Research Report

Laboratory Assay to Evaluate Fungicide Efficacy for Control of Rapid Blight of Cool Season Turfgrass

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Abstract

Rapid blight of turfgrass, caused by Labyrinthula terrestris, has become a chronic disease of cool season turfgrasses wherever turf is irrigated with elevated salinity irrigation water and/or in poorly leached soils with increased levels of sodium chloride. Control strategies include using alternative sources of irrigation water with less sodium chloride, leaching soils, using salt tolerant grasses and applying effective fungicides. Because of the widespread and sporadic occurrence of rapid blight, a quick and reliable laboratory assay was developed to test efficacy of chemical treatments. The laboratory assay gives results comparable to field trials in less than four weeks, greatly increasing our ability to determine the efficacy of chemical treatments in a timely and cost effective manner. The laboratory assay correlates highly to results in comparable field trials of selected chemicals, and is valuable for screening chemicals and turfgrass varieties before, or in place of, lengthy and costly field trials.

Introduction

Salinity stress has been associated with a new and unique disease of cool-season turfgrasses known as "rapid blight". Rapid blight symptoms were first observed in turf in California in 1995, but the causal agent was unknown until 2003 when the pathogen was shown to be a new *Labyrinthula* species (Olsen et al., 2003). It was named *Labyrinthula terrestris* by researchers at The University of Arizona (Bigelow, et al., 2005). *L. terrestris* is unusual as a plant pathogen since members of the genus *Labyrinthula* have previously been associated with marine plants and algae. Their classification has been difficult, and they are commonly referred to as the marine net slime molds although they are not related to true slime molds. Investigations of different isolates of *Labyrinthula* species indicate that the isolates from turfgrass are closely related to each other but not to the isolates from marine systems (Olsen, 2007). Rapid blight has been confirmed in at least ten states, including Arizona, California, New Mexico and Texas in the southwest United States, and the United Kingdom and Ireland (Stowell et al., 2005).

Since its discovery, rapid blight has become a chronic problem on cool-season turfgrasses irrigated with high salinity water (> about 2.0 dS/m). Affected turf yellows and dies. In a few cases, entire fall overseeding have failed due to pre- or post-emergence infections by *L. terrestris* (Kerrigan et al., 2012; Olsen, 2007). In hot climates it affects cool-season turfgrass varieties used for overseeding Bermuda including: rough bluegrass (*Poa trivialis*) and perennial rye (*Lolium perenne*); while in warm and temperate locations it can be found on perennial cool-season turfgrasses including annual bluegrass (*Poa annua*), creeping bentgrass (*Agrostis paulustris*) and colonial bentgrass (*Agrostis tenuis*).

Field trials to determine the effects of fungicides or other control strategies for rapid blight on turfgrasses are not only slow and costly, but also very limited. In Arizona, only one location has been available where trials can be conducted, and trials have been initiated only once a year at overseeding (Olsen et al., 2011). Field trials also are

limited since the dual effects of salinity damage and rapid blight disease cannot be differentiated. The development of a laboratory assay has become an important avenue to make progress in determining new chemical controls and the susceptibility of different grasses.

The purpose of this project was to develop an assay that was quick and reliable, was standardized so that it could be repeated from one assay to another, and that gave results that matched those in field trials. Due to constraints in field evaluations, a laboratory assay that gives quick results and screening of potential chemicals in a small space under controlled conditions would be a valuable asset. For example, space to conduct trials on a golf course has been difficult to arrange; and if it rains enough to lower the salinity of soils, rapid blight disappears. The lab assay enables manipulation of salinity, dosage of chemicals, varieties of turfgrass. It also allows us to determine effects of treatments in non-infected plants so that we can establish if damage is due to a specific treatment: salinity alone, salinity and infection with *Labyrinthula terrestris*, or infection with *Labyrinthula terrestris* alone.

The development of this assay is based on a series of preliminary trials over several years in which parameters for the assay were determined. This report does not contain data from all preliminary trials. It is not meant to be an analysis of how the assay reached its current stage, but rather a detailed description of its components for future studies of rapid blight. The intent is to report in detail an assay for rapid blight that can be used by other researchers and turfgrass managers to screen chemicals and grass varieties and to manipulate assay parameters such as chemical rates, salinity, *Labyrinthula* isolates and turfgrass varieties.

Materials and Methods

Laboratory trial development

A series of preliminary trials were conducted to determine parameters for the assay including plant growth techniques; growth chamber environment including temperature and day length; salinity, pH and nutrient components of the irrigation solution; inoculum preparation and storage, inoculation density and timing and method of inoculations; and when and how to make disease ratings.

Plant growth techniques

Poa trivialis was used for establishing assay protocol because it is very susceptible, popular as an overseed, and fast growing. It was chosen over perennial ryegrass because it is much more susceptible (Olsen et al., 2011), and its symptom development is faster in laboratory assays. "Sabre III" variety was used throughout assay development. Field trials with "Sabre III" were conducted at the same time as laboratory trials were being developed, so it was possible to make correlations of efficacy ratings in the lab assay compared to field.

Seed germination and growth

Seed was planted into #30 silica sand in 4 fl oz polystyrene cups by placing about 0.25 g seed on the surface to create a "lawn" of the turfgrass (Fig. 1). The level of sand was constant in each cup to make a uniform volume and to facilitate a uniform cutting height at seven days when treatments began (see below). Four holes were made in the bottom of each cup with a sharp pencil allowing good drainage but small enough to prevent sand leakage. Cups were wetted gently with tap water to moisten seed thoroughly and placed in a tray inside a plastic bag at room temperature (22^{o}-24^{o}C). After 5 days, cups were removed from the bags and placed on elevated stainless steel screens in plastic trays to allow complete drainage and maintain constant salinity (Fig. 1). They were irrigated daily to excess with elevated salinity irrigation solution (described below). All experiments were conducted in a growth chamber at 23^{o}C day/20 ^{o}C night with a 12 hr light/dark photoperiod.

Irrigation solution

Nutrients: Half-strength Hoagland's solution was the base nutrient solution used in the trials (Maynard and Hochmuth, 1997). The EC of the solution was about 1.05 dS/m before adjustment to a standard EC using NaCl. EC was measured using a Milwaukee pH/EC/TDS meter "Combined Smart Meter" (Spectrum Technologies, Inc) which was reliable, easy to calibrate and relatively inexpensive.

pH: In preliminary trials using pH of 5.0, 7.0 and 8.5, there was no difference in disease development. The pH 7.0 was chosen for the lab assay to maintain a more neutral environment. The pH of irrigation solutions was adjusted using 1.0 N NaOH before the salinity was adjusted (Combined Smart Meter).

Salinity: Preliminary trials using salinities ranging from 2.0 to 8.0 dS/m resulted in disease development in inoculated plants. In *P. trivialis* seedlings used in laboratory assays, irrigation water adjusted with NaCl to salinities above 5.0 dS/m caused reduced growth compared to controls adjusted to EC 0.7-1.2 dS/m. In plants irrigated with nutrient solution with an EC below 2.0 dS/m, disease symptoms developed slowly or little at all (Fig. 2). Using results from preliminary trials at 2.0, 2.5 3.5, 4.0 , 5.0 dS/m, a salinity of 3.5 was chosen for assays since consistent disease symptoms developed in 7-10 days after inoculation without plant growth reduction.

Pathogen culture and inoculation techniques

Pathogen culture: An isolate of *Labyrinthula terrestris* from the golf course at which field trials were conducted (see field trial below) was used in all assays. It was maintained both on (1) SIA+ medium (Olsen et al., 2003) amended with 0.5 – 1.0 cm long pieces of sterilized perennial ryegrass leaf pieces at room temperature, and (2) in *P. trivialis* plants inoculated and irrigated with 3.5 dS/m irrigation solution from which fresh isolates were easily recovered by placing symptomatic seedlings on to SIA+ medium and re-isolating.

Inoculation: Inoculations were made by applying one ml of a 80-100,000 cells/ml suspension of *Labyrinthula terrestris* cells to each cup. Cell suspensions were made by flooding 7-10 day old culture plates with 3.5 dS/m water (diluted seawater) and dislodging the cells from the agar surface with a wide bacterial cell spreader. The cell suspension was poured into a 100 ml sterilized bottle, examined in a counting cell (Sedgewick rafter cell), and adjusted to the known suspension with the same water. The *L. terrestris* cells were applied using a one ml pipette tip and adding the cells drop-wise over the "lawn" of *P. trivialis*.

Timing of inoculation: In preliminary assays, inoculations with the rapid blight pathogen, *Labyrinthula terrestris*, were made at seeding, on the day of fungicide treatment, or up to 3 days before fungicide treatments. For comparison, other treatments were either not treated with any fungicides (NTC) or treated with Insignia which is known from previous assays and field trials (Olsen and Gilbert, 2010) to give excellent control. Cups treated with fungicides but not inoculated were used to determine any phytotoxic effects of fungicides or salinity. Since there was no difference in the final ratings (described below) among the different inoculation times, inoculations were made on the same day as fungicide treatments for the standard assay.

Fungicide application

Fungicides were applied to 7 day old seedlings that created a "lawn" in each cup. Plants were cut with sterilized scissors to make each cup a uniform height. Aqueous solutions of fungicides using the label field rate of each fungicide were made in one liter batches so that the appropriate amount of product was applied to each cup individually in 5 ml water. Each fungicide was applied to each replication of each treatment using a 5 ml polypropylene pocket mister (Lotioncrafter.com) that made it possible to spray exactly 5 ml over plants on each cup. Control cups were sprayed with water. The misters are disposable and were used only once. Five fungicides with different modes of action were selected for statistical analyses from data from two field trials and three laboratory trials: Insignia (pyraclostrobin) 0.5 and 0.9 fl oz/1000 ft^2, Disarm 480SC (fluoxastrobin) 0.36 fl oz/1000 ft^2, Secure (fluazinam) 0.5 fl oz/1000 ft^2, Fore (mancozeb) 4.0 and 8.0 oz/1000 ft^2, and Clearys 3336 (thiophanate methyl) 6.0 oz/1000 ft^2.

Experimental design and evaluation

There were 3 replications of each treatment (3 cups each). A completely randomized experimental design was used in all trials in which each cup represented a replication. Lab assays were repeated at least twice. Evaluations of fungicide efficacy were conducted using a disease rating scale of 1-9 with 1 = cups in which all plants were collapsed or dead and 9 = cups in which all plants were healthy. These ratings were also used in the field. In both lab and field, plots with no symptoms were rated as 9 while plots or cups that were completely dead were rated as 1. Ratings were made based on visual inspection using these two poles of reference. Disease symptoms were observed in inoculated, non-treated cups first, about 7 days after the first inoculation. Ratings were then made at 8, 15 and 21 days after fungicide treatment. Preliminary trials were also conducted to investigate techniques using the wet and/or dry weight of cuttings from plants to rate disease. Results were very promising, but this technique takes a great deal more time. Results were not significantly different from the visual ratings, and it was not used further.

Field trials

Site

Fungicide trials were established at Estrella Mountain Ranch Golf Club in Goodyear, AZ in fall 2010 and 2011 to evaluate efficacy of fungicides on rapid blight disease in fall overseed turfgrasses (Olsen and Gilbert, 2010). Trials were conducted on a practice tee that has a history of severe rapid blight. Tifway '419' Bermudagrass was overseeded with *Poa trivialis* 'Sabre III' at 10 lb seed/1000 ft^2. The Bermudagrass was prepared for overseeding the first week of October according to standard practices by lightly verticutting, followed by scalping and removal of clippings.

Fungicide application

Fungicides were applied to 3x5 ft^2 plots arranged in a randomized complete block design with 4 replications. Fungicide treatments were applied in water equivalent to 2 gal/1000 ft^2 with a CO2 powered sprayer at 30 psi using TeeJet 8002VS nozzles. The plots were mowed at 0.5-in. and maintained according to standard practices for the practice tee. Salinity of the irrigation water was about 4.0-4.5 dS/m throughout the trial. Application dates and amount of product applied are shown in Table 1. Treatments were applied 10 days after overseeding just before first mow and again 14 days later. Plots were rated using a scale of 1-9 for plot area killed by rapid blight where 1= 0-10% coverage and 9 = 90-100% coverage of *Poa trivialis* overseed. *Poa trivialis* plots were rated for percent of surviving turfgrass in each replication of each treatment 28 days after the first application.

Comparison of results of laboratory assays and field trials

The likelihood of the lab assay results being different from field trials results for application of the same chemicals was tested using a Two-sample T-Test with method (field trial or lab assay) as the categorical variable (Statistix 9.0, Analytical Software, Tallahassee, FL, 2008).

Results and Discussion

Results of all trials combined in either the laboratory or the field are shown in Table 1. Comparisons to field trials using a Two-sample T-Test for rating by method (null hypothesis: difference = 0) show that disease ratings for the five fungicides combined were not significantly different (unequal variances assumed, P=0.9484) as shown in Table 2. Results indicate that the laboratory assay used for this comparative study will be useful for quick, repeatable assays for early evaluation of chemical control products.

Several components of the assay that were determined during preliminary trials will make it possible to run assays without the use of specialized laboratory equipment as well as save time. For example, the small misters worked well for this quick assay and proved to be ideal for this application. They were both accurate and precise, dispensing only the 5 ml each spray. They are inexpensive; making them disposable for each assay, so cross contamination from other products was eliminated. The #30 silica sand was an excellent medium for plant growth. It contains no salts, so salinity adjustments were easy to maintain, and after early trials in which the sand was autoclaved before use, any sterilization or pasteurization was found to be unnecessary. Growing plants in individual cups made handling the inoculated and non-inoculated treatments much easier. *L. terrestris* can move from plant to plant easily by handling or when plants touch one another, so inoculated and non-inoculated plants must be carefully handled.

Other work with rapid blight has shown that there are several *Labyrinthula* species that may cause rapid blight, (Douhan et al., 2009; Olsen et al., 2013), so it may be that local isolates should be used in laboratory assays. However, in one preliminary trial with another pathogenic *Labyrinthula*, Laby 31 (Olsen et al., 2013), there was no difference in assay results. Since the majority of *Labyrinthula* species isolates have been identified as *Labyrinthula terrestris*, are genetically identical, and have been isolated from symptomatic plants on golf courses, results from trials using the single isolate from central Arizona are probably representative. However, this assay is a unique opportunity to evaluate the efficacy of fungicide activity toward different *Labyrinthula* isolates and to test isolates for resistance development. The assay also is a quick and easy way to evaluate susceptibility of seedlings of new cultivars of turfgrass. Early work indicated that varieties advertised as salt tolerant were less susceptible to rapid blight, but that effect has not always been consistent in field or laboratory trials with varieties of *P. trivialis* or

perennial rye (Olsen, et al., 2011). The lab assay would afford the opportunity to determine effects of different salinities, inoculum levels or other parameters before conducting field trials.

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Table 1. Comparison of lab assays and field trials for chemical control of rapid blight of *Poa trivialis*.

Fungicide	Product /1000 ft^2	[1,2]Avg lab rating	[1,2]Avg field rating
Insignia	0.9 fl oz	8.8	8.4
Insignia	0.5fl oz	8.0	8.0
Disarm 480SC	0.36 fl oz	3.8	4.8
Secure	0.5 fl oz	3.3	5.7
Fore	8.0 oz	7.7	7.3
Fore	4.0 oz	3.3	3.5
Clearys 3336	6.0 oz	3.0	3.8
Non-treated control + Laby	NA	1.8	2.5
Non-treated control - Laby	NA	9.0	NA

[1]Average of 4 replications in each field trial and 6 replications in laboratory assays. Ratings are a scale of 1-9 with 1 = all plants collapsing or dead, 9 = all plants healthy.
[2]Lab ratings are from 15 days after treatment and inoculation; field ratings are 28 days after treatment.

Table 2. Results of Two-sample T-Test for rating of fungicide efficacy by two methods, laboratory assay and field trial, with the null hypothesis: difference =0.

Method	[1]N	mean	SD	SE
Lab assay	48	4.98	2.63	0.38
Field trial	32	5.02	2.34	0.41
	P=0.9484			

[1] The number of replications combined for all trials within each method.

Figure 1. *P. trivialis* growing in silica sand in polystyrene cups placed on screens in trays so that cups can be individually irrigated to excess daily.

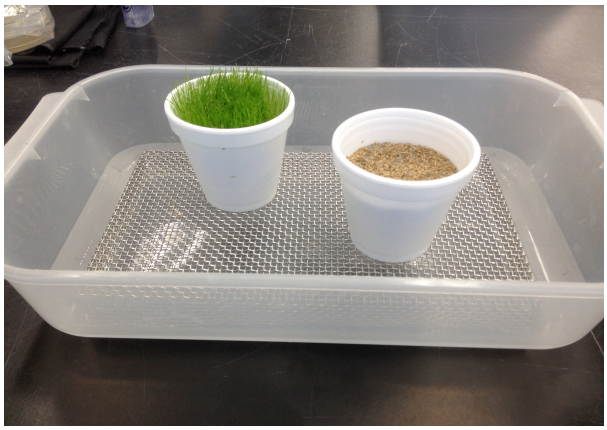

Figure 2. Preliminary assays were used to determine effects of salinity and Insignia fungicide. In this trial, all plants were inoculated 7 days after seeding. The tray on the left marked with a red tag was irrigated with 4.0 dS/m irrigation water, and cups in the right row (showing no symptoms) were treated 10 days earlier with Insignia at 0.5 fl oz/1000 ft^2. The tray on the right marked with a green tag was irrigated with 2.0 dS/m irrigation water, and cups in the left row (showing no symptoms) were treated with Insignia at 0.5 fl oz/1000 ft^2.