Silicon-Induced Systemic Defense Responses in Perennial Ryegrass Against Infection by *Magnaporthe oryzae*

Alamgir Rahman, Christopher M. Wallis, and Wakar Uddin

First and third authors: Department of Plant Pathology and Environmental Microbiology, The Pennsylvania State University, University Park 16802; and second author: United States Department of Agriculture–Agricultural Research Service, San Joaquin Valley Agricultural Sciences Center, Parlier, CA 93648.

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ABSTRACT

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Sustainable integrated disease management for gray leaf spot of perennial ryegrass may involve use of plant defense elicitors with compatible traditional fungicides to reduce disease incidence and severity. Silicon (Si) has previously been identified as a potential inducer or modulator of plant defenses against different fungal pathogens. To this end, perennial ryegrass was inoculated with the causal agent of gray leaf spot, *Magnaporthe oryzae*, when grown in soil that was nonamended or amended with three different levels of calcium silicate (1, 5, or 10 metric tons [t]/ha). When applied at a rate of 5 t/ha, calcium silicate was found to significantly suppress gray leaf spot in perennial ryegrass, including a significant reduction of disease incidence

(39.5%) and disease severity (47.3%). Additional studies observed non-penetrated papillae or cell-wall appositions harboring callose, phenolic autofluorogens, and lignin-associated polyphenolic compounds in grass grown in the Si-amended soil. Regarding defense-associated enzyme levels, only following infection did grass grown in Si-amended soil exhibit greater activities of peroxidase and polyphenol oxidase than equivalent inoculated control plants. Also following infection with *M. oryzae*, grass levels of several phenolic acids, including chlorogenic acid and flavonoids, and relative expression levels of genes encoding phenylalanine ammonia lyase (*PALa* and *PALb*) and lipoxygenase (*LOXa*) significantly increased in Si-amended plants compared with that of nonamended control plants. These results suggest that Si-mediated increase of host defense responses to fungal pathogens in perennial ryegrass has a great potential to be part of an effective integrated disease management strategy against gray leaf spot development.

Gray leaf spot caused by *Magnaporthe oryzae* (Catt.) B. C. Couch is a devastating disease of perennial ryegrass (*Lolium perenne* L.) (Uddin et al. 2004) as well as several other cereal crops such as rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.) (Debona et al. 2012). Perennial ryegrass is a widely used cool-season turf type and forage grass species because of its superior agronomic attributes such as color, upright growth, and rapid germination (Uddin et al. 2003). However, the devastating nature of gray leaf spot has seriously affected turfgrass industries due to the increasing requirements for greater fungicide usage in order to keep perennial ryegrass fairways disease free (Uddin et al. 2004). Greater fungicide use has resulted in undesirable consequences such as development of fungicide-resistant *M. oryzae* strains (Vincelli and Dixon 2002) and nontarget effects on beneficial microorganisms (Viji et al. 2003). Although perennial ryegrass cultivars resistant to gray leaf spot have been developed using conventional breeding techniques (Han et al. 2006), its complicated genetic control of desirable traits and highly self-incompatible nature makes these breeding approaches extremely difficult (Bonos and Huff 2013). Therefore, a need exists for novel management options, including the possibility of using silicon (Si) for the management of gray leaf spot of perennial ryegrass.

Si-amended soil has been reported to be effective in suppressing diseases in several plants, including blast disease (Rodrigues et al. 2004) and sheath blight of rice (*O. sativa* L.) (Rodrigues et al. 2003); bacterial wilt of tomato (*Solanum lycopersicum* L.) (Ghareeb et al. 2011); and powdery mildew of wheat (*T. aestivum* L.), barley (*Hordeum vulgare* L.), cucumber (*Cucumis sativus* L.), rose (*Rosa*

hybrida L.), and *Arabidopsis* (Chain et al. 2009; Fauteux et al. 2006; Liang et al. 2005; Shetty et al. 2012; Wiese et al. 2005). Si amendments have also been reported to suppress several fungal diseases on turfgrasses such as gray leaf spot on perennial ryegrass (Nanayakkara et al. 2008), powdery mildew on Kentucky bluegrass (*Poa pratensis* L.) (Hamel and Heckman 1999), dollar spot (*Sclerotinia homoeocarpa*) and brown patch (*Thanatephorus cucumeris*) on creeping bentgrass (*Agrostis stolonifera* L.) (Fry et al. 2006), and leaf spot or melting out diseases caused by *Bipolaris cynodontis* on bermudagrass (*Cynodon dactylon* L.) (Datnoff and Rutherford 2004). Si-mediated multilayered defense responses in plants have been identified such as deposition of Si in the epidermal cell wall providing a physical barrier and, thereby, preventing *M. oryzae* appressorial penetration on rice leaves (Hayasaka et al. 2008). Also, formation of Si-deposited impenetrable papilla on wheat leaf epidermal cells against powdery mildew (*Blumeria graminis* f. sp. *tritici*), and augmented generation or deposition of phenolics or phytoalexins that include flavonoids in cucumber (Bélanger et al. 2003) and rose (Shetty et al. 2011) and phytoalexins (momilactones) in rice (Rodrigues et al. 2004), have been noted. Furthermore, following infection with *M. oryzae*, rice plants grown on Si-amended soil exhibited increased activity of defense-associated enzymes such as peroxidase (PRX), polyphenol oxidase (PPO), and phenylalanine ammonia lyase (PAL) (Cai et al. 2008), as well as augmented expression of pathogenesis-related (PR) genes, including glucanase (*Glu*) and *PR-1* (Brunings et al. 2009; Rodrigues et al. 2005).

Previously, gray leaf spot disease incidence and severity on perennial ryegrass significantly decreased following soil Si amendment (Nanayakkara et al. 2008; Rahman and Uddin 2012). Furthermore, leaf Si content of perennial ryegrass was found to increase with the increase of calcium silicate amendments (Nanayakkara et al. 2008). Despite the widespread research supporting the disease-suppressive abilities of Si, the mechanisms of Si-induced defense

Corresponding author: W. Uddin; E-mail address: wxu2@psu.edu

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responses in perennial ryegrass remains unknown. Understanding the mechanisms of induced resistance in perennial ryegrass conferred by Si amendments of soil would help to design a better Si formulation as well as the timing of applications. Recently, we demonstrated the induction of host resistance in perennial ryegrass using salicylic acid and benzothiadiazole against *M. oryzae* infection, which is mediated through frequent papilla deposition as well as augmented defense-related gene expression (Rahman et al. 2014). This suggested that enhanced host defense responses in this grass species could be exploited for better protection against gray leaf spot development.

Our current investigation focuses on identifying the underlying mechanisms of the prophylactic effect of Si against gray leaf spot on perennial ryegrass. Physiological and cytological changes indicative of defense responses in perennial ryegrass plants grown in Si-amended and nonamended soils, followed by challenged inoculation with *M. oryzae*, were analyzed. Development of cell-wall appositions (papillae), accumulation of phenolic- or lignin-associated polyphenolic compounds, and relative expression levels of defense-related genes provided a comparative view of the cellular defense responses in *M. oryzae*-infected perennial ryegrass with and without Si treatment.

MATERIALS AND METHODS

Plant materials. All experiments were conducted in controlled-environment chambers (model PGR 1; 5CONVIRON, Canada). 'Legacy-II' perennial ryegrass (*L. perenne* L.), a cultivar highly susceptible to infection by *M. oryzae*, was seeded at a rate of 20 g/m² in plastic pots (10.2 cm in diameter) in peat Fafard mix number 2 (Griffin Greenhouse and Nursery Supplies, Leola, PA). Fafard number 2 contains 55% peat moss, 20% perlite, and 25% vermiculite.

Si amendment of soil-mix. Addition of Si to Fafard mix number 2 was carried out as described previously (Nanayakkara et al. 2008). Si was supplied as calcium silicate (Excellerator: 39.3% Si, 5.8% CaMgSiO₄, 17.9% Ca₃Mg(SiO₄)₂, 8.0% CaMg-SiO₇, and 7.6% Ca₂MgSiO₄; Harsco Minerals Inc., Pittsburgh). In brief, calcium silicate rates added to each pot of Fafard mix number 2 were calculated based on field application rates. Different rates of Si were incorporated by hand mixing the Si product thoroughly with approximately 630 cm³ of Fafard mix number 2 that filled a single plastic pot (10.2 cm in diameter) seeded with perennial ryegrass Legacy-II, as described previously (Nanayakkara et al. 2008). Three-week-old plants were fertilized with water-soluble fertilizer (24% N, 8% P, 16% K Miracle-Gro all-purpose water-soluble plant food; Scott's Miracle-Gro Products, Inc., Marysville, OH) to a field capacity (0.7 g/liter water) of the planting medium. All Si-amended experimental pots contained calcium silicate at 5 metric tons [t]/ha (6.48 mg/cm³); however, disease incidence and severity experiments also included two additional rates, 1 and 10 t/ha (1.29 and 12.96 mg/cm³, respectively). Agricultural lime (CaCO₃) was applied to adjust the calcium (Ca) level to that of the treatments with Excellerator, which contains 24% Ca according to the manufacturer's product label.

Inoculation of the plants with *M. oryzae* and disease rating. An isolate of *M. oryzae* (PgPA 05T-01), maintained on filter paper strips at -80°C, was used for all inoculations in this study (Tosa et al. 2007). Preparation of *M. oryzae* conidia was the same as describe previously (Nanayakkara et al. 2008; Rahman et al. 2014). Briefly, potato dextrose agar (BD Difco, NJ) blocks containing actively growing fungal mycelia of *M. oryzae* were transferred to full-strength oatmeal agar (BD Difco). After 5 days of incubation at 25 to 26°C under fluorescent light (72 µE·m⁻² s⁻¹), autoclaved (15 min at 121°C at 121 psi) alfalfa (*Medicago sativa* L.) stems were placed in the culture plates. After 12 to 14 days of incubation, conidia were harvested and adjusted to 3 to 4 × 10⁴ conidia/ml with sterile milliQ water (0.01% Tween 20). Four-week-old perennial ryegrass plants grown at 24°C were inoculated with the conidial suspension by spraying the leaves until run-off. Plants treated with

sterile milliQ water containing 0.01% Tween 20 (Sigma-Aldrich, St. Louis) served as noninoculated control plants. Immediately after inoculation, plants were placed under continuous fluorescent light (77 to 85 µE m⁻² s⁻¹) at 28°C and relative humidity of >95% in a Percival dew chamber (model E 54U-DL; Boone, IA). Plants containing pots were arranged in a randomized complete block design with three replications for each treatment.

Disease incidence and severity were assessed 5 days after inoculation with *Magnaporthe oryzae* as described previously (Rahman et al. 2014). Disease incidence was assessed by quantifying percent symptomatic leaves with each treatment. Disease severity was assessed using an index of 0 to 10 (0 = no necrosis and 10 = >90% necrotic leaf area on individual blades). The experiments were repeated once. Data from each experiment were subjected to analysis of variance (ANOVA) using MINITAB (version 16.1.0; Minitab Inc., State College, PA). Multiple comparisons of means for disease incidence and severity were made using the Student-Newman-Keuls test ($P \leq 0.05$).

Visualization of defense responses. For analyses of callose deposition at the infection sites, cleared leaves were stained with 0.05% aniline blue as described previously (Rahman et al. 2014). Briefly, leaf segments of 3 cm in length were transferred to a bleaching solution of ethanol/acetic acid (3:1, vol/vol) for at least 24 h until all tissues were completely cleared of chlorophyll. Following three washes in 0.067 M K₂HPO₄ buffer (pH = 9), leaf segments were incubated for 2 h in 0.067 M K₂HPO₄ buffer containing 0.05% aniline blue. The samples were then mounted in 60% (vol/vol) glycerol for microscopic analysis. Callose-stained segments were visualized using bright-field microscopy with UV filter (Olympus BX-61; excitation filter, 350/50 nm and DM 400 dichroic beam splitter and emission filter, 460/50 nm).

Phenolic compounds were analyzed as autofluorescence in cleared leaf segments mounted in 60% (vol/vol) glycerol and visualized under blue light epifluorescence (Nikon Eclipse E600; GFP filter set, 480/30 nm and DM 400 dichroic beam splitter 505 and emission filter, 535/40 nm), whereas lignin-associated polyphenolic compounds were analyzed by their blue or turquoise appearance when stained in a solution of 0.05% toluidine blue in 50 mM citrate buffer (pH 3.5) visualized under bright-field microscopy, as described above (Mellersh et al. 2002).

In each experiment, 75 to 100 single-cell interaction sites were inspected and scored independently. All experiments were repeated twice. All bright-field and UV-filtered images were acquired digitally (Hamamatsu cooled digital camera: ORCA ER model C4742-80 and Olympus DP71) and further processed with the CellSens Dimension software. All epifluorescence images were acquired using Nikon Digital Sight DS-5M camera and analyzed using Nikon Digital Sight DL software. For statistical analysis, percentages were transformed to arcsine square roots (transformed value = $180/\pi \times \arcsin [\sqrt{(\%/100)}]$) to normalize data and stabilize the variance of data (Ahrens et al. 1990; Troch et al. 2014). Data were analyzed using MINITAB (version 16.1.0; Minitab Inc.) for ANOVA followed by means comparison using Tukey's honest significant difference (HSD) test ($P \leq 0.05$).

Enzyme activity assays. PRX and PPO enzyme activity in plant leaf extracts were measured at 0, 24, 48, 72, and 96 hours postinoculation (hpi), as previously described by Liang et al. (2005). Briefly, 0.2 g of freshly collected leaf tissue was homogenized in liquid nitrogen using an ice-cold mortar and pestle. Ground leaves were then transferred to a 1.5-ml microcentrifuge tube, and 1 ml of ice-cold buffer (50 mM borate buffer [pH 8.7], 5.0 mM sodium hydrogen sulfite, and polyvinylpyrrolidone at 0.2 g/ml) solution was added. After thoroughly mixing for 1 min using a vortex, the suspension was centrifuged at 9,000 × g for 15 min at 4°C. The supernatant was collected as enzyme extract.

PRX enzyme activity was measured using a substrate mixture containing 790 µl of 100 mM acetate buffer (pH 5.4) and 100 µl of 0.25% guaiacol (in ethyl alcohol), and 100 µl of 0.5 M H₂O₂ was

added to 10 µl of enzyme extracts. Changes in absorbance at 470 nm were recorded after 10 min and the activity of PRX was expressed as change in absorbance at 470 nm (ΔA_{470}) per milligram of protein per minute. PPO enzyme activity was measured using a substrate mixture containing 880 µl of 50 mM potassium phosphate buffer (pH 6.8), 100 µl of 0.5 ml of 15 mM catechol, and 20 µl of enzyme extracts. Following incubation at room temperature for 10 min, changes in absorbance at 420 nm were recorded spectrophotometrically and the activity of PPO was expressed as change in absorbance at 420 nm (ΔA_{420}) per milligram of protein per minute. The protein assay was performed using a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). For all samples, enzyme activity at each time-point was compared with that of nonamended *M. oryzae* inoculated control plant leaf tissue enzyme activity at 0 hpi. All experiments were repeated once. Data from each experiment were subjected to ANOVA using MINITAB (version 16.1.0; Minitab Inc.). Multiple comparisons of means were made using the Student-Newman-Keuls multiple range test ($P \leq 0.05$).

RNA extraction and analysis of gene expression using quantitative real-time polymerase chain reaction. Procedures used for collection, storage, and analyses of leaf samples were identical, as described previously (Rahman et al. 2014). Briefly, total RNA was isolated using 1.0 ml of TRIzol reagent (Life Technologies, Grand Island, NY) from 0.1 g of leaf tissue following the manufacturer's instructions. Two-step real-time polymerase chain reaction (PCR) was performed using the Applied Biosystems 7300 Real-Time PCR System (Life Technologies). An aliquot of 2 µg of total RNA was treated with DNase I (NEB, Ipswich, MA) followed by generation of cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies) with oligo (dT) primers, according to the manufacturer's instructions. To quantify altered gene expression, specific primers for four perennial ryegrass genes—*PALa*, *PALb*, lipoxygenase (*LOXa*), and stably expressed eukaryotic elongation factor 1 α (*eEF1A(s)*)—were used for each cDNA sample (Table 1). Each primer pair was chosen based on the amplification efficiency between a target and reference gene *eEF1A(s)*, which was determined as described by Livak and Schmittgen (2001). The specification of each pair of primers was also confirmed by randomly sequencing PCR products. The real-time quantitative PCR was conducted using the SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies). PCR reactions were carried out in triplicate in 96-well plates. After denaturation at 95°C for 15 min, a two-step amplification occurred: 15 s of denaturation at 95°C, 30 s of annealing at 60°C, and 30 s of extension at 72°C, with a total of 45 cycles. Identical thermal cycling was used for all targets. The transcript levels were normalized against perennial ryegrass *eEF1A(s)* gene. For each gene, the sample value at the 0 h time of nonamended healthy plants was defined as the calibrator sample (Livak and Schmittgen 2001). Average threshold cycle (Ct) normalized to the *eEF1A(s)* transcript for each condition as $2^{-\Delta Ct}$ and fold changes were calculated as $2^{-\Delta \Delta Ct}$ (Livak and Schmittgen 2001). Negative controls lacking cDNA were routinely included. Melting curve analysis of the amplification products was performed at the end of each PCR reaction to ensure that a single PCR

was detected. All experiments were performed three times. Data were analyzed using MINITAB (version 16.1.0; Minitab Inc.) for ANOVA followed by means comparison at each time point using Tukey's HSD test ($P \leq 0.05$).

Extraction, identification, and quantification of plant leaf phenolic metabolites. Leaf samples at five time points (0, 24, 48, 96, and 120 h relative to pathogen inoculation) were collected, weighed, and frozen in liquid nitrogen before storage at -80°C. Phenolic metabolites from plant leaf samples were extracted and analyzed as described previously (Wallis and Chen 2012). An aliquot of 100 mg fresh weight of leaf tissue was ground to powder in liquid nitrogen with a mortar and pestle, followed by an overnight extraction in 100% methanol (0.5 ml) at 4°C. The extract was then centrifuged at 12,000 $\times$ g for 15 min. The pellet was reextracted and supernatants from both extractions were combined together. All extracts were then filtered through a 0.45-µm polyvinylidene difluoride membrane (PALL Life Sciences, New York) and stored at -80°C until analysis by high-performance liquid chromatography (HPLC). Three independent extractions were carried out for analysis from each of the two independent experiments.

For HPLC analysis, 50 µl of each sample was injected in a Shimadzu (Columbia, MD) LC-20AD dual-pump system equipped with an SIL-20A HT auto-sampler cooled to 4°C, a Shimadzu Shim-Pack XR-ODS 4.6-by-100-mm column in a CTO-20 column oven set at 50°C, and an SPD-20A photodiode array detector. A step-gradient elution was performed using water with 2% (vol/vol) acetic acid (Sigma-Aldrich) as solvent A and methanol with 2% (vol/vol) acetic acid as solvent B, with a flow rate of 1.0 ml/min. Separation conditions were an initial 2-min hold of 95% solvent A, changing to 52.5% solvent B over the next 20 min, followed by 100% solvent B during the next 10 min and holding for 3 min before reequilibration at 95% solvent A for 5 min to prepare for the next run. The phenolic constituents in extracts of plant leaves were monitored with SPD-20A scanning at 280 and 320 nm. Different peaks were putatively identified by matching against UVOL/VOLis spectra, relative retention times, and masses that were determined by running each sample on a Shimadzu LCMS-2020 HPLC mass spectrometer using the same gradient program as that of HPLC analyses, thus making the comparison of chromatograms and spectra completely reliable. The LC mass spectrometry analysis used an electrospray ionization probe and masses for each peak matched with previously identified plant derived phenolic compounds (Wallis and Chen 2012). Identification of 3-*O*-caffeoylquinic acid (CQA) (neochlorogenic acid), 5-*O*-CQA (chlorogenic acid [CGA]), quinic acid, and ellagitannin were made by matching retention times, UVOL/VOLis spectra, and masses with those of authentic standards. All other phenolic compounds remain to be putatively identified. In order to quantify compounds, a standard curve was generated from authentic standards at an appropriate wavelength. For putatively identified phenolic compounds, flavonol hexoside was quantified using a standard curve of quercetin-3- β -D-glucoside, flavonol cinnamate using kaempferol, and lignan cinnamate using chichoric. Differences in phenolic compounds accumulated between Si-amended and nonamended plants at 24, 48, and 96 hpi were determined using Student's *t* test ($P \leq 0.05$). Other data were analyzed using MINITAB (version 16.1.0; Minitab Inc.) for ANOVA followed

TABLE 1. DNA primers used to assay the gene expression by quantitative real-time polymerase chain reaction

Gene	Primer	Primer sequence (5'–3')	Product size (base pair)	Reference
<i>PALa</i>	Forward	GCTTGAAGGGTGCTGAGATT	131	Sawbridge et al. 2003
	Reverse	AGAGATGAGACCGAGAGAGTTG	...	...
<i>PALb</i>	Forward	GCCGAGTTAGAGCAGAACATC	110	Sawbridge et al. 2003
	Reverse	CGGCATTCTTGATCCTGTT	...	...
<i>LOXa</i>	Forward	TCGCTGAATCGCTTGAACA	136	Eaton et al. 2010
	Reverse	TCCAGACCATCATCGGGATA	...	...
<i>eEF1As</i>	Forward	CCGTTTTGTGCGAGTTTGGT	113	Lee et al. 2010
	Reverse	AGCAACTGTAACCGAACATAGC	...	...

by means comparison using the Student-Newman-Keuls multiple range test ($P \leq 0.05$).

RESULTS

Effect of Si on gray leaf spot incidence and severity. Different rates of calcium silicate (1, 5, and 10 t/ha) application varied considerably in suppressing gray leaf spot incidence in perennial ryegrass. Although calcium silicate at rates of 1 and 5 t/ha significantly suppressed disease incidence compared with non-amended control ($P = 0.0049$ and 0.0005 , respectively), calcium silicate at 10 t/ha was insignificant ($P = 0.3586$) (Fig. 1). Additionally, calcium silicate at 5 t/ha was significantly more efficient than at 1 and 10 t/ha ($P = 0.0285$ and 0.0009 , respectively) while an application rate of 1 t/ha was significantly better than 10 t/ha ($P = 0.0057$) in suppressing gray leaf spot incidence (Fig. 1).

Disease severity at a calcium silicate rate of 1 and 5 t/ha was significantly ($P = 0.0004$ and $P < 0.0001$, respectively) lower, but not at 10 t/ha ($P = 0.2804$), compared with that of nonamended control plants (Fig. 1). Furthermore, calcium silicate at 5 t/ha was significantly more efficient in reducing disease severity than at 1 and 10 t/ha ($P < 0.0001$ in both cases) while an application rate of 1 t/ha exhibited significant better suppression of gray leaf spot than that of 10 t/ha ($P = 0.0014$) (Fig. 1).

Fig. 1. Effects of different rates of calcium silicate amendments (1, 5, and 10 t/ha) in Fafard mix number 2 on gray leaf spot incidence (DI) and severity (DS) development in perennial ryegrass in a controlled-environment chamber study.

Fig. 2. Representative images of aniline-blue-stained callose deposition inside cell-wall appositions (CWAs) during attempted penetration by conidia of *Magnaporthe oryzae* on perennial ryegrass epidermal cells at 48 h postinoculation (hpi). Nonpenetrated papillae showing localized deposition of callose beneath the attempted penetration sites as seen by epifluorescent microscopy with **A**, white light and **B**, UV filter. Penetrated callose deposited papilla on the epidermal cell, as viewed by epifluorescent microscopy with **C**, white light and **D**, UV filter, in which *M. oryzae* has established intracellular hyphae (IH). Bars = 20 μ m. Ap = appressorium. **E**, Frequency of callose deposition inside CWAs at the attempted fungal penetration sites in leaf epidermal cells of 4-week-old perennial ryegrass with and without Si amendments at different time intervals. In all, 75 to 100 single epidermal cell–conidia interactions were examined per replication. Values represent the means $\pm$ standard deviation. Presence of asterisks indicates significant differences among plants using Tukey's honest significant difference test ($P < 0.05$).

Because Si at the rate of 5 t/ha (6.48 mg/cm^3) exhibited the maximum level of protection against *M. oryzae*, this concentration was used throughout the rest of the study for analyzing mechanism of host defense in perennial ryegrass.

Cytological analyses of the perennial ryegrass defense responses against *M. oryzae*. *Callose deposition.* Callose deposition was observed in both Si-amended and nonamended plants by 24 hpi with no significant difference (Fig. 2E). However, a significantly higher proportion of callose deposition was observed in perennial ryegrass leaves with Si amendment by 48 and 72 hpi, compared with that of nonamended control plants ($P < 0.05$) (Fig. 2E). Furthermore, a significantly higher proportion of all callose-deposited sites remained nonpenetrated by the fungal penetration peg in Si-amended plants (79% sites remained nonpenetrated) compared with that of nonamended control plants (35.6% remained nonpenetrated) after 48 hpi ($P < 0.05$) (data not shown).

Accumulation of phenolic or polyphenolic compounds. Auto-fluorescent phenolic compounds were found to accumulate at attempted pathogen entry sites or whole cell autofluorescence following infection with *M. oryzae* in both nonamended and Si-amended plants (Fig. 3A–C). Although autofluorescence was detectable regardless of the treatment, a significantly higher percentage of interaction sites in Si-amended plants exhibited augmented deposition of phenolic compounds at 24 and 48 hpi compared with that of nonamended control plants ($P < 0.05$) (Fig. 3D). The early appearance of autofluorescence surrounding the appressorium (Fig. 3D) in Si-amended plant epidermal cells indicated a rapid development of cell wall appositions (CWAs) harboring phenolic compounds. Such CWAs containing phenolic or polyphenolic compounds were also detected using toluidine blue staining (Fig. 4A–D). By 48 and 72 hpi, significantly more CWAs exhibited toluidine blue positive lignin or polyphenolic deposition in Si-amended treated perennial ryegrass compared with that of non-amended control plants ($P < 0.05$) (Fig. 4E). Also, more enhanced and stronger deposition of lignin or polyphenolic compounds was observed in Si-amended plants (Fig. 4A–B) compared with the nonamended controls (Fig. 4C and D).

Enzyme activity. Induction of PRX activity was observed within 24 h of *M. oryzae* inoculation in Si-amended inoculated plants compared with nonamended inoculated control plants, becoming significant at 48 hpi ($P < 0.05$) (Fig. 5A). However, for nonamended inoculated control plants, PRX activity became significantly elevated at 72 hpi ($P < 0.05$). On the other hand, in

Si-amended inoculated plants, the onset of elevated PPO activity initiated at 24 hpi and remained significantly greater throughout rest of the study period ($P < 0.05$) (Fig. 5B). However, nonamended inoculated control plants exhibited a delayed onset, not before 48 hpi, of PPO activity compared with Si-amended inoculated plants (Fig. 5B). Although PPO activity continued to be elevated up to 96 hpi in nonamended inoculated control plants, enzyme activity never reached the same level as that of Si-amended inoculated plants (Fig. 5B). Neither of the enzymes exhibited any increase of activity in Si-amended noninoculated control perennial ryegrass plants.

Defense-related gene expression analysis. Basal levels of all three defense genes (*LpPALa*, *LpPALb*, and *LpLOXa*) before pathogen inoculation ($t = 0$) were found to be low, without any significant difference among experimental plants.

In the present study, a rapid induction of both *PALa* and *PALb* (Fig. 6A) was associated with Si-amended (4.3- and 3.9-fold, respectively) inoculated plants at 24 hpi as compared with non-amended inoculated plants. Although relative expression of *PALb* was more significant in Si-amended inoculated plants than non-amended inoculated plants at 24 hpi, *PALa* was significantly elevated only at 48 hpi ($P < 0.05$). By 72 hpi, relative expression levels of both genes were comparable in all plants with no significant difference. An induction of *LOXa* expression was observed in Si-amended inoculated plants by 48 hpi, which was more than 3.5-fold higher in comparison with that of nonamended control plants ($P < 0.05$) (Fig. 6B). By 72 hpi, however, the relative expression level of *LpLOXa* was comparable among all the treatments.

Phenolic acid and flavonoid expression analyses. Several phenolics and flavonoids were putatively identified in the methanol extracts of perennial ryegrass leaves (Table 2) based on UVOL/VOL is spectra, relative retention times, and masses, as previously described by Wallis and Chen (2012).

In the control, noninoculated plants, no significant changes in any of the phenolic acids or flavonoids identified were noticed at 0 and 120 h following pathogen challenge (Table 3). On the other hand, a significant ($P < 0.05$) decrease in the concentration of all four phenolic acids—namely, neochlorogenic acid (3-*O*-CQA), neochlorogenic acid isomer 1, CGA (5-*O*-CQA), and CGA derivative 1—was observed for nonamended inoculated plants at 120 hpi compared with control noninoculated and Si-amended inoculated plants (Table 3). Apart from CGA, the rest of the phenolic acid concentrations were replenished by 120 hpi in Si-amended inoculated plants to the level similar to or greater than that of 0 hpi (basal level). Although the concentration of CGA at 120 hpi in Si-amended inoculated plants was significantly lower compared with that of 0 hpi nonamended noninoculated, Si-amended inoculated, and control inoculated plants, it was still significantly higher than that of 120 hpi in nonamended inoculated plants (Table 3). Similarly, upregulation of all phenolic acids was significantly higher by 24 hpi in Si-amended inoculated plants compared with nonamended inoculated plants, with the exception of CGA (Fig. 7).

Concentrations of different flavonoids, on the other hand, in Si-amended plants were significantly ($P < 0.05$) higher at 120 hpi compared with those of nonamended and healthy plants (Table 3). Additionally, significant ($P < 0.05$) increases of all flavonoids were observed by 24 hpi for Si-amended plants (Fig. 7). Apart from flavonol cinnamate isomer 2, which reached its summit at 96 hpi,

Fig. 3. Representative epifluorescence images of **A**, nonamended control and **B**, and **C**, Si-amended perennial ryegrass epidermal cells with attempted penetration sites by conidia of *Magnaporthe oryzae* at 24 h postinoculation (hpi). **B**, Cell wall appositions and **C**, fluorescent epidermal cells beneath appressorium (Ap) are visible. Bars = 20 μ m. **D**, Frequency of autofluorescent phenolics deposition at attempted penetration sites by conidia of *M. oryzae* in leaf epidermal cells of 4-week-old perennial ryegrass with and without Si amendment at different time intervals. In all, 75 to 100 single epidermal cell–conidia interactions were examined per replication. Values represent the means $\pm$ standard deviation. Presence of asterisk indicates significant differences among plants using Tukey's honest significant difference test ($P < 0.05$).

Fig. 4. Representative images of toluidine blue staining for lignin-associated polyphenolics inside cell-wall appositions and cell walls of epidermal cells with attempted penetration sites by conidia of *Magnaporthe oryzae* at 24 h postinoculation (hpi). **A**, White light and **B**, UV filter images from nonamended control plants; **C**, white light and **D**, UV filter images from Si-amended perennial ryegrass leaf epidermal cells infected by *M. oryzae*. Bars = 20 μ m and Ap = appressorium. **E**, Frequency of lignin-associated polyphenolics deposition at the attempted penetration sites by conidia of *M. oryzae* on leaf epidermal cells of 4-week-old perennial ryegrass with and without Si amendments at different time intervals. In all, 75 to 100 single epidermal cell–conidia interactions were examined per replication. Values represent the means $\pm$ standard deviation. Presence of asterisks indicates significant differences among plants using Tukey's honest significant difference test ($P < 0.05$).

the rest of the flavonoids reached their peak by 24 hpi and gradually tapered off (Fig. 7). No significant ($P > 0.05$) difference in the concentration of quinic acid or ellagitannin was observed among the plants (data not shown).

DISCUSSION

Although calcium silicate has been demonstrated previously by Nanayakkara et al. (2008) to suppress gray leaf spot development, this is the first time Si-mediated suppression of *M. oryzae* in perennial ryegrass was elucidated in depth. Suppression of disease incidence and severity appeared to be significantly higher at a rate of calcium silicate at 5 t/ha compared with 1 and 10 t/ha. On the other hand, 10 t/ha was found not to be effective in suppressing the disease compared with that of nonamended control. The Si formulation used in this study was calcium magnesium silicate, and it is not known whether or not higher concentrations (>5 t/ha) have any detrimental effect on plant physiology due to high alkalinity of this Si formulation. It is possible that, at a high concentration, this Si formulation might increase the pH of Fafard mix number 2 (pH 6.3) (Nanayakkara et al. 2008). High alkalinity of Si formulations is not uncommon and has been noted by others (Shetty et al. 2012). Notably, perennial ryegrass (4 week old) growing on soil mix amended with calcium silicate at 10 t/ha was visibly stunted in

growth compared with plants amended with calcium silicate at 1 and 5 t/ha (Fig. 8).

Previously, we reported that deposition of impenetrable papillae or CWAs at the attempted appressorial penetration sites on perennial ryegrass leaf epidermal cells plays an important role as a preinvasive defense response against *M. oryzae* (Rahman et al. 2014, 2015). Deposited callose at the infection sites is known to either prevent penetration of an appressorial penetration peg (Fig. 2A and B) or form a haustorial collar neck (Fig. 2C and D) following penetration by impeding fungal structure (Zeyen et al. 2002). One of the most prominent constituents in CWAs is callose (β -1,3-glucan), which is thought to play a key role in encasing fungal invasive

Fig. 5. Time course of **A**, peroxidase and **B**, polyphenol oxidase enzyme activity in Si-amended (Si+) and nonamended (Si-) perennial ryegrass leaf tissue followed by inoculation with *Magnaporthe oryzae* (M+) at different time intervals. Si-amended (Si+) but noninoculated (M-) healthy control is also included. Values represent the means $\pm$ standard deviation. Presence of asterisk indicates significant differences compared with that of nonamended *M. oryzae* inoculated control plant leaf tissue enzyme activity at 0 h postinoculation using the Student-Newman-Keuls test ($P < 0.05$).

Fig. 6. Expression pattern of perennial ryegrass **A**, *PALa*, **B**, *PALb*, and **C**, *LOXa* genes following inoculation with *Magnaporthe oryzae*. Solid white bars = nonamended control; black bars = Si-amended plants. Each bar represents average relative expression ($\pm$ standard deviation) of PR genes normalized to the housekeeping gene *eEF1A(s)* for three replicates. Fold changes were calculated by calibrating data to the 0 h postinoculation of healthy control sample. Presence of asterisk indicates significant differences between Si-amended and nonamended control plants using Tukey's honest significant difference test ($P < 0.05$).

structures, thereby limiting water and nutritional availability (Huckelhoven 2007). However, by 24 hpi, nonamended and Si-amended plants exhibited similar rates of CWAs deposition, and Si-amended plants exhibited continued CWA deposition as the time progressed, with significantly higher frequencies of papillae deposited by 72 hpi compared with nonamended plants (Fig. 2). *M. oryzae* has been documented to initiate penetration through an appressorial infection peg into the first leaf epidermal cell after 24 h following landing and secure attachment to the leaf surface in grass species, including rice (Parker et al. 2009), *Brachypodium distachyon* (Routledge et al. 2004), and goosegrass (*Eleusine indica* L.) (Heath et al. 1990). Furthermore, the percentage of intracellular hyphae (IH) generated from appressorium was found to be similar between 48 and 72 hpi but always higher than that at 24 hpi (Heath et al. 1990), suggesting that blocking IH development plays an important role in these grass species' defense against fungal invasion. Although papilla-associated penetration resistance in response to *M. oryzae* infection is not commonly observed in rice (Heath et al. 1990; Rodrigues et al. 2005), appressorial penetration resistance via Si deposited in the rice leaf epidermis cell wall has been documented (Hayasaka et al. 2008; Kim et al. 2002). On the other hand, *B. distachyon* (Routledge et al. 2004) and goosegrass (Heath et al. 1990, 1992) leaf epidermal cells exhibited papilla-associated penetration resistance in response to *M. oryzae* infection similar to that observed in our study. Deposition of Si inside penetration resistant papillae has been observed in barley–*Blumeria graminis* f. sp. *hordei* (Zeyen et al. 2002) and goosegrass–*M. oryzae* interactions. The remarkable similarity between the insoluble biogenic opal silica ($\text{SiO}_2 \cdot n\text{H}_2\text{O}$) containing papillae in the Si-amended barley–*B. graminis* f. sp. *hordei* interaction (Zeyen et al. 2002) and Si-amended perennial ryegrass–*M. oryzae* interaction

(Fig. 2B) suggests similar deposition of Si inside the CWAs in Si-amended plants.

Accumulation of autofluorescent phenolic compounds inside CWAs or development of fluorescent epidermal cells following fungal infection have been demonstrated to be associated with plant disease resistance (Faivre-Rampant et al. 2008; Shetty et al. 2012). As a hemibiotrophic pathogen, *M. oryzae* proliferates biotrophically during the first 48 h of infection and later switches to necrotrophic growth by 72 hpi, which leads to the initiation of necrosis of the primary infected cell (Parker et al. 2009), resulting in the degradation of cell components and giving rise to whole cell autofluorescence. Our current study supports this, because significant accumulation of autofluorescent phenolic compounds was observed in Si-amended plants at 24 and 48 hpi, thereby preventing fungal progression compared with nonamended control plants (Fig. 3D).

In Si accumulator plants such as barley, rice, and wheat, Si has been found to be absorbed as monosilicic acid and later polymerizes into silica gel (Zeyen et al. 2002). Furthermore, monosilicic acid exhibits a strong affinity for organic polyhydroxyl compounds such as ortho-diphenols in cell wall lignification during maturation and pathogen attack (Diogo and Wydra 2007). In an attempt to identify lignin-associated polyphenols in deposited CWAs, leaf blades were stained with toluidine blue following *M. oryzae* inoculation (Mellersh et al. 2002; O'Brien et al. 1964). Deposition of similar lignin-associated polyphenolic compounds in CWA was found to be effective in thwarting *M. oryzae* progression in perennial ryegrass epidermal cells in our previous study (Rahman et al. 2015). Therefore, the observation that significantly higher accumulation of lignin-associated polyphenolic compounds in CWAs in Si-amended plants than in nonamended control plants provides another facet of

TABLE 2. Phenolic compounds quantified in the methanol extracts from Si-amended and nonamended perennial ryegrass leaves using high-performance liquid chromatography^x

Compound	Retention time (min)	UV/Vis max	Mass
Quinic acid ^y	1.97	276	192
Neochlorogenic acid (3- <i>O</i> -caffeoylquinic acid) ^y	6.40	280, 324	354
Neochlorogenic acid isomer 1 ^z	7.07	280, 325	354
Chlorogenic acid (5- <i>O</i> -caffeoylquinic acid) ^y	7.50	295, 326	354
Chlorogenic acid derivative 1 ^z	11.68	312	433, 354, 168
Flavonol hexoside ^z	12.99	265, 339	610
Lignan cinnamate ^y	14.65	265, 346	696
Ellagitannin ^y	16.58	265, 337	812
Flavonol cinnamate (kaempferol cinnamate hexoside) ^y	16.90	265, 337	594
Flavonol cinnamate isomer 1 ^z	20.14	264, 336	772
Flavonol cinnamate isomer 2 ^z	20.63	265, 342	534
Flavonol cinnamate isomer 3 ^z	21.35	267, 333	858

^x Putative identifications were made based on matching of UV/Vis spectra (λ_{max}) or masses from liquid chromatography–mass spectrometry (LC-MS).

^y Identified by comparison with authentic standard and based on comparison of retention time, UV, and LC-MS data from the literature (Wallis and Chen 2012).

^z Tentatively identified by UV and mass spectral data.

TABLE 3. Individual phenolic compound mean levels present in perennial ryegrass leaves at 0 and 120 h post inoculation with *Magnaporthe oryzae*^y

Compound	Mean ($\pm$ standard deviation) ($\mu\text{g/g}$ fresh weight)					
	Control ^z		Nonamended		Si-amended	
	0 h	120 h	0 h	120 h	0 h	120 h
Neochlorogenic acid	38.31 $\pm$ 4.05 a	36.95 $\pm$ 6.29 a	36.55 $\pm$ 4.97 a	11.01 $\pm$ 3.32 b	33.54 $\pm$ 4.59 a	33.51 $\pm$ 2.04 a
Neochlorogenic acid isomer 1	107.16 $\pm$ 17.93 a	86.58 $\pm$ 12.12 a	119.77 $\pm$ 16.99 a	13.24 $\pm$ 4.03 b	91.86 $\pm$ 23.3 a	61.07 $\pm$ 10.06 a
Chlorogenic acid	32.37 $\pm$ 7.99 a	23.51 $\pm$ 7.09 a	49.32 $\pm$ 10.37 a	4.70 $\pm$ 1.6 c	45.01 $\pm$ 9.03 a	18.66 $\pm$ 4.57 b
Chlorogenic acid derivative 1	10.43 $\pm$ 2.22 a	15.63 $\pm$ 2.98 a	11.27 $\pm$ 2.28 a	3.59 $\pm$ 0.85 b	10.32 $\pm$ 2.56 a	12.95 $\pm$ 1.31 a
Flavonol hexoside	18.74 $\pm$ 4.43 a	18.05 $\pm$ 1.82 a	23.72 $\pm$ 6.35 a	11.27 $\pm$ 4.46 a	12.56 $\pm$ 5.75 a	33.36 $\pm$ 1.18 b
Lignan cinnamate	2.03 $\pm$ 1.65 a	1.73 $\pm$ 1.05 a	3.85 $\pm$ 1.29 a	1.33 $\pm$ 0.73 a	3.82 $\pm$ 0.33 a	19.54 $\pm$ 2.51 b
Flavonol cinnamate isomer 1	3.67 $\pm$ 0.36 a	2.19 $\pm$ 2.17 a	6.43 $\pm$ 4.9 a	5.06 $\pm$ 1.72 a	5.52 $\pm$ 3.69 a	70.57 $\pm$ 11.5 b
Flavonol cinnamate isomer 2	4.92 $\pm$ 4.7 a	3.03 $\pm$ 0.51 a	7.56 $\pm$ 6.6 a	8.12 $\pm$ 3.77 a	2.38 $\pm$ 0.76 a	75.62 $\pm$ 5.94 b
Flavonol cinnamate isomer 3	1.80 $\pm$ 1.36 a	1.97 $\pm$ 0.4 a	3.78 $\pm$ 2.9 a	2.27 $\pm$ 0.99 a	2.13 $\pm$ 0.8 a	44.56 $\pm$ 6.28 b

^y Numbers followed by different letters within a row are significantly different according to the Student-Newman-Keuls multiple range test ($P < 0.05$).

^z Healthy noninoculated control perennial ryegrass plants.

Fig. 7. Mean ($\pm$ standard deviation) concentrations ($\mu\text{g/g}$ fresh weight [FW]) of **A**, neochlorogenic acid; **B**, neochlorogenic acid isomer 1; **C**, chlorogenic acid; **D**, chlorogenic acid derivative 1; **E**, flavonol hexoside; **F**, lignin cinnamate; **G**, flavonol cinnamate isomer 1; **H**, flavonol cinnamate isomer 2; and **I**, flavonol cinnamate isomer 3 in Si-amended (Si+) and nonamended (Si-) perennial ryegrass leaves at 0, 24, 48, and 96 h postinoculation (hpi) of *Magnaporthe oryzae* (MO+). Presence of asterisks indicates significant differences among plants using Student's *t* test ($P < 0.05$).

Fig. 8. Perennial ryegrass plants grown in Fafard mix number 2 with and without calcium silicate amendments; **A**, nonamended healthy plants; **B**, 1 t/ha; **C**, 5 t/ha; and **D**, 10 t/ha amended plants.

Si-mediated CWA-associated penetration resistance in perennial ryegrass (Fig. 4E).

PRX and PPO have been implicated in the generation of phenolic antimicrobial compounds and lignification of plant cells during microbial invasion and cellular protection (Mohammadi and Kazemi 2002). In forage plants, including perennial ryegrass, members of hydroxycinnamic acids such as 3-, 4-, and 5-CQA are considered to be precursor molecules in the generation of *ortho*-quinones (antimicrobial compound) by PPO through a multistep oxidative pathway (Parveen et al. 2010). Both PRX and PPO have been implicated in the production of monolignol radicals which are subsequently cross-linked to generate lignin subunits through oxidative coupling during microbial invasion (Huttermann et al. 2001; Mohammadi and Kazemi 2002; Parveen et al. 2010). Significant increase of activity of both PRX and PPO in Si-amended perennial ryegrass following *M. oryzae* inoculation (Fig. 5), together with a strong affinity of Si for *ortho*-diphenolic compounds (Diogo and Wydra 2007), suggested that Si might play an important role in the augmented host defense. Additionally, in our previous study, increased PRX activity in leaf tissue was found to be associated with gray leaf spot suppression in perennial ryegrass (Rahman et al. 2015).

To investigate the resistance mechanisms at a molecular level, gene expression of selected defense-related genes *LpPALa*, *LpPALb*, and *LpLOXa* was analyzed using real-time quantitative reverse-transcription PCR. PAL is the key enzyme in the phenylpropanoid pathway; both *PALa* and *PALb* were significantly up-regulated in Si-amended perennial ryegrass plants compared with nonamended control plants by 48 hpi (Fig. 6A and B). Induced expressions of *PAL* have been reported to be associated with suppression of gray leaf spot in perennial ryegrass (Rahman et al. 2015). Octadecanoid pathway, initiated by *LOX* (13-lipoxygenase), is another major defense-related metabolic process that leads to the generation of jasmonic acid (JA)-mediated defense response in plants (Kachroo and Kachroo 2012). Significantly faster and higher induction of *LOX* in Si-amended perennial ryegrass following *M. oryzae* inoculation suggests the initiation of such metabolic pathway as a result of robust defense response activation (Fig. 6C). Collectively, these gene expression analyses indicate that plants grown in Si-amended soil and challenge inoculated with *M. oryzae* exhibit an attacker-induced enhanced expression of defense-related genes. Recently, salicylic acid pathway-independent resistance of *Arabidopsis* against powdery mildew (Vivancos et al. 2014) and JA-responsive antiherbivore defense in rice (Ye et al. 2013) were demonstrated to be associated with Si supplement. Based on these recent findings, together with the gene expression analyses in current study, it is tempting to speculate that Si might function as a modulator of JA-mediated defense response pathways in perennial ryegrass to restrict *M. oryzae* proliferation. However, further analysis of JA-responsive marker genes as well as quantification of endogenous JA in perennial ryegrass would be necessary to confirm such a hypothesis.

Si-mediated resistance of rice against blast disease has also been found to be associated with a rapid accumulation of phenolics and phytoalexins (Rodrigues et al. 2003, 2004, 2005). In this study, Si-primed perennial ryegrass was also found to exhibit increased production of many but not all phenolic compounds, including neochlorogenic acid (3-*O*-CQA) and CGA (5-*O*-CQA), compared with the nonamended control (Fig. 7; Table 3). Notably, CGA has been suggested to be an endogenous substrate for PPO in perennial ryegrass, due to its relatively high basal level compared with other forage plant species, for generating quinones and semiquinones, which are powerful antioxidants with potential toxic properties against microbial invasion (Lattanzio et al. 2012; Parveen et al. 2010; Shetty et al. 2012). Simultaneous upregulation of both PPO activity and CGA expression following pathogen challenge in Si-amended perennial ryegrass strongly indicates their synergistic activity in suppressing of *M. oryzae* proliferation, possibly through the generation of toxic phenolic compounds. Recently, in switchgrass

(*Panicum virgatum* L.), which also has high basal levels of CGA, *p*-coumaroyl Co-A (a precursor in lignin biosynthesis) together with quinic acid were found to serve as substrates for CGA biosynthesis (Escamilla-Treviño et al. 2014). Given that the CGA pool at 120 hpi in Si-amended plants was significantly lower than that of the basal level (0 hpi) (Table 3), mobilization of CGA into other induced resistance-related metabolic pathway is plausible. Therefore, it is conceivable that Si amendment has resulted in a primed state that resulted in augmented accumulation of defense-related antimicrobial-phenolics or lignin-associated polyphenolic compounds through the enhanced expression of PAL and PPO activity following pathogen attack, thereby suppressing gray leaf spot development in perennial ryegrass.

In addition to CGA, another phenolic compound class called flavonoids was also primed by Si for enhanced expression following pathogen challenge. Flavonoids were observed to significantly inhibit *M. oryzae* (Lattanzio et al. 2006) and *Podosphaera pannosa* (Shetty et al. 2011) spore germination. Similar inhibition of *M. oryzae* in Si-amended perennial ryegrass by the putatively identified flavonol cinnamate isomers could be anticipated, although definite identification and purified extracts of these flavonol compounds would be necessary to conclusively prove such assumptions.

In conclusion, Si evidently plays a substantive role in perennial ryegrass defense responses against *M. oryzae* by delaying fungal ingress and colonization. These results demonstrate that Si does this by activating additional defense mechanisms, notably through induction of defense-related enzymes and phenolics, which assist in the host overcoming fungal infection. Therefore, use of Si in an integrated disease management program along with conventional chemical fungicides holds an untapped potential for gray leaf spot management on perennial ryegrass.

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