VI. EVALUATON OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY METHOD FOR BROWN PATCH DETECTION IN PERENNIAL RYEGRASS

SYNOPSIS

Enzyme-linked immunosorbent assay (ELISA) technology is a molecular-based plant disease diagnostic tool, which may be used to measure plant pathogen populations. Information is limited regarding the influence of sampling method on the ability of ELISA to predict brown patch (Rhizoctonia solani Kühn). This field investigation was conducted during 1991 and 1992 to determine the effectiveness of ELISA to predict R. solani infection events in 'Caravelle' perennial ryegrass (Lolium perenne L.). Samples were collected either between 7 and 8 AM or 4 and 5 PM, and from plots mowed to a height of either 1.7 or 4.5 cm. A pathogen detection reading ≥ 30 reflectance units within 72 hrs prior to an infection event was considered a successful prediction. Pathogen detection levels were generally higher in AM-sampled turf, and in plots mowed to a height of 4.5 cm. In the two-year period, only 7 of 15 infection events were predicted from samples collected from high-cut turf, but only four events were predicted from samples collected from low-cut turf. While this technology is useful for confirming the presence of R. solani, it was judged unreliable for predicting infection events.

INTRODUCTION

Molecular biology and biotechnology research have led to the development of anitbodies that detect proteins or nucleic acids of plant pathogens (Clark, 1981). As a result, enzyme-linked immunosorbent assay (ELISA) methods were developed for plant disease diagnosis and detection (Miller and Martin, 1988). As described by Miller and Martin (1988), an ELISA detects fungal pathogens by a double-sandwich antibody reaction. Miller and Martin (1988) discuss the potential of ELISA to detect plant pathogens and quantify their populations prior to the appearance of disease symptoms. Plant pathogen levels, as determined from ELISA techniques, can be compared to established disease thresholds, and therefore facilitate disease management decisions (Miller, 1982).

Commercial ELISA-based turfgrass disease detection kits are available for brown patch (Rhizoctonia solani Kühn), dollar spot (Sclerotinia homoeocarpa Bennett), and Pythium blight (Pythium spp.) (Rittenberg et al., 1988). Turfgrass disease diagnosis and detection using ELISA was demonstrated by Shane (1991), Baldwin (1993), and Schumann et al. (1994). Shane (1991) concluded that pathogen quantification was not consistent, however, ELISA was useful to confirm Pythium blight diagnosis. Baldwin (1993) used ELISA technology to support fungicide spray decisions for dollar spot. He (1993) reported a reduction in fungicide applications from ELISA detection results when compared to fungicide sprays based on visual disease assessment. For brown patch, fungicide sprays were reduced and acceptable brown patch control was acheived by combining weather-based

disease forecasts with ELISA-based confirmation of pathogen levels (Schumann et al., 1994).

While ELISA technology has been shown to improve fungicide use for turfgrass disease management, the influence of mowing height and sampling time on detection of pathogen levels by ELISA have not been investigated. Therefore, the objectives of this study were to monitor R. solani levels in 'Caravelle' perennial ryegrass (Lolium perenne L.) using leaf tissue samples collected from turf mowed at two heights of cut (1.7 and 4.5 cm) and two collection timings (AM or PM). The ELISA system, sold under the trade name of Reveal® Turf Disease Detection Kit by Neogen Corporation, Lansing, MI, was used.

MATERIALS AND METHODS

In 1991 and 1992, the ELISA method was evaluated using leaf samples from a mature stand of 'Caravelle' perennial ryegrass. The field experiments were conducted at the University of Maryland Cherry Hill Turfgrass Research and Education Facility, Silver Spring, MD. The study site was subjected to two mowing heights (1.7 or 4.5 cm), and plot size measured 1.5 by 1.5 m. The plots were mowed three times weekly with a reel mower, and clippings always were removed.

From 21 June to 19 July 1991 (i.e., 29 sampling days), two assays were conducted using samples from 1.7 and 4.5 cm-high plots, and samples were collected between 7:00 and 8:00 AM or between 4:00 and 5:00 PM. Approximately 2 g of primarily symptomless turfgrass clippings were randomly sampled at four locations per plot (i.e., four sub-samples) from two

replicate plots at both mowing heights. That is, there were two replicate samples from each mowing height. At both mowing heights, the four sub-samples per plot were combined, and approximately 2 g of leaf tissue were extracted to represent the plot sample. The study area received no fertilizer or fungicide treatments during the study period.

Turfgrass clippings were sampled and extractions prepared according to the instructions provided by Neogen Corporation. Briefly, plant tissue sap was extracted by grinding leaf blades on abrasive pads and placed in an extraction solution. Several drops from the extraction solution were placed on the surface of the detector, which is a porous device that contains specific antibodies attached to its surface. Next, the detection, rinse, color, and finishing solutions were added to the detector in sequence, resulting in a color reaction on the surface of the detector. The color reaction was quantified with a portable, battery-powered reflectometer (AgriMeter II™, Neogen Corporation, Lansing, MI).

Based on the intensity of the color reaction on the detector, the AgriMeter II™ provides a numerical value (i.e., pathogen level) that corresponds to a disease risk threshold. Disease risk was determined according to the Reveal® Interpretation Guide, which defines risk levels as follows: 0 to 19 (low); 20 to 29 (caution); 30 to 37 (danger); and > 38 (extreme). A low range value indicates that the pathogen was not detected or that the pathogen was present at a low population level. The caution range indicates that the pathogen was present and disease development is possible if favorable weather conditions occur. The danger range indicates that the pathogen is present and disease symptoms are likely, and a preventive disease management program is suggested. The extreme range

indicates that disease symptoms are likely, and a curative disease management program is suggested.

The ability of ELISA to forecast brown patch outbreaks was determined by comparing observed events when foliar R. solani mycelium were either present or absent versus the daily risk levels detected. Detector levels corresponding to the danger (i.e., 30 - 37) or extreme (i.e., > 38) risk ranges were considered necessary to forecast a brown patch outbreak or R. solani infection event. Periodically throughout the study, turfgrass clippings with disease symptoms were examined microscopically to confirm the presence of R. solani. The R. solani biotype at the study site was identified as belonging to anastomosis group AG-1 IA.

Because detection levels were typically greater from AM versus PM-sampled turf in 1991, leaves were only collected between 7 and 8 AM in 1992. From 6 June to 31 August 1992 (i.e., 87 sampling days), two assays were conducted on samples from plots maintained at 1.7 and 4.5 cm mowing heights. The assay methods in 1992 were the same as previously described for 1991.

To statistically compare the four levels of disease risk, the results from the ELISA tests were standardized as follows: 0 - 19 (low range) = 10; 20 - 29 (caution range) = 25; 30 - 37 (danger range) = 34; and > 38 (extreme range) = 59. All data were subjected to repeated measures analysis of variance to evaluate the treatment effects over time (Rowell and Walters, 1976; SAS Institute, 1985). The significantly different means were separated using Fisher's protected least significant difference test at $P \leq 0.05$ (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

1991 Results. The assay consistently tested positive for R. solani throughout the duration of the field study. Actual pathogen assay levels for 1991 are listed in Table 17. Analysis of the data revealed that pathogen levels were influenced by mowing height and sampling time (Table 18). Detection levels were greater from plots maintained at 4.5 versus 1.7 cm. Mean detection levels for turf mowed at 4.5 and 1.7 cm were 25 and 19, respectively (Table 18). When comparing AM and PM sampling times averaged over mowing height, higher detection levels were observed from AM-sampled turf on 22 of 29 days (Table 17). Mean detection levels were 26 and 18 for turf sampled in the AM and PM timings, respectively (Table 18).

Brown patch outbreaks, as determined by visual observation of foliar R. solani mycelium, were observed on 3, 8, and 11 July (Table 17). A brown patch outbreak was considered predicted or forecast when detection levels ≥ 30 were recorded within 72 hrs of an observed infection event. Pathogen levels ≥ 30 were observed in plots maintained at the 4.5 cm mowing height prior to the infection events on 3 and 11 July, but not on 8 July. Samples collected from 1.7 cm-high turf predicted the infection events observed on 3 July (sample from AM on 1 July) and 11 July. The 11 July infection event was predicted from samples collected from both timings and heights of cut on 10 July. In low-cut turf, pathogen levels in the danger range were observed on 24 and 29 June and 17 to 18 July, but no foliar mycelium was evident within 72 hrs. Hence, these were considered false predictions.

Data suggested that the ELISA method was somewhat more effective in predicting brown patch disease outbreaks using high-cut rather than low-

cut turf samples (Table 17). On high-cut turf, detection levels reached and maintained the danger/extreme ranges for 96 hrs prior to the appearance of foliar mycelium on 3 July, and 48 hrs before the disease outbreak on 11 July. Detection levels, however, steadily declined below the danger range prior to the disease outbreak on 8 July. When taking into consideration all detections ≥ 30, there were 9 false predictions and two out of three possible correct predictions were achieved in 1991.

1992 Results. Samples were collected only between 7 and 8 AM from 6 June to 31 August in 1992 (Table 19). Detection levels were generally higher from samples collected from plots mowed to a height of 4.5 cm (i.e., 27 of 87 days) when compared to low-cut samples, but detection levels were in the same risk range regardless of mowing height on 43 of 87 days (Table 19). For data averaged over the season, detection levels were significantly greater in turf mowed to a height of 4.5 cm (Table 18).

Foliar mycelium was observed on 1, 2, 8, 14, 15, 22, 24, 28, and 29 July, and 18, 19, and 26 August (Table 19). Quantifying pathogen levels with the ELISA method is 1992 resulted in more successful brown patch forecasts in turf mowed to a height of 4.5 cm. In high-cut turf, disease risk values ≥ 30 coincided with brown patch outbreaks on 1, 2, 8, 14, 22, and 24 July (Table 19). On 1 and 8 July, however, the prediction (i.e., detection level ≥ 30) came on the day foliar mycelium was observed, but detection levels ≥ 30 were not recorded for the previous 72 hr period. In high-cut turf, assay values in the danger or extreme range were observed 48 hrs prior to brown patch outbreaks on 14 and 22 July, and 24 hrs prior to a disease outbreak on 24 July 1992. On low-cut turf, disease risk in the danger and extreme range recorded on 22 and 23 July, coinciding with infection events on 22 and 24

July. Hence, only the 24 July infection event was predicted prior to the disease outbreak. There was a false prediction from 1.7 cm-sampled plots on 15 June. Hence, the ELISA method failed to predict infection events within 72 hrs on five dates (i.e., 28 and 29 July, and 18, 19, and 26 August). Although detection levels ≥ 30 were recorded on 1 and 8 July when foliar mycelium was evident, the risk levels 72 hrs prior to these dates were low. Hence, only five of twelve infection events were predicted within 72 hrs prior to the appearance of foliar mycelium in 1992.

SUMMARY AND CONCLUSIONS

Over the two-year period, 7 of 15 infection events were predicted within 72 hrs prior to the appearance of foliar R. solani mycelium. In both years, detection levels were higher from samples collected in turf mowed to a height of 4.5 cm, when compared to turf maintained at 1.7 cm. Of the seven predicted infection events, only two in each year were successfully determined from samples collected from plots mowed to a height of 1.7 cm. Golf course fairways, tees and greens are mowed ≤ 1.7 cm. Hence, the data indicated that there would be little or no value in using the Reveal® kits for predicting brown patch outbreaks on golf courses, at least according to the forecast guidelines imposed in this study. It should be noted that Schumann et al. (1994) used a prediction threshold risk value of ≥ 23. This threshold would have increased prediction accuracy in this study, but it also would have resulted in 17 and 24 false predictions in 1991 and 1992, respectively.

These kits may be of limited value on higher-cut turfgrass sites, but the 47% success rate among samples from 4.5 cm plots is not reliable enough

for commercial turfgrass management. These results were similar to the findings of Shane (1991). Monitoring Pythium blight epidemics, Shane (1991) reported that increases in detection levels coincided with, but typically did not precede the development of disease symptoms. He (1991) concluded that while advanced detection of disease development was unsatisfactory, the ELISA method was useful for verifying the diagnosis of the disease. The greatest value of the R. solani assay also would be its ability to effectively identify R. solani as the pathogen from infected tissues. This is important, because the foliar mycelium produced by Pythium spp. is not distinguishable from R. solani without the aid of a microscope.

Table 17. Mean pathogen detection levels in perennial ryegrass as influenced by mowing height and sampling time in 1991.

	Detector level†				
	Mowing height				
	1.7 cm		4.5 cm		
	Time of assay‡				*R. solani*
Date	AM	PM	AM	PM	mycelium§
21-Jun	19	07	13	10	-
22-Jun	16	07	20	09	-
23-Jun	21	06	20	14	-
24-Jun	31	13	28	30	-
25-Jun	25	13	33	10	-
26-Jun	20	16	36	21	-
27-Jun	17	13	12	13	-
28-Jun	10	05	18	11	-
29-Jun	32	15	51	18	-
30-Jun	27	15	33	36	-
1-Jul	30	29	38	60	-
2-Jul	26	28	32	49	-
3-Jul	25	26	34	44	+
4-Jul	20	22	28	31	-
5-Jul	07	20	20	24	-
6-Jul	14	09	12	12	-
7-Jul	19	17	10	10	-
8-Jul	23	28	07	08	+
9-Jul	30	13	43	10	-
10-Jul	35	45	41	39	-
11-Jul	33	12	38	06	+
12-Jul	24	09	27	11	-
13-Jul	10	07	18	05	-
14-Jul	08	06	09	02	-
15-Jul	21	05	29	16	-
16-Jul	15	13	10	10	-
17-Jul	34	03	21	18	-
18-Jul	37	19	37	30	-
19-Jul	21	14	35	14	-

†Detector level indicates brown patch risk as follows: 0 to 19 (low), 20 to 29 (caution), 30 to 37 (danger), and > 38 (extreme).
‡Samping time was either 7 to 8 AM or 4 to 5 PM.
§Response of '+' and '-' indicates mycelium visually observed or not observed on turfgrass foliage, respectively.

Table 18. Effect of mowing height and sampling time on pathogen detection levels in perennial ryegrass in 1991, and the effect of mowing height on pathogen detection levels in perennial ryegrass in 1992.

1991				1992	
Mowing height	Detector level[†]	Sampling time[‡]	Detector level[†]	Mowing height	Detector level[†]
1.7 cm	19 b[§]	AM	26 a[§]	1.7 cm	13 b[§]
4.5 cm	25 a	PM	18 b	4.5 cm	17 a

[†]Detector level indicates brown patch risk as follows: 0 to 19 (low), 20 to 29 (caution), 30 to 37 (danger), and > 38 (extreme).
[‡]Sampling time was either 7 to 8 AM or 4 to 5 PM.
[§]Means followed by the same letter are not significantly different ($P \leq 0.05$) according to Fisher's protected least significant difference test.

Table 19. Mean pathogen detection levels in perennial ryegrass as influenced by mowing height in 1992.

Date	Detector level† —Mowing height— 1.7 cm	4.5 cm	*R. solani* mycelium‡	Date	Detector level† —Mowing height— 1.7 cm	4.5 cm	*R. solani* mycelium‡	Date	Detector level† —Mowing height— 1.7 cm	4.5 cm	*R. solani* mycelium‡
6-Jun	02	01	-	1-Jul	21	30	+	1-Aug	02	07	-
7-Jun	04	02	-	2-Jul	17	31	+	2-Aug	04	13	-
8-Jun	21	07	-	3-Jul	09	02	-	3-Aug	04	25	-
9-Jun	04	01	-	4-Jul	04	29	-	4-Aug	13	13	-
10-Jun	07	09	-	5-Jul	09	25	-	5-Aug	07	03	-
11-Jun	23	04	-	6-Jul	19	27	-	6-Aug	05	03	-
12-Jun	05	24	-	7-Jul	12	29	-	7-Aug	04	01	-
13-Jun	03	06	-	8-Jul	19	34	+	8-Aug	03	02	-
14-Jun	01	03	-	9-Jul	16	27	-	9-Aug	04	03	-
15-Jun	31	17	-	10-Jul	32	22	-	10-Aug	03	02	-
16-Jun	28	25	-	11-Jul	22	29	-	11-Aug	01	05	-
17-Jun	20	23	-	12-Jul	12	38	-	12-Aug	13	03	-
18-Jun	12	24	-	13-Jul	10	49	-	13-Aug	05	00	-
20-Jun	08	06	-	14-Jul	13	31	+	14-Aug	01	01	-
19-Jun	05	12	-	15-Jul	10	24	+	15-Aug	04	06	-
21-Jun	06	02	-	16-Jul	05	11	-	16-Aug	13	17	-
22-Jun	13	07	-	17-Jul	10	16	-	17-Aug	21	25	-
23-Jun	10	09	-	18-Jul	10	19	-	18-Aug	11	17	+
24-Jun	14	26	-	19-Jul	16	28	-	19-Aug	04	10	+
25-Jun	08	07	-	20-Jul	25	41	-	20-Aug	01	01	-
26-Jun	06	16	-	21-Jul	21	34	-	21-Aug	06	04	-
27-Jun	16	07	-	22-Jul	54	30	+	22-Aug	02	02	-
28-Jun	05	01	-	23-Jul	39	32	-	23-Aug	03	03	-
29-Jun	09	14	-	24-Jul	27	22	+	24-Aug	05	11	-
30-Jun	04	06	-	25-Jul	27	23	-	25-Aug	05	06	-
				26-Jul	25	20	-	26-Aug	18	21	+
				27-Jul	29	18	-	27-Aug	15	20	-
				28-Jul	19	16	+	28-Aug	09	26	-
				29-Jul	29	18	+	29-Aug	09	15	-
				30-Jul	18	17	-	30-Aug	06	13	-
				31-Jul	30	18	-	31-Aug	04	13	-

†Detector level indicates brown patch risk as follows: 0 to 19 (low), 20 to 29 (caution), 30 to 37 (danger), and > 38 (extreme).

‡Response of '+' and '-' indicates mycelium visually observed on turf foliage.