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Influence of Infection of Cotton by *Rotylenchulus Reniformis* and *Meloidogyne Incognita* on the Production of Enzymes Involved in Systemic Acquired Resistance

Sudarshan K. Aryal,[1] Richard F. Davis,[2] Katherine L. Stevenson,[1] Patricia Timper,[2] Pingsheng Ji[1]

Abstract: Systemic acquired resistance (SAR), which results in enhanced defense mechanisms in plants, can be elicited by virulent and avirulent strains of pathogens including nematodes. Recent studies of nematode reproduction strongly suggest that *Meloidogyne incognita* and *Rotylenchulus reniformis* induce SAR in cotton, but biochemical evidence of SAR was lacking. Our objective was to determine whether infection of cotton by *M. incognita* and *R. reniformis* increases the levels of P-peroxidase, G-peroxidase, and catalase enzymes which are involved in induced resistance. A series of greenhouse trials was conducted; each trial included six replications of four treatments applied to one of three cotton genotypes in a randomized complete block design. The four treatments were cotton plants inoculated with i) *R. reniformis,* ii) *M. incognita*, iii) BTH (Actigard), and iv) a nontreated control. Experiments were conducted on cotton genotypes DP 0935 B2RF (susceptible to both nematodes), LONREN-1 (resistant to *R. reniformis*), and M-120 RNR (resistant to *M. incognita*), and the level of P-peroxidase, G-peroxidase, and catalase activity was measured before and 2, 4, 6, 10, and 14 d after treatment application. In all cotton genotypes, activities of all three enzymes were higher ($P \leq 0.05$) in leaves of plants infected with *M. incognita* and *R. reniformis* than in the leaves of control plants, except that *M. incognita* did not increase catalase activity on LONREN-1. Increased enzyme activity was usually apparent 6 d after treatment. This study documents that infection of cotton by *M. incognita* or *R. reniformis* increases the activity of the enzymes involved in systemic acquired resistance; thereby providing biochemical evidence to substantiate previous reports of nematode-induced SAR in cotton.

Key words: BTH, catalase, *Meloidogyne incognita,* peroxidase, reniform nematode, root-knot nematode, *Rotylenchulus reniformis*, systemic acquired resistance.

Meloidogyne incognita (the southern root-knot nematode) and *Rotylenchulus reniformis* (the reniform nematode) are two major root parasites attacking cotton across the U.S. production belt (Robinson and Cook, 2001). Both nematodes may be present in the same field, and when *Meloidogyne* and *Rotylenchulus* are feeding on the same host, the interaction can be antagonistic for either nematode (Singh, 1976; Kheir and Osman, 1977; Taha and Kassab, 1980; Mishra and Gaur, 1981; Thomas and Clark, 1981; Stetina et al., 1997). In cotton, concomitant infection with *M. incognita* and *R. reniformis* reduced the population density of the species that was applied at the lower initial inoculum level (Diez et al., 2003). Most of these studies proposed competition as a mechanism to explain the antagonistic interaction between *M. incognita* and *R. reniformis*. In contrast, a recent study concluded that prior infection of cotton with either *R. reniformis* or *M. incognita* can elicit enhanced defense against the other species through the induction of systemic acquired resistance (SAR) (Aryal et al., 2011).

Induced resistance is the physiological state of enhanced defensive response by the plant which is stimulated by specific environmental stimuli that provides both qualitative and quantitative expression of defense mechanisms against subsequent infections (Van Loon, 1997). Systemic acquired resistance is a type of induced resistance that involves the salicylic acid (SA)-mediated signaling pathway (Van Loon et al., 2006) that enhances the natural defense systems of plants and provides broad spectrum of resistance to a range of pathogens including plant-parasitic nematodes. Onset of SAR requires the accumulation of SA, which operates in the signaling pathway for plant defense, and the systemic or coordinated expression of pathogenesis related (PR) proteins (Ward et al., 1991; Hammerschmidt, 1999; Sticher et al., 1999). The expression of SAR does not require the presence of pathogen-specific resistance genes, although the defense mechanisms activated are those used in other forms of plant resistance to pathogens (Kuc, 1982; Heath, 2000; Walters et al., 2005).

Systemic acquired resistance against *M. hapla* in tomato (Ogallo and McClure, 1996) and *Bursaphelenchus xylophilus* in pine (Kosaka et al., 2001) has been reported following inoculation with avirulent strains of the nematodes. Similarly, an avirulent population of *M. incognita* induced SAR against a virulent *M. arenaria* in soybean (Ibrahim and Lewis 1986) and grape (McKenry and Anwar, 2007). Several abiotic compounds have been reported to induce SAR against plant-parasitic nematodes, including acibenzolar-S-methyl or benzothiadiazole (ASM or BTH), DL-α-amino-n-butyric acid (AABA), DL-β-amino-n-butyric acid (BABA), DL-γ -amino-n-butyric acid (GABA), p-aminobenzoic acid (PABA), riboflavin and salicylic acid (SA) (Kempster et al., 2001; Oka and Cohen, 2001; Chinnasri et al., 2006).

Plants have evolved an array of defense mechanisms to protect themselves against a range of pathogens and pests (Maleck and Dietrich, 1999). Systemic acquired resistance has been associated with the synthesis and post-infection accumulation of SA, PR proteins and enzymes such as peroxidase and catalase in different plants (Zacheo et al., 1983; Yu et al., 1999; Ramamoorthy et al., 2001). Accumulation of salicylic acid after pathogen infection is

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[1]Department of Plant Pathology, University of Georgia, Tifton, GA 31793
[2]USDA-ARS, Crop Protection and Management Research Unit, Tifton, GA 31793
E-mail: saryal@ufl.edu
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involved in multiple defense pathways (Pieterse and Van Loon, 1999). Application of benzo (1,2,3) thiadiazole-7-carbothioic acid (S) methyl ester (BTH), an SA analog, elicited increased-peroxidase activity both locally and systemically in cotton seedlings (Inber et al., 2001). Peroxidases and catalases are involved in the defense mechanisms of plants either by their direct participation in cell wall reinforcement or by their antioxidant role in the oxidative stress generated during plant pathogen infections (Mehdy, 1994). Our objective was to determine whether infection of cotton by *R. reniformis* or *M. incognita* increases the activity of three enzymes involved in SAR: P-peroxidase, G-peroxidase, and catalase.

Materials and Methods

Experimental plants and nematode inocula: Cotton genotypes used in these experiments were Deltapine DP 0935 B2RF, a cotton cultivar susceptible to both *M. incognita* and *R. reniformis*; LONREN-1, a germplasm line that is resistant to *R. reniformis* but susceptible to *M. incognita*; and M-120 RNR, a germplasm line resistant to *M. incognita* but susceptible to *R. reniformis*. Seedlings were grown in plastic pots (12 cm deep, 950 cm^3) containing 750 cm^3 of steam-pasteurized soil (sand 85%, silt 11%, clay 4%). Plants were allowed to grow for 4 to 5 wk until the 5 to 6 leaf stage before applying treatments. Plants were watered as needed up to twice a day. After germination, each plant was supplied with 10 g of slow releasing fertilizer (NPK-14:14:14).

Vermiform stages of *R. reniformis* or *M. incognita* were added as nematode inoculum. Both species were obtained from greenhouse cultures maintained on eggplant (*Solanum melongena* var. *esculentum*) cv. Florida Market. Second-stage juveniles of *M. incognita* were obtained from the infected roots of eggplant by the mist extraction technique (Viglierchio and Schmitt, 1983). Mixed vermiform stages of *R. reniformis* were extracted from soil by using gravity screening and centrifugal sugar flotation technique (Jenkins, 1964).

Experimental design, inoculation techniques and leaf sampling: In a greenhouse experiment, four treatments were applied to one of the three previously mentioned cotton genotypes (DP 0935, LONREN-1, and M-120 RNR) in a randomized complete block design with six replications. The four treatments were i) plants inoculated with *R. reniformis*, ii) plants inoculated with *M. incognita*, iii) plants treated with BTH (Actigard, Syngenta Crop Protection, Greensboro, NC), and iv) a non-treated control. Nematode inoculum consisting of 7,000 vermiform stages for each species was divided into three 3-cm-deep holes in the plant's root zone. Fifty milliliters of 50 ppm (50 mg a.i./liter) BTH (Actigard) was applied as a soil drench to each pot receiving the BTH treatment to serve as a positive control. In each trial, one leaf (the first true leaf) from each plant was collected immediately before applying treatments (day 0), and the oldest remaining leaf on each plant was collected 2, 4, 6, 10 and 14 d after applying treatments. The entire experiment was conducted twice.

Enzyme extraction: The activities of pyrogallol peroxidase (P-peroxidase), guaiacol peroxidase (G-peroxidase) and catalase enzymes in the leaf samples were measured using a spectrophotometer as described below, and units of protein per milligram were calculated for each enzyme. The leaf collected from each plant was rinsed with running tap water, blotted dry with a paper towel, and 1-cm leaf discs were removed using a cork borer. For each enzyme assay, a 100-mg sample of leaf tissue was homogenized in 1 ml of ice-cold (crushed ice maintained the temperature between 0 to 4°C) 0.1 M potassium phosphate buffer (pH 6.0) for P-peroxidase, 1.2 ml of 0.1 M sodium phosphate buffer (pH 6.2) for G-peroxidase, and 1.2 ml of 0.05 M potassium phosphate buffer (pH 7.0) for catalase.

P-peroxidase activity: The homogenates were centrifuged at 14,000g for 20 min at 4°C, and the supernatants (enzyme extracts) were used to determine peroxidase activity. Peroxidase activity was assessed by measuring the formation of purpurogallin from a pyrogallol substrate at 20°C (method by Sigma-Aldrich, St. Louis, MO). The reaction mixture contained 160 µl of 0.1 M potassium phosphate buffer (pH 6.0), 80 µl of .0147 M hydrogen peroxide solution, 160 µl of 5% (w/v) pyrogallol solution, and 1.05 ml of distilled H_2O (ddH_2O) in a 1.5-ml cuvette. The reaction mixture was equilibrated at 20°C using a Spectronic GENESYS 10 spectrophotometer (Spectronic Instruments Inc., Rochester, NY). The initial reference absorbance of the blank (ddH_2O) was monitored at 420 nm until it reached a constant baseline. Fifty microliters of ice-cold enzyme extract from each sample was then transferred into individual cuvette containing the reaction mixture and the absorbance at 420 nm was recorded every 20 sec for 3 min. The sum of the change in absorbance was used to calculate the units of protein per milligram using the following formula (Chance and Maehly, 1955):

$$\text{Units/mg solid} = \frac{\Delta A420\text{nm}/20\,\text{sec}}{12 \times (\text{mg enzyme as solid/ml reaction mix})}$$

G-peroxidase activity: Homogenates were centrifuged at 14,000g for 35 min at 4 °C. Peroxidase activity was determined by using guaiacol as the hydrogen donor substrate (described in Jagdale et al., 2009). The reaction mixture contained 300 µl of 0.1 M sodium phosphate buffer, 150 µl of 0.18 M guaiacol, 170 µl of 0.88 M hydrogen peroxide solution and 855 µl ddH_2O in a 1.5-ml cuvette. Following an initial reading of the blank (ddH_2O), the reaction was initiated by adding 25 µl of enzyme extract at 0°C from each sample into individual cuvette containing the reaction mixture. The absorbance at 470 nm at 25°C was recorded every minute for

3 min. The sum of the change in absorbance was recorded by monitoring the formation of tetraguaiacol and the reading was used to calculate the units/mg of protein using the following formula (Bergmeyer, 1974):

$$\text{Units/mg solid} = \frac{\Delta A470\text{nm/min} \times 1000}{25.5 \times (\text{mg enzyme as solid/ml reaction mix})}$$

Catalase activity: Catalase activities in cotton leaves were determined from 100 mg leaf tissue as described in the Worthington enzyme manual (1988). Homogenates were centrifuged at 14,000g for 20 min at 4 °C and the supernatants were collected. The reaction mixture contained 500 µl of 0.059 M hydrogen peroxide substrate prepared in 0.05 M potassium phosphate buffer and 950 µl ddH_2O in a 1.5-ml cuvette. The reaction mixture was equilibrated at 25°C, and after an initial reading of the ddH_2O blank, the reaction was initiated by adding 50 µl of ice-cold enzyme extract. The absorbance at 240 nm was recorded every minute for 3 min. The sum of the change in absorbance was used to calculate the units of protein per milligram using the following formula (Worthington, 1988):

$$\text{Units/mg} = \frac{\Delta A240\text{nm/min} \times 1000}{43.6 \times \text{mg enzyme/ml reaction mix}}$$

Data analysis: Data from each enzyme assay were analyzed by repeated measures analysis of variance using the mixed model (GLIMMIX) procedure of SAS (Version 9.2, SAS Institute Inc., Cary, NC). Treatment replications within a trial and repetition of the trials were considered random effects, and least squares treatment means were compared using the PDIFF option in the GLIMMIX procedure ($P \leq 0.05$).

Results

The mixed models analysis of data pooled from the two trials indicated that the treatment effects were consistent between trials (i.e., no trial × treatment interaction). Therefore, data from the pooled analysis were presented.

Catalase activity: In the susceptible DP 0935, the application of BTH, *R. reniformis* and *M. incognita* increased the activity of catalase relative to the nontreated control. The activity of catalase was significantly increased in all treatments 4 d after treatment (DAT) application. At every sampling time, *R. reniformis* induced numerically higher catalase activity than *M. incognita*, but the differences were not significant (Fig. 1). In the reniform-resistant LONREN-1, BTH and *R. reniformis* caused increased catalase activity compared to the control beginning 6 DAT and continuing until 14 DAT, but *M. incognita* had no effect compared to the control and had lower catalase activity than the *R. reniformis* treatment (Fig. 1). In the root-knot resistant M-120 RNR, BTH and *M. incognita* increased catalase activity beginning 6 DAT and continuing until 14 DAT, but *R. reniformis* increased activity only at 10 DAT (Fig. 1).

P-peroxidase activity: Application of BTH, *R. reniformis* and *M. incognita* increased the activity of P-peroxidase in all cotton genotypes. In the susceptible DP 0935, P-peroxidase activity increased beginning 4 DAT with BTH and 6 DAT with *R. reniformis* or *M. incognita*. *Rotylenchulus reniformis* resulted in greater P-peroxidase activity than *M. incognita* only at 6 DAT (Fig. 2). In LONREN-1, all treatments resulted in greater activity 6 DAT and continued until 14 DAT, and *R. reniformis* and *M. incognita* had similar effects (Fig. 2). In M-120 RNR, *M. incognita* and BTH increased P-peroxidase activity 2 DAT and 4 DAT, respectively, but *R. reniformis* had no effect until 10 DAT. For all treatments, once an effect was elicited, increased enzyme activity greater than the control was observed continually until 14 DAT (Fig. 2).

G-peroxidase activity In the susceptible DP 0935, BTH increased G-peroxidase activity 4 DAT whereas *R. reniformis* and *M. incognita* had increased activity beginning 6 DAT. *Meloidogyne incognita* had a greater effect than *R. reniformis* at 14 DAT (Fig. 3). In LONREN-1, both BTH and *M. incognita* increased G-peroxidase activity 4 DAT, and *R. reniformis* increased activity 6 DAT, but *M. incognita* and *R. reniformis* treatments did not differ from each other at any sampling time. Once enzymatic activity increased, the effect lasted until 14 DAT (Fig. 3). In M-120 RNR, increased G-peroxidase activity in response to BTH treatment was evident at 4 DAT, *M. incognita* at 6 DAT, and *R. reniformis* at 10 DAT. On days 4 and 10, the *M. incognita* treatment had greater activity than the *R. reniformis* treatment (Fig. 3).

Discussion

The synthesis and accumulation of peroxidases and catalase are frequently associated with plant defense against various pathogens where they are catalysts for the oxidation of substrates like phenol and its derivates by hydrogen peroxide (Buonario and Montalbini, 1993; Lebeda et al., 1999). Catalase occurs in peroxisomes and decomposes hydrogen peroxide to water and oxygen. Higher concentrations of H_2O_2 orchestrate programmed cell death but lower concentrations of H_2O_2 participate in many resistance mechanisms, including reinforcement of the plant cell wall, phytoalexin production, and enhancement of resistance to various stresses (Dempsey and Klessig, 1995; Dat et al., 2000; Mittler, 2002). The role of peroxidases in plant defense systems is to remove the toxic effect of hydrogen peroxide from tissues and to participate in the synthesis of phenolic compounds and the building of intermolecular bonds to fortify cell walls at the sites of pathogen invasions (Repka and Slovakova, 1994; Passardi et al., 2004).

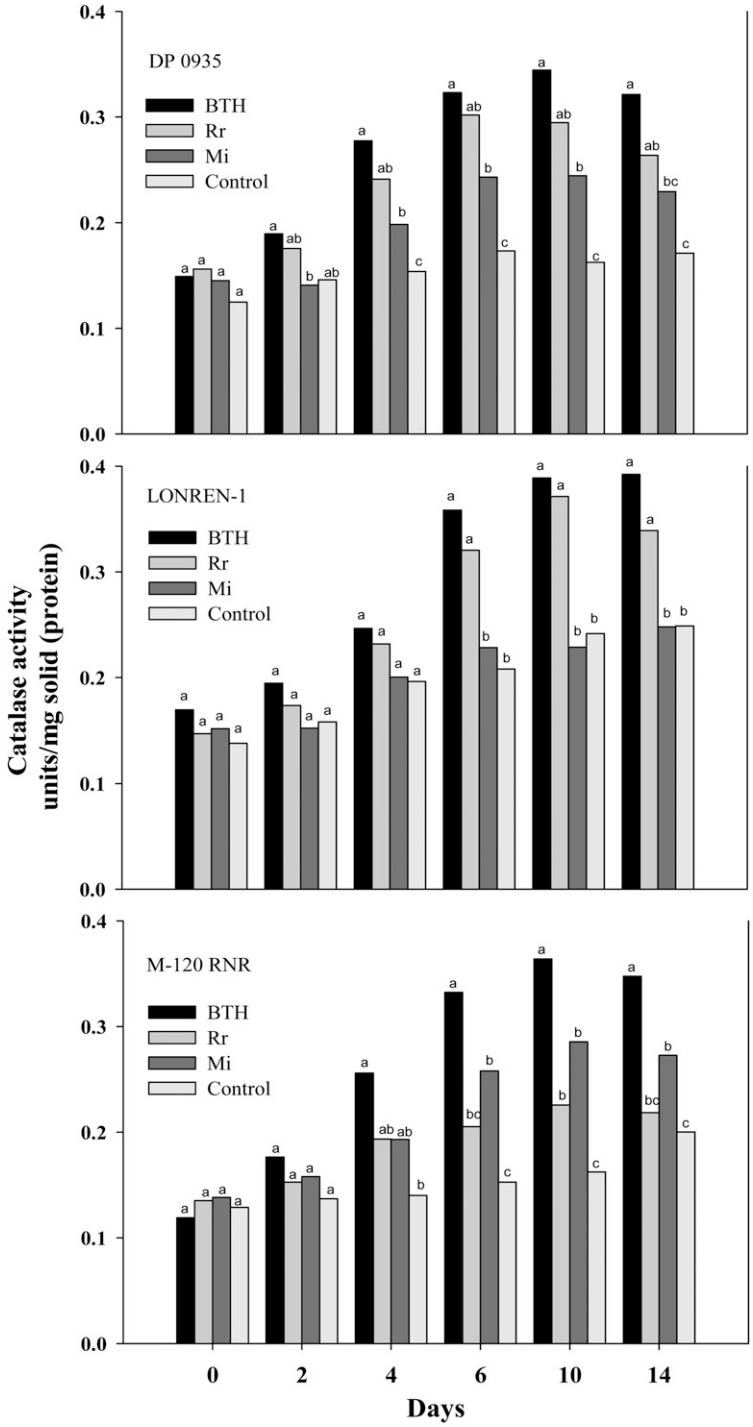

FIG. 1. Effect of BTH, *Rotylenchulus reniformis* (Rr), and *Meloidogyne incognita* (Mi) on the activity of catalase in the leaves of susceptible DP 0935, Rr-resistant LONREN-1, and Mi-resistant M-120 RNR cotton. Means are pooled from two trials, and bars within a genotype on the same day with the same letter are not significantly different according to a comparison of least squares means ($P \leq 0.05$).

Salicylic acid and its analogs have been shown to increase the activities of defense-related enzymes in plants. Our findings are similar to those of Jagdale et al. (2009) who reported that salicylic acid (SA) and the entomopathogenic nematode (EPN) *Steinernema carpocapsae* with its symbiotic bacterium *Xenorhabdus nematophila* induced defense mechanisms in *Hosta* and *Arabidopsis thaliana* that increased the production of catalase, P-peroxidase and G-peroxidase. In another study, a different SA analog, β-aminobutyric acid (BABA) increased G-peroxidase activity in tomato plants, although catalase activity was not increased (Sahebani and Hadavi, 2009). Increased peroxidase activities also have been observed in SA-treated cowpea (Fernandes et al., 2006) and broadleaf dock (Moore et al., 2003). Similarly, elevated catalase activity was observed on SA-treated bean (Clarke

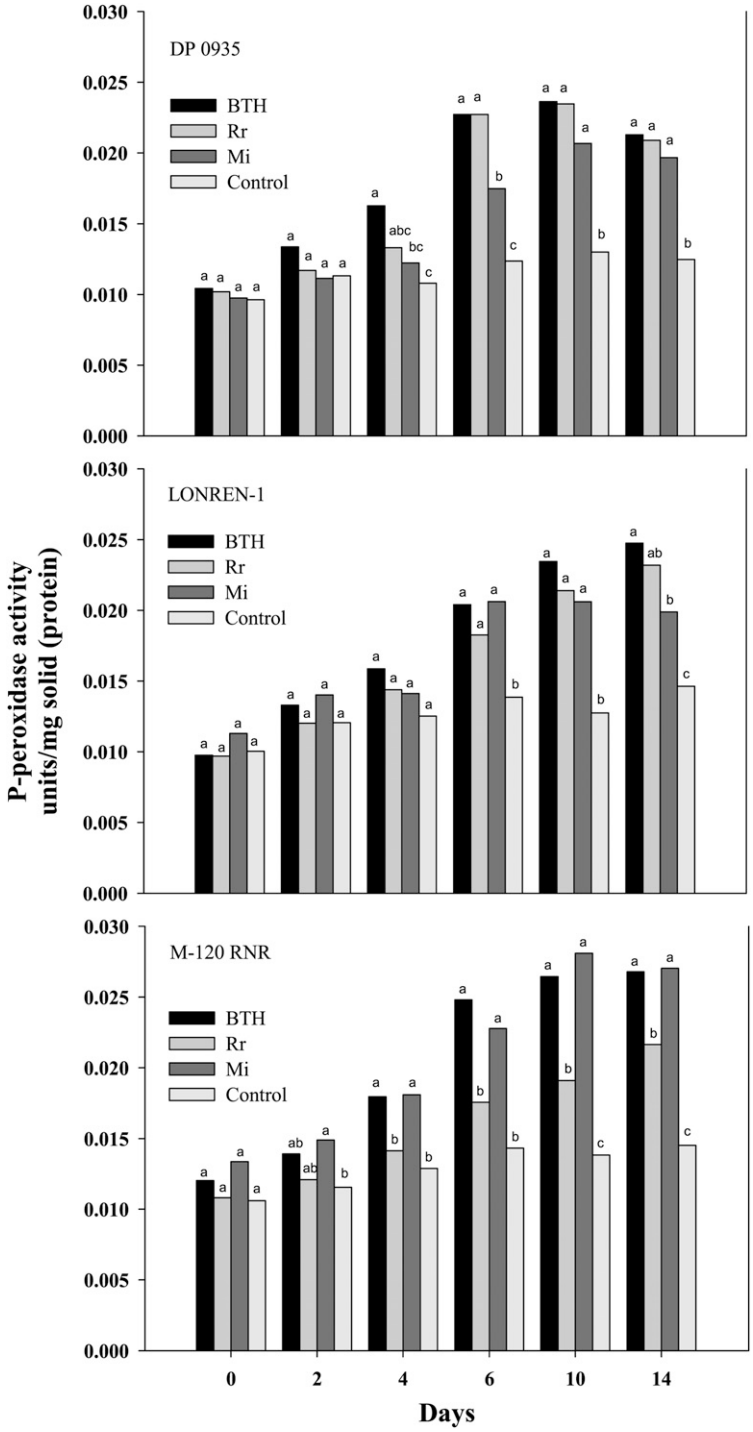

FIG. 2. Effect of BTH, *Rotylenchulus reniformis* (Rr), and *Meloidogyne incognita* (Mi) on the activity of P-peroxidase in the leaves of susceptible DP 0935, Rr-resistant LONREN-1, and Mi-resistant M-120 RNR cotton. Means are pooled from two trials, and bars within a genotype on the same day with the same letter are not significantly different according to a comparison of least squares means ($P \leq 0.05$).

et al., 2002) and tobacco (Dorey et al., 1998; Yu et al., 1999). Acibenzolar-S-methyl (ASM or BTH) is an SA analog that triggered the expression of defense genes in wheat (Pasquer et al., 2005) and tomato (Herman et al., 2007).

Infection by nematodes also has been shown to increase peroxidase and catalase levels in plants (Zacheo et al., 1983; Lambert, 1995; Niebel et al., 1995). Genes with homology to several known plant-defense genes (including peroxidase, chitinase, lipoxygenase, and proteinase inhibitors) were expressed locally within 12 hr of inoculation with *M. incognita* (Lambert, 1995). Similarly, a gene encoding for catalase production was induced both locally and systemically in potato after infection with *M. incognita* or *Globodera pallida* (Niebel et al., 1995). The post-infection accumulation of peroxidase enzymes in tomato plants resistant to *M. incognita* reached maximum

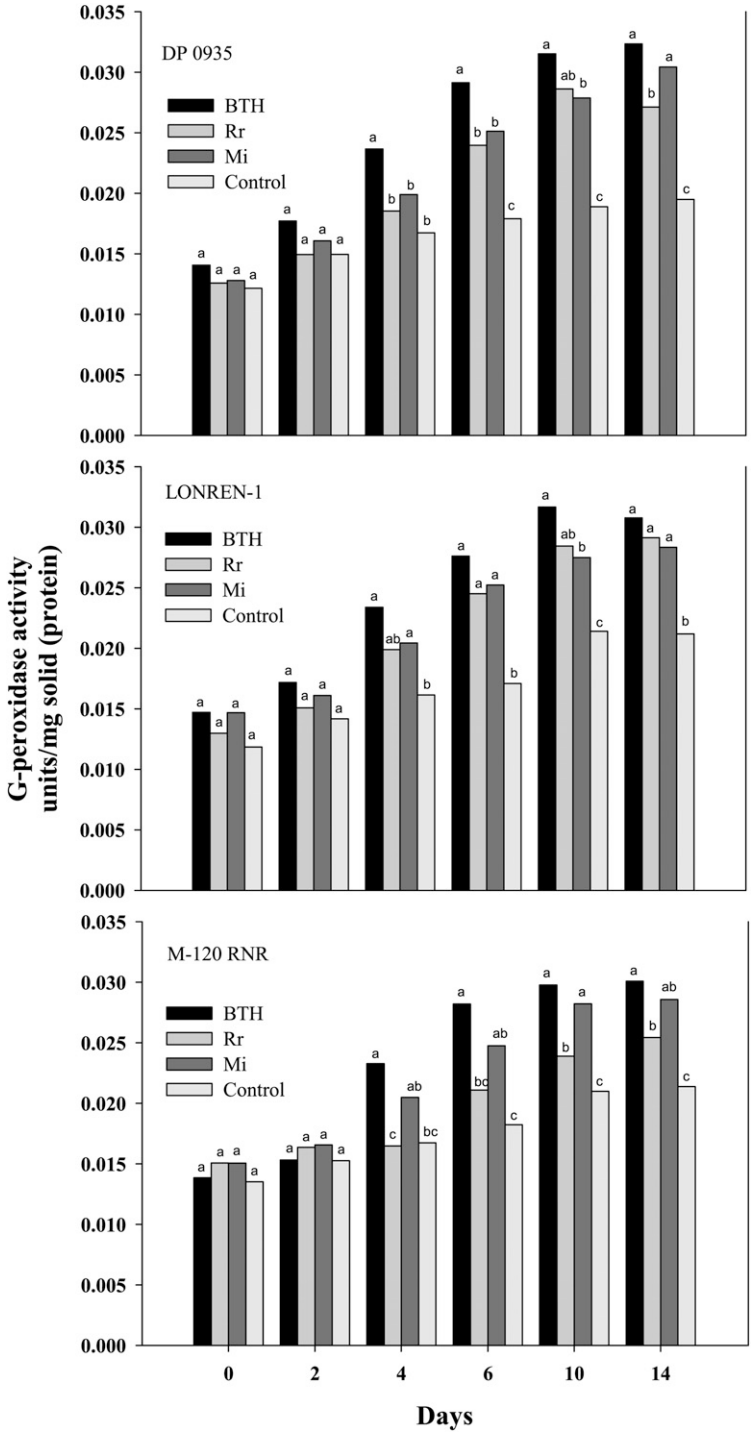

FIG. 3. Effect of BTH, *Rotylenchulus reniformis* (Rr), and *Meloidogyne incognita* (Mi) on the activity of G-peroxidase in the leaves of susceptible DP 0935, Rr-resistant LONREN-1, and Mi-resistant M-120 RNR cotton. Means are pooled from two trials, and bars within a genotype on the same day with the same letter are not significantly different according to a comparison of least squares means ($P \leq 0.05$).

levels 10 d after inoculation with an avirulent *M. incognita* population (Zacheo et al., 1983).

Plants react to pathogen attack through a range of active and passive defense mechanisms. Systemic acquired resistance (SAR) is an active defense system associated with increased expression of a large numbers of defense-related genes encoding phytoalexins, biosynthetic enzymes, anti-microbial factors, proteinase inhibitors, peroxidases, hydrolytic enzymes, and other PR-proteins (Wobbe and Klessig, 1996). The results from the enzyme assays support the hypothesis that infection of cotton by *M. incognita* or *R. reniformis* induce the components of SAR (Aryal et al., 2011). We found that application of BTH (which is a functional analog of salicylic acid), *R. reniformis* or *M. incognita* systemically enhanced the activities of H_2O_2-scavenging enzymes

compared to the nontreated cotton plants in three different cotton genotypes. We found that all treatments, including the nontreated control, generally resulted in increasing levels of defense enzyme activity over time. That could be due to increasing plant age during the study, but it may be due to the unavoidable injuries plants suffered as leaves were removed for our assays. Relative to the nontreated control, the levels of enzyme activity generally increased following treatment with BTH, *R. reniformis* or *M. incognita*. Increased enzyme activity was typically observed 4 to 6 DAT. The consistently increasing activity of the three enzymes caused by BTH was typically more rapid than that caused by nematodes, which could be because BTH is an SA analog and therefore directly involved in signaling; the nematodes must stimulate SA synthesis before enzyme activity is increased.

Previous studies have shown that SAR may be induced if a nematode tries to parasitize a plant that has constitutive host-plant resistance against that nematode (Kosaka et al., 2001; McKenry and Anwar, 2007; Anwar and McKenry, 2008). We included cotton genotypes with resistance to the potential inducer species because it was unknown if that would have an effect on our results. We found only limited evidence of significant effects of cotton genotype on enzymatic activity elicited by *M. incognita* or *R. reniformis*. In DP 0935, enzyme activity was affected equally by *M. incognita* and *R. reniformis* except for G-peroxidase 14 DAT. However, *R. reniformis* caused greater catalase activity than *M. incognita* on reniform-resistant LONREN-1, and *M incognita* caused greater P-peroxidase activity than *R. reniformis* on root-knot resistant M-120 RNR.

Induction of SAR typically takes several days after the application of an inducing agent, but the duration of the effect can be variable. Thaler et al. (1999) found that BTH consistently induced SAR in field grown tomatoes 5 DAT. Genes to produce PR proteins were activated in tobacco soon after application of SA (12 hr for acidic PR-1 and 3 d for basic PR-1), and high levels of expression were maintained for up to 20 DAT (Friedrich et al., 1996). BTH induced PR-1 in canola starting 1 DAT, and the stimulation continued for up to 3 wk (Potlakayala et al., 2007). In contrast, ASM (BTH)-induced defense gene expression in three tomato cultivars decreased to pretreatment levels 7 d after application (Herman et al., 2007). In our study, the effects of BTH, *M. incognita*, and *R. reniformis* were consistent among trials and increased defense enzyme activity levels 4 to 6 DAT, and the effect continued for the remainder of the 14-day-long experiment.

This study documents that infection of cotton by *R. reniformis* or *M. incognita* enhances the activation of defense-related enzymes in a similar manner to SA and its functional analogs. This study also provides biochemical evidence that supports the conclusion that infection by *R. reniformis* or *M. incognita* elicited a SAR response in cotton (Aryal et al., 2011). Knowledge that infection by nematodes, and possibly other pathogens, can elicit SAR in cotton opens new avenues for investigation. We do not know the range of organisms that can induce SAR in cotton, nor do we know how effective it may be against various nematodes and other pathogens. However, the practical implications of this study suggest that the use of SAR-inducing chemicals such as BTH can contribute to the control of plant-parasitic nematodes in cotton fields. Additionally, when two or more damaging nematode species are present in the same field, the use of a cultivar with resistance to one species may help to reduce the population densities of the other nematodes through the induction of SAR.

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