c Cylinders of bentgrass 'Pennlinks' (11 cm diam) were removed from a standard 2-yr old putting green and placed in 15 cm clay pots. Each cylinder consisted of sand and an extensive root

d 12 pots of bentgrass were inoculated and incubated in a growth room maintained at 28° C/18° C, 14h/10h light/ dark cycle for 5 d, three cylinders were sampled each day.

e Distance - sampling cores(1 cm diam) were removed at three distances;1.0, 2.5, and 4.0 cm along radii from the central core.

f Depth - sampling plugs were separated into thatch and 1 cm intervals (1 - 3 cm).

g The presence or absence of populations of *B. cepacia* on thatch were determined by directly plating 10 pieces of grass onto modified selective medium.

h Densities of *B. cepacia* below the thatch were determined by dilution plating of the sand/root zone from three cylinders on modified -selective - medium and are reported as mean log cfu/g.

systems. Because these organisms appear to inhabit different niches, it may be possible to use LA with another bacterium and achieve additive or synergistic control of pathogens.

In order to successfully use biological control agents in the field to control soilborne pathogens, we must increase our understanding of their biology and ecology (*28*). We believe that our experimentation adds to this understanding by identifying niches where these organisms colonize and should aid in the developing control strategies to target specific pathogen and increase the control potential of each biological control agent.

TABLE 2. Horizontal and vertical distribution of *Burkholderia cepacia* [s] inoculated in a central core[b] of a cylinder of bentgrass [c].

Day[d]	Depth[e]	Distance[f]		
		1.0	2.5	4.0
1	thatch[g]	0.0	0.0	0.0
	1.0[h]	0.0	0.0	0.0
	2.0	0.0	0.0	0.0
	3.0	0.0	0.0	0.0
2	thatch	0.0	0.0	0.0
	1.0	5.9	4.4	3.8
	2.0	5.2	4.2	2.5
3	thatch	0.0	0.0	0.0
	1.0	5.0	2.5	2.5
	2.0	5.6	0.0	0.0
	3.0	5.5	4.1	0.0
4	thatch	0.0	0.0	0.0
	1.0	5.7	3.9	0.0
	2.0	5.7	4.1	0.0
	3.0	5.8	4.5	0.0
5	thatch	0.0	0.0	0.0
	1.0	6.3	3.4	0.0
	2.0	6.0	4.7	0.0
	3.0	5.3	4.5	0.0

a *B. cepacia* was grown on king's Medium B and diluted to 10^8 cfu/ml using sterile 0.85% NaCl and determined with a Septronic 20 at an absorbance value of 0,1 at 660 nm. This suspension was mixed with sterile white builder's sand to provide a final concentration of 10^7 cfu/g.

b A central plug (2cm diam X 9cm deep) was removed from each bentgrass cylinder and filled with 40g of *B. cepacia* sand mixture. Watering was from the bottom of the pots.

c Cylinders (11 cm diam) of bentgrass 'Pennlinks' were removed from a standard 2-yr-old putting green and placed into 15 cm clay pots and the space between the cylinder and the pot filled with sterile sand. Each cylinder consisted of sand and an extensive root system.

d 12 pots of bentgrass were inoculated and incubated in a growth room maintained at 28° C/18° C, 14h/10h light/ dark cycle for 5 d, three cylinders were sampled each day.

e Distance - sampling cores(1 cm diam) were removed at three distances;1.0, 2.5, and 4.0 cm along radii from the central core.

f. Depth - sampling plugs were separated into thatch and 1 cm intervals (1 - 3 cm).

g The presence or absence of populations of *B. cepacia* on thatch were determined by directly plating 10 pieces of grass onto modified selective medium.

h Densities of *B. cepacia* below the thatch were determined by dilution plating of the sand/root zone from three cylinders on modified -selective - medium and are reported as mean log cfu/g.

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