INDUCED RESISTANCE TO *MELOIDOGYNE INCOGNITA* AND *ROTYLENCHULUS RENIFORMIS* IN COTTON

by

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(Under the Direction of Katherine L. Stevenson)

ABSTRACT

Systemic acquired resistance, which results in enhanced defense mechanisms in plants, can be elicited by biotic or abiotic factors such as pathogens causing a hypersensitive necrotic reaction, virulent and avirulent pathogenic strains, and certain chemicals. Induced resistance against plant-parasitic nematodes has been documented, but is not as well understood as induced resistance to other pathogen groups. Split-root experiments conducted on susceptible, reniform resistant and root-knot resistant cotton showed that prior infection with one nematode species could induce systemic resistance against the other nematode species. The rate of nematode reproduction was evaluated to quantify the level of systemic acquired resistance. In a different project, infection by reniform and root-knot nematodes systemically enhanced the activities of pyrogallol peroxidase, guaiacol peroxidase and catalase enzymes in the leaves of cotton plants. This study documents for the first time that infection of cotton by a nematode can elicit enhanced defense to another nematode species through induction of systemic acquired resistance.

INDEX WORDS: Catalase, cotton, induced resistance, *Meloidogyne incognita*, peroxidase, reniform nematode, root-knot nematode, *Rotylenchulus reniformis*, split-root system, systemic acquired resistance

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DEDICATION

This thesis is dedicated to my father, who taught me that the best kind of knowledge to have is that which is learned for its own sake. It is also dedicated to my mother, who taught me that even the largest task can be accomplished if it is done one step at a time. They are always being the source of my inspiration and pride. They also have been there giving me love and support.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Economic importance of cotton: Cotton (*Gossypium hirsutum*), a soft, staple fiber crop, is an important cash crop. Worldwide, its returns are exceeded only by wheat, rice, soybean, and corn. A closely compressed rectangular bale of lint weighing 218 kg (480 pounds) is the production unit in the United States (Robinson, 2007).The U.S. cotton production is estimated to reach up to 3.9 million metric tons with a US$104 billion farm gate value (price based on current trade in New York) for the year 2010/11(U.S. Cotton Market Monthly Economic Letter, 2011). The world's top five cotton producers are China, India, United States, Pakistan, and Brazil, producing 6.4, 5.4, 4.0, 1.9, and 1.9 million metric tons, respectively (USDA, 2010), which is nearly two third of the world's production.

The land area used for cotton production in the United States reached its highest point in 1926 at approximately 18 million hectares (Starr et al., 2007). After World War II, the introduction of mechanized farming and the availability of effective, relatively low-cost fertilizers, pesticides and improved cotton cultivars ensured the production of considerably greater yields per unit area and the total hectares cropped were significantly decreased. Besides lint, cotton seed is a valuable source of oil, ruminant animal feed and other feed products. The U.S. cotton belt covers the southern United States from the San Joaquin valley of California to southeastern Virginia. Major producing areas are found in the southeastern coastal plain, the lower Mississippi valley and Texas. In 2009, a total of 12.8 million bales of cotton lint were

harvested from more than 3 million ha of land (USDA, 2009). Cotton is produced on different soil types ranging from coarse loams to fine alluvial clays. Large areas of the cotton-producing belt are infested with one or more species of plant-parasitic nematodes that are damaging to cotton.

Nematode problems and distribution in cotton fields: Nematode parasites of cotton are of great importance and pose serious economic threats to the cotton industry worldwide. Four nematodes species, *Meloidogyne incognita, Rotylenchulus reniformis, Hoplolaimus columbus* and *Belonolaimus longicaudatus* are significant pathogens of cotton in the U.S., but *M. incognita* (races 3 and 4) and *R. reniformis* are of primary concern to the U.S. cotton industry (NCCA, 2010). For the year 2009, the Cotton Disease Council estimated that 4% of the U.S. cotton crop was lost to damage from these two nematodes with root-knot causing 2.6% loss and reniform causing 1.4% loss (Cotton disease loss estimate committee report, 2009).

Meloidogyne incognita (the southern root-knot nematode) and *Rotylenchulus reniformis* (the reniform nematode) thrive in tropical, subtropical and warm temperate soils throughout most of the world, generally within 35° of the equator (Robinson and Cook, 2001). One or both species are present in most cotton-producing areas and are considered to be serious problems in cotton production wherever they occur. *Meloidogyne incognita* causes the greatest damage and is prevalent in California, Texas, and the southeastern states (Blasingame, 2006). In recent years, the range of *R. reniformis* has increased significantly throughout the mid-south region and has caused greater economic losses than the root-knot nematode in the states of Alabama, Arkansas, Louisiana, and Tennessee (Heald and Robinson, 1990; Blasingame, 2006).

Meloidogyne incognita races 3 and 4 can reach high population levels and cause the greatest damage when cotton is planted year after year in sandy soils. These nematodes are best

adapted to coarse-textured, sandy soils rather than fine textured silt or clay based soils (Robinson, 2007). The presence of root-knot nematodes in a field does not always result in significant yield loss. Cotton plants can usually tolerate a small amount of damage from root-knot and other plant-parasitic nematodes before yield loss occurs. Many factors influence the amount of crop damage that will occur with a given population of root-knot nematodes. Factors to be considered include soil type, soil fertility levels, moisture availability, soil temperature and presence of other pathogens or pests (Daulton and Nusbaum, 1961). Losses may exceed 47% of the potential yield in severe infestations (Davis and May, 2005).

Over the last decade, *Rotylenchulus reniformis* is emerging as a serious threat to cotton production in the United States, with yield losses ranging from 10 to 70% in infested fields (Kirkpatrick and Lorenz, 1997). Reniform nematodes are widely distributed in tropical, sub-tropical and warm temperate zones in South America, North America, the Caribbean Basin, Africa, southern Europe, the Middle East, Asia, Australia, and the Pacific (Ayala and Ramirez, 1964). Reniform nematodes are common in sandy, sandy loam, sandy clay and clay loam soils (Kinloch and Sprenkel, 1994). In the U.S., infestations of reniform nematode have become established in 11 out of 16 cotton-producing states. In Mississippi and Louisiana, more than 500,000 ha of cotton fields were infested by this nematode in 1997 (Overstreet and McGawley, 1997). Worldwide, cotton yield losses due to nematodes were estimated to be 10.7% by Sasser and Freckman (1987), which was equivalent to 1.9 million metric tons of cotton lint worth $US 4 billion at 1987 prices. In 1999, approximately 13.7 million US dollars in losses were attributable to this nematode in the U.S. (Mueller, 2000), but the losses increased to approximately 80 million in 2001 (McLean et al., 2001) to 130 million in 2006 (Robinson, 2007).

In the U.S., both *M. incognita* and *R. reniformis* can occur in the same field. Interactions between both nematode species in cotton fields have not been fully characterized, but it is believed that *R. reniformis* has a competitive advantage over *M. incognita* (Diez et al., 2003; Robinson, 2007). Greenhouse studies have shown that interactions can be antagonistic for either nematode when *M. incognita* and *R. reniformis* are feeding on the same plant. In concomitant inoculations, *M. incognita* inhibited reproduction of *R. reniformis* on black gram (*Vigna mungo*) (Mishra and Gaur, 1981). *Meloidogyne incognita* also inhibited *R. reniformis* reproduction on soybean (Singh, 1976) and sweet potato (Thomas and Clark, 1981), but *M. incognita* was not affected by *R. reniformis* in either study. In contrast, *R. reniformis* inhibited *M. incognita* on tomato (Kheir and Osman, 1977) and cowpea (Taha and Kassab, 1980). In cotton, both *M. incognita* and *R. reniformis* were capable of reducing the population density of each other when the amount of primary inoculum was higher than that of the other nematode species (Diez et al., 2003).

CURRENT STRATEGIES FOR MANAGEMENT OF NEMATODES IN COTTON FIELDS

Cultural practices: Cultural practices that have been documented to minimize nematode survival and reproduction include crop rotation with non-hosts, resistant or antagonistic cover crops, mixing of soil with plant material or animal manure, and destruction or removal of cotton stalks and roots (Barker and Koenning, 1998; Davis et al., 2003). Vegetable crops and tobacco in rotation with cotton should be avoided in fields infested with root-knot or reniform nematode. Grain crops such as corn or grain sorghum support little or no reproduction of *R. reniformis* (Robinson et al., 1997). Crops of corn or peanuts suppress reniform nematodes and peanuts or resistant soybean cultivars suppress root-knot nematode for a succeeding cotton crop (Noe et al.,

1991). Soybean cultivars with resistance to the reniform nematode can be incorporated in rotation with cotton to reduce the population density of *R. reniformis* and increase cotton yield (Davis et al., 2003). However, suppression of reniform nematode by rotation with non-host crops is often only effective for one year (Davis et al., 2003). Small grains including wheat (*Triticum aestivum*), rye (*Secale cereale*) and oats (*Avena sativa*), are used as winter cover crops in the Southeast to control soil erosion (Snapp et al., 2005). Many winter cover crops, usually small grains, are poor hosts and many legumes (e. g., vetches and clovers) are good hosts for *M. incognita*, *H. columbus* and *B. longicaudatus*, but normally not for *R. reniformis* (Timper et al., 2006). The cyclic hydroxamic acids DIBOA (2,4-dihydroxy-(2H)-1,4-benzoxazin-3(4H)-one), and DIMBOA (2,4-hydroxy-7-methoxy-(2H)-1,4-benzoxazin-3(4H)-one) that are produced by members of the grass family are toxic to plant-parasitic nematodes (Zasada et al., 2005).

Chemical control (nematicides): Initially, fumigants (any volatilizable chemical compound used to control insects/pests) such as chloropicrin were used for control of soil-borne pests on high value crops (Johnson and Feldmesser, 1987). Later on, the concept of soil fumigation was established as a practical means of nematode control leading to the discovery of the nematicidal properties of methyl bromide, ethylene dibromide (EDB) and the mixture of 1,3-dichloropropene and 1,2-dichloropropane (DD). The discovery of 1,2-dibromo-3-chloropropane (DBCP) in 1954 increased interest in the application of nematicides to cotton fields because of its lower phytotoxic effects compared to other available fumigants and because it was relatively easier to apply (Raski, 1954).

In the U. S., aldicarb has been the single most widely used nematicide in cotton for more than 20 years (Koenning et al., 2004), but it will be phased out by 2014 because it does not meet food safety standards and may pose unacceptable dietary risks, especially to infants and young

children (Landrigan and Goldman, 2011). Application of a low concentration of non-fumigant nematicides as a seed dressing protects emerging roots from nematode infection for only a limited period of time (Monfort et al., 2006). Abamectin, produced by *Streptomyces avermictilus*, was registered for use on cotton in 2006. Another compound, thiodicarb, was registered for use as a seed treatment nematicide in 2007. However, cotton plants in fields with high nematode levels may not be adequately protected from nematode damage by seed treatments alone. Some recently developed techniques such as variable-rate technology and use of aerial imagery for mapping nematode population distribution within fields and site-specific delivery of nematicides to infested fields may improve nematode management (Overstreet et al., 2007). Use of nematicides is likely to remain the first line of defense against plant-parasitic nematodes in cotton in the U.S. until effective nematode-resistant cotton cultivars or other tools for mitigating nematode damage in cotton are available.

Biological control: During the past thirty years, many studies have been undertaken to investigate the use of microorganisms as biocontrol agents against plant-parasitic nematodes. Many species of bacteria and fungi have been identified as pathogens of plant-parasitic nematodes and have shown suppressive effects on nematode pest populations (Stirling, 1991). However, biological control often does not meet the grower's expectation for management due to lower efficacy or less consistency compared to chemical control (Pal and McSpadden Gardener, 2006).

RESISTANCE: A DIFFERENT APPROACH TO PEST/DISEASE MANAGEMENT

The term resistance in nematology is defined as the ability of a plant to inhibit the reproduction of a nematode species relative to reproduction on a plant lacking such resistance

(Cook and Evans, 1987). Every plant has developed a wide range of physical and chemical defense mechanisms to protect themselves against fungi, bacteria, viruses, nematodes, and insects through metabolic, biochemical, and molecular defenses, as well as physical or structural barriers such as thorns or waxy leaves (Hammerschmidt, 2007). Constitutive or host-plant resistance is expressed independently of injury. Host-plant resistance has great potential for managing nematodes in cotton because it is economical and highly effective. The use of resistant cultivars is easy to implement and more predictable in effect than cultural tactics such as multi-year rotations (Ogallo et al., 1999). Resistant cotton cultivars have an added benefit of protecting susceptible crops grown in rotation (Ogallo et al., 1999).

Resistance to Rotylenchulus reniformis: Since the 1980s, more than 3,000 accessions of the genus *Gossypium* have been evaluated to discover sources of resistance against the reniform nematode. Only weak to moderate resistance has been reported in *G. hirsutum*, but high to very high levels of resistance have been found in other *Gossypium* species, including *G. anomalum, G. arboreum, G. barbadense, G. herbaceum, G. longicalyx, G. raimondii, G. somalense, G. stocksii* and *G. thurberi* (Yik and Birchfield, 1984; Stewart and Robbins, 1995; Robinson et al., 1997; Robinson et al., 2004). Reniform nematode resistance in accessions of *G. barbadense*, which hybridizes freely with *G. hirsutum*, usually suppresses 70% to 90% of nematode populations (Robinson et al., 2004). Many accessions of *G. arboreum*, from which genes are introgressed via bridging species, are highly resistant to the reniform nematode and the most resistant *G. arboreum* accessions suppress reproduction by 95% or more compared to susceptible *G. hirsutum* (Stewart and Robbins, 1995). *Gossypium longicalyx*, from which genes can be transferred to *G. hirsutum* only with great effort, is almost immune to *R. reniformis*. Two reniform-nematode-resistant lines, LONREN-1 and LONREN-2 (source of resistance: *G.*

longicalyx), are reported to suppress *R. reniformis* population densities by 85% to 98% (Robinson et al., 2007) in field tests at multiple locations and were released by the USDA in 2007.

Resistance to Meloidogyne incognita: Only a few root-knot-resistant cotton cultivars have been developed with yield potential and fiber quality comparable to popular susceptible cultivars. Stoneville 5599BR and LA 887 had moderate levels of nematode resistance (Gutierrez et al., 2010). Phytogen PH98-3196, a moderately resistant line which suppressed populations of *M. incognita* by 77%, could be used in rotation with a susceptible cultivar along with nematicides to reduce nematode damage (Davis and Kemerait, 2009). PHY 367 WRF also has been used by some growers for its excellent early-season vigor, root-knot nematode tolerance, high yield potential, and good fiber quality (Phytogen, 2011). Acala NemX is adapted to western cotton production areas, and *M. incognita* population densities were suppressed and yields were higher when Acala NemX was planted in fields with moderate to severe infestations (Ogallo et al., 1997). Similarly, when Acala NemX was planted in the same infested plots for three consecutive years, the yield was stable, while the yield in plots planted to a root-knot susceptible cultivar declined around 30% from the first year to the third year of the test (Ogallo et al., 1999). In addition to protecting the yield potential of the crop in infested fields, resistance to root-knot nematodes also suppressed final nematode population densities. The reduced nematode population densities in fields planted with Acala NemX was also beneficial for successive crops.

Induced resistance in plants: Plant defense mechanisms can be either preformed (constitutive) or active (induced) resistance; induced resistance is activated or expressed only after the plant is attacked or otherwise injured (Huang, 1998). The general understanding that plants can actively guard themselves and have induced resistance against virulent pathogens has

been known for over 100 years (Beauverie, 1901). Almost every plant exhibits various defense mechanisms that interfere with the host-parasite relationship, either physiologically or anatomically, to inhibit pathogen invasion (McKenry and Anwar, 2007). In addition to specific defense responses based on so-called constitutive resistance or R-genes, plants have broad-spectrum defense responses against certain strains of pathogens that can be induced locally or systemically by biotic or abiotic components in nature. Such post-infection expression of defense mechanisms is considered to be induced resistance, which is characterized by the subsequent release of a variety of pathogenesis-related (PR) compounds and a hypersensitive reaction (HR) that consists of rapid and restricted necrosis around the infection sites (Kuc, 1982, 1983). Van Loon (1997) defined induced resistance as the physiological state of defensive response by the plant which provides both qualitative and quantitative expression of defense mechanisms once triggered appropriately.

Defense activators stimulate the natural defense mechanisms of plants against the attack of pathogens and diseases. Pathogens (bacteria, fungi and viruses) producing hypersensitive necrotic lesions, avirulent pathogenic strains, insects, entomopathogenic nematodes (EPNs), abiotic elicitors or chemical products such as benzothiadiazole (BTH), β-aminobutyric acid (BABA), 2,6-dichloroisonicotinic acid (INA), salicylic acid, jasmonic acid, and various inorganic salts are some examples of successful plant defense activators (Edreva, 2004; McKenry and Anwar, 2007; Jagdale et al., 2009, Kone et al., 2009). The induced plant is resistant to virulent pathogens and other pests as the result of enhanced expression of defense responses induced by infection or, in some cases, as the result of an inducing treatment (Van Loon et al., 1998). The overall phenomenon of induced resistance that results from the interaction of a plant with a suitable inducing agent requires i) the presence of all necessary

genes to mount an effective defense (Heath, 2000) and ii) the ability to activate defenses directly by the inducing treatment (Conrath et al., 2002). In the latter case, the inducing treatment primes or sensitizes the plant, which allows increased expression of a wide range of defenses upon attack by a pathogen. The inducing or resistance-activating treatment in systemic resistance results in a change in cells at a distance from the induction site that allows rapid defense activation called priming (Conrath et al., 2002).

Induced resistance can be local or systemic. Local induced resistance refers to cases where the response is local whereas systemic induced resistance describes resistance that is induced in a part of the plant that is spatially separated from the point of induction (Oka et al., 1999). Although they differ spatially, both local and systemic resistance require some time to develop after application of the inducing treatment and both are non-specific in nature. Reduced population growth of the pathogen in locally induced resistance may be due to the production of PR proteins (defensive plant proteins specifically induced in pathological or related situations) and cell wall alterations that prevent or inhibit growth and development of the inducing inoculum as well as challenge pathogens (Hammerschmidt, 1999). Resistance responses such as the onset, intensity and durability of defense activation are dependent on the quantitative relationship between concentration of inducers and number of host cells that are affected (Sequeira, 1983). The intensity of resistance may vary greatly depending on the location and distance of the challenge inoculation in relation to the inducing treatment (Sequeira, 1983).

Much of our understanding of induced resistance comes from studies of resistance induced by application of pathogens or other microbes. Inoculating stems of tobacco plants with sporangia of *Peronospora tabacina* induced resistance in the leaves to subsequent inoculation with the same pathogen. However, despite having induced resistance, the plants were visibly

stunted (Cruikshank and Mandryk, 1960). Ross (1961) published two papers on acquired resistance of tobacco to tobacco mosaic virus (TMV). He used tobacco plants with the N-gene for resistance against TMV and demonstrated that the tissues around the TMV-induced local lesions were highly resistant to infection by both TMV and tobacco necrosis virus (Ross, 1961a). In another experiment, he confirmed that the infection of N-gene tobacco with TMV resulted in increased systemic resistance to challenge TMV and the systemic resistance was also effective against tobacco necrosis virus, turnip mosaic virus, and tobacco and tomato ring-spot viruses (Ross, 1961b).

Cucumber is an excellent model system for induced resistance. The inoculation of an anthracnose-susceptible cucumber leaf with the cucumber anthracnose fungus *Colletotrichum orbicularae* induced systemic resistance to that pathogen (Kuc et al., 1975), and similar to *Peronospora tabacina* on tobacco, a virulent isolate that caused a necrotic lesion was capable of inducing systemic resistance in cucumber. Hammerschmidt and Yang-Cashman (1995) demonstrated that resistance in cucumber could be induced against and by a broad range of necrotic-lesion-inducing pathogens as well as the HR induced by bacterial pathogens of hosts other than cucumber.

At least two forms of induced resistance, systemic acquired resistance (SAR) and induced systemic resistance (ISR) have been described (Fig. 1.1) as distinct phenomena based on the type of inducing agents and host signaling pathways that result in resistance expression (Sticher et al., 1997; Van Loon et al., 1998). Induced resistance (SAR and ISR) involves the coordinated expressions of multiple genes and/or defense signaling pathways (Vallad and Goodman, 2004). Although the downstream components are similar in SAR and ISR, upstream components differ, mainly involving the salicylic acid pathway for SAR and the jasmonic acid/ethylene pathways

for ISR (Pieterse and Van Loon, 2007). It is believed that pathogenic organisms trigger SAR and nonpathogenic rhizobacteria activate ISR (Wubben et al., 2007). Both result in similar phenotypic responses but involve different mechanisms (signaling) and are effective against a wide range of pathogens (Pieterse and Van Loon, 2004). Like SAR, ISR has been confirmed to act systemically against fungi, bacteria, and viruses in many plants including *Arabidopsis*, bean, carnation, cucumber, radish, tobacco, and tomato (Van Loon et al., 1998). Both SAR and ISR signaling are modulated by NPR1, a key regulatory protein (Pieterse and Van Loon, 2004).

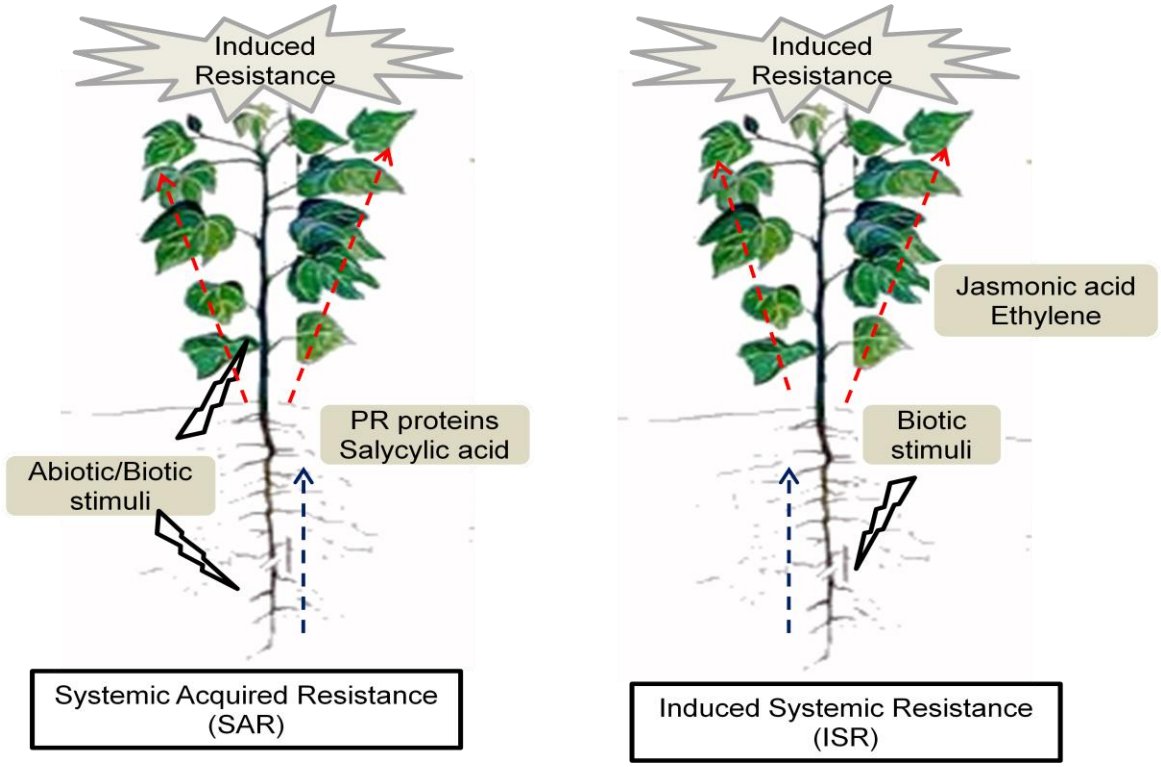

Figure 1.1. A graphical representation of the two best characterized forms of induced resistance, SAR and ISR, in plants (modified from Vallad and Goodman, 2004).

Onset of SAR requires the accumulation of salicylic acid (SA) and the systemic or coordinated expression of PR proteins (Hammerschmidt, 1999; Sticher et al., 1999). ISR depends on ethylene and jasmonic acid (JA) signaling pathways instead of the SA pathway. In

Arabidopsis, ISR triggered by *Pseudomonas fluorescens* WCS417r acts independently of SA but requires an intact NPR1 protein and sensitivity to JA and ethylene (Pieterse et al., 1998). Moreover, activation of ISR is associated with priming of gene expression, which is a physiological state in which plants respond more rapidly and strongly upon pathogen attack because their defense genes are expressed more rapidly after pathogen attack. These responses may include some PR proteins associated with both SA-dependent and the JA-dependent pathways (Conrath et al., 2006). Both SAR and ISR result in broad spectrum resistance.

The history of induced resistance research: Reports from the early 20th century indicate what appears to be induced resistance to disease when Bernard demonstrated that prior infection of orchid embryos with a less virulent strain of mycorrhizal *Rhizoctonia* resulted in an enhanced capacity of the embryo to resist infection by a more virulent strain of the same pathogen (Allen, 1959). Prior inoculation of the cut surface of a potato tuber with an avirulent race of *Phytophthora infestans* resulted in locally induced resistance to virulent races of the same pathogen (Muller and Burger, 1940). They also suggested that the healthy tissue immediately below the HR necrotic tissue was also resistant to the virulent strain of *P. infestans.* In the 1950s, initial biochemical evidence for inducible defenses was reported, including induced resistance (Allen, 1959; Muller, 1959). Application of D- or DL-phenylalanine induced resistance in apple leaves against *Venturia inaequalis* (Kuc et al., 1959). Hijwegen (1963) demonstrated that phenylserine could induce resistance in cucumber, and White (1979) reported that salicylic acid was an inducer of resistance against tobacco mosaic virus in tobacco. Many synthetic and natural compounds have since been reported to induce resistance. The first synthetic resistance elicitor, benzothiadiazole (BTH), was commercialized as Actigard in the 1990s and many other resistance activators have been identified (Kessman et al., 1994; Cohen, 2002). By far, the most

effective chemical elicitors available are BTH and 2,6-dichloroisonicotinic acid (INA); both are considered as functional analogs of SA. The practical use of resistance elicitors has increased greatly because they can easily be applied via conventional application equipment.

Several studies have been conducted to evaluate the elicitation of ISR by PGPR in many plants. The application of *Pseudomonas fluorescens* strain WCS 417r induced systemic resistance to Fusarium wilt caused by *Fusarium oxysporum* f. sp. *dianthi* in carnation (Van Peer et al., 1991). *Pseudomonas putida* strain 89B-27 and *Serratia marcescens* strain 90-166 reduced the severity of Fusarium wilt of cucumber caused by *Fusarium oxysporum* f. sp. *cucumerinum* by inducing systemic resistance (Liu et al., 1995). In a screening test, some PGPR strains applied as a seed treatment against *Colletotrichum orbiculare* in cucumber resulted in a significant reduction of anthracnose (Wei et al., 1991, 1996). In rice, seed treatment followed by a root dip and a foliar spray with *P. fluorescens* strains Pf1 and Pf7 expressed a high degree of ISR against *Rhizoctonia solani*, the sheath blight pathogen (Vidhyasekaran and Muthamilan, 1999). In sugarcane, PGPR-mediated ISR was observed against *Colletotrichum falcatum* causing red rot disease (Viswanathan and Samiyappan, 1999).

Seed treatment of cucumber with *P. putida* strain 89B-27, *Flavomonas oryzihabitans* strain INR-5, *S. marcescens* strain 90-166 or *Bacillus pumilus* strain INR-7 induced systemic protection against angular leaf spot caused by *Pseudomonas syringae* pv. *lachrymans* as evidenced by reduced total lesion diameter compared to non-treated plants (Wei et al., 1996). Halo blight of bean, caused by *Pseudomonas syringae* pv. *phaseolicola* was controlled when seeds were treated with *P. fluorescens* strain 97 (Alstrom, 1991), whereas seed treated with *P. putida* strain 89B-27 and *S. marcescens* strain 90-166 decreased the incidence of bacterial wilt disease in cucumber, caused by *Erwinia tracheiphila* (Kloepper et al., 1993). Similarly, PGPR-

mediated ISR against viral diseases has been reported in cucumber and tobacco plants. Seed treated with *P. fluorescens* strain 89B-27 and *S. marcescens* strain 90-166 reduced the intensity of cucumber mosaic virus (CMV) and delayed symptom development in cucumber and tomato (Raupach et al., 1996). A soil drench with *P. fluorescens* strain CHAO also induced systemic protection following inoculation with tobacco necrosis virus (TNV) in tobacco (Maurhofer et al., 1998).

Induced resistance to plant-parasitic nematodes has not been as extensively studied as that to fungi and bacteria, but induced resistance (both ISR and SAR) has been documented for plant-parasitic nematodes. *P. fluorescens* induced ISR against *M. javanica* (Siddiqui and Shaukat, 2004) and *Heterodera schachtii*, the sugar beet cyst nematode, and also reduced early root penetration (Oostendrop and Sikora, 1989, 1990). In a split-root assay, *Bacillus sphaericus* B43 and *Rhizobium etli* G12 triggered ISR against potato cyst nematode by reducing the juvenile penetration of the roots on the responder side when the bacteria were applied as an inducer to the other half of root system (Hasky-Gunther et al., 1998). These two bacteria also caused ISR against *M. incognita* on tomato by reducing the J2 penetration on the responder root side (Schafer et al., 2006). Application of *P. chitinolytica* reduced the penetration rate of juveniles of root-knot nematodes in tomato (Spiegel et al., 1991). Sikora (1988) demonstrated that *Bacillus subtilis* induced systemic protection against *M. incognita* in cotton. Root and soil populations of the rice root nematode, *Hirschmanniella oryzae*, were reduced following the application of PGPR (*P. fluorescens*) as a seed treatment (Swarnakumari et al., 1999). Fewer galls and egg masses of *M. incognita* were observed following treatment of tomato roots with *P. fluorescens* strain PF1 (Santhi and Shivakumar, 1995).

More recently, entomopathogenic nematodes (EPN) were reported to induce components of ISR in plants. Jagdale et al. (2009) reported that the EPN *Steinernema carpocapsae* and its symbiotic bacterium *Xenorhabdus nematophila* induced defense mechanisms in *Hosta* and *Arabidopsis thaliana* by activating the production of key defense enzymes. They also found expression of the plant resistance protein promoter PR1-gene in *A. thaliana* leaves through a GUS (β-glucoronidase) activity assay when treated with EPNs.

Ibrahim and Lewis (1986) demonstrated that Centennial soybean, which is normally susceptible to *M. arenaria*, expressed increased resistance to this nematode after prior inoculation with *M. incognita.* SAR to the root-knot nematode *M. hapla* was observed in tomato and pyrethrum plants following prior inoculation with more than one species (*M. incognita* or *M. javanica*) to which the plants were resistant (Ogallo and McClure, 1995). In a split-root assay, SAR against *M. hapla* was obtained on tomato by pre-inoculation with an avirulent strain of *M. incognita* (Ogallo and McClure, 1996). In pine, prior inoculation with an avirulent strain of *Bursaphelenchus xylophilus* induced SAR to the virulent strain of *B. xylophilus* (Kosaka et al., 2001). In split-root experiments, McKenry and Anwar (2007) demonstrated that an avirulent population of *M. incognita* induced SAR to a virulent population of *M. arenaria* in Harmony grape rootstock, which is resistant to *M. incognita.* The induction of resistance became apparent when plants were exposed to the virulent population 7 d after inoculation with the avirulent population. They also reported that the higher the inoculum density of the avirulent population, the higher the level of SAR. In tomato it has been documented that challenge inoculations with a virulent population of *M. incognita* to half of the root system 7 d after inoculating the other half with an avirulent population of same species significantly reduced the reproduction rate of the virulent population (Anwar and McKenry, 2008).

Several abiotic compounds have been reported to induce SAR against plant-parasitic nematodes. Acibenzolar-S-methyl (benzothiadiazole 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) have been reported to induce SAR to major genera of plant-parasitic nematodes (Kempster et al., 2001; Oka and Cohen, 2001; Chinnasri et al., 2006).

Activation of defense related enzymes during induced resistance: The expression of induced resistance does not involve the presence of major pathogen-specific resistance genes, although the defense mechanisms activated are those used in other forms of plant resistance to pathogens (Heath, 2000). Once plants are exposed to the inducing treatment, direct activation of defense mechanisms may occur or they may occur only after pathogen introduction. A cascade of molecular and biochemical reactions trigger the expression of SAR. During the onset of potential plant defense against challenge inoculation, recognition inducers result in signal molecules being generated and translocated long distances where they switch on diverse processes. Recognition of inducers results from the binding of pathogen-derived molecules (elicitors) or synthetic products with receptor sites on plant membranes or cell walls (Edreva, 2004). SAR induction is associated with both localized and systemic accumulation of SA, which is a phytohormone that plays a key role in defense signaling (Vlot et al., 2009), and PR proteins (Van Loon, 1997). Phenylalanine ammonia-lyase (PAL), peroxidase, and polyphenoloxidase are other key defense enzymes that are generally present constitutively and increase after pathogen attack and which are often referred to as PRs (Van Loon et al., 2006). A set of genes that encode PRs, called SAR genes, and the timing of PR-gene expression correlates with the onset and duration of SAR (Ward et al., 1991). PR-1 proteins are often used as markers of the enhanced

defense associated with pathogen-induced SAR, but their biological activity has remained obscure (Van Loon and Van Strien, 1999). Peroxidases are a well-known class of PR proteins (PR-9) that are produced in plant tissues during pathogen infection (Van Loon et al., 2006). Peroxidase can enhance cell wall fortification by catalyzing lignifications, which boosts resistance against pathogens (Passardi et al., 2004b).

Several proteins activated during plant defense play a key role in different metabolic responses. Class III plant peroxidases (EC 1.11.1.7, EC number is a numerical classification scheme for enzymes, based on the chemical reactions they catalyze) have been abbreviated in previous literature as POD, POX, Prx, Px, and PER. Peroxidases are heme-containing glycoproteins encoded by a large family of genes, with 138 members in rice (Passardi et al., 2004a) and 73 members in *Arabidopsis* (Welinder et al., 2002). Peroxidases are often involved in a wide range of physiological and biochemical processes throughout the plant life cycle (Fig. 1.2) probably due to the high number of enzymatic isoforms (isoenzymes) and their multi-faceted enzyme-catalyzed reactions (Passardi et al., 2005). Plant peroxidases have a proposed role in auxin metabolism, lignin and suberin formation, cross-linking of cell wall components, phytoalexin synthesis, and the metabolism of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Mehdy, 1994; Almagro et al., 2009). Several peroxidase isoenzymes in plants are activated by pathogen infection and tissue injury showing the importance of peroxidases in plant defense (Van Loon et al., 2006).

Catalases (EC 1.11.1.6) are a second superfamily of peroxidases found in peroxisomes of nearly all aerobic cells that protect the cell from the toxic effects of hydrogen peroxide (H_2O_2). Catalase is a major enzymatic system for removal of H_2O_2 from plant cells. Catalases catalyze the dismutation of H_2O_2 into O_2 and H_2O which prevents the damaging effects of H_2O_2

accumulation and protects cells from oxidative stress (Chelikani et al., 2004). A model was proposed to explain SA-mediated SAR in which SA binds and inactivates catalase resulting in increased intracellular H_2O_2 that in turn acts as an inducer of defense genes like the PR-1 gene (Chen et al., 1993). Assessment of almost 40 SA and 2,6-dichloroisonicotinic acid (INA) analogs have confirmed both qualitative and quantitative correlation between the capacity to inhibit catalase and the ability to induce PR gene expression and enhance disease resistance (Conrath et al., 1995). Catalases and peroxidases have been shown to specifically bind SA and therefore may also contribute to the action of SA in plant defense (Du and Klessig, 1997).

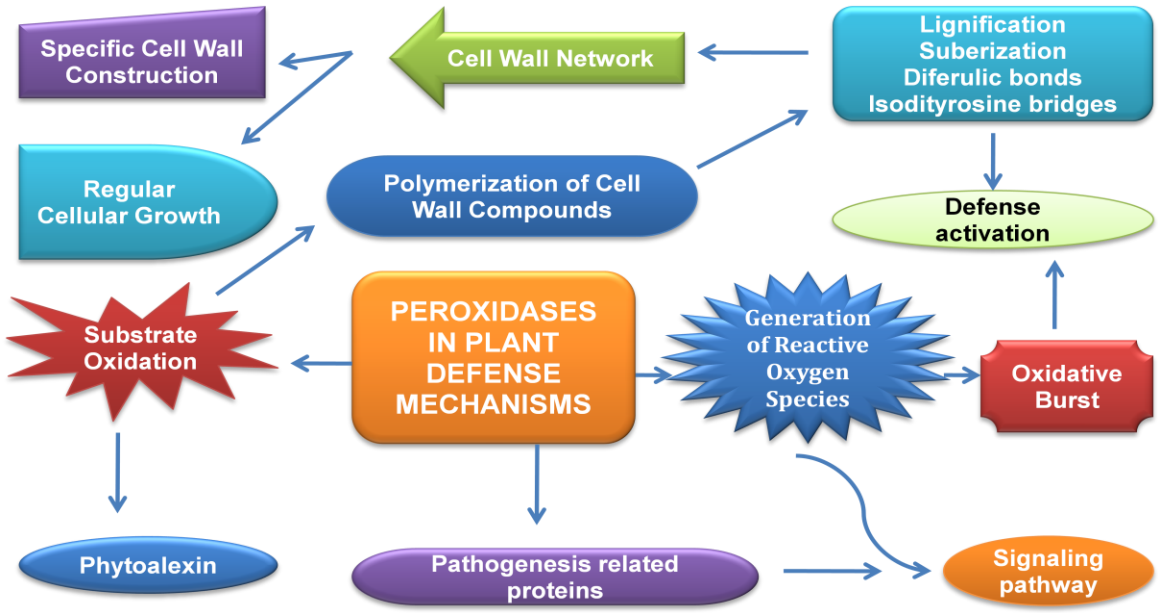

Figure 1.2. Pictorial of the specific roles of plant peroxidases in defense reactions (modified from Almagro et al., 2009).

Catalase and peroxidase are induced by oxidative stress and they convert H_2O_2 to H_2O. It has been demonstrated that H_2O_2 activates acid-benzoic 2-hydroxylase (Leon et al., 1995), an enzyme required for salicylic acid (SA) biosynthesis. Catalase and peroxidase are also induced at

the commencement of pathogen infection and the induction is coincident in time with the transient enhancement of free SA levels (Blilou et al., 2000).

Most PR proteins are capable of generating hydrogen peroxide that can be toxic to pathogens or could directly or indirectly stimulate plant-defense responses. During SAR, the production of ROS may play a key role in plant defense systems. The predominant species detected during plant-pathogen interactions are superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH) (Mehdy, 1994), and the process is referred to as an oxidative burst. For example, H_2O_2 can induce the expression of defense-related genes where H_2O_2 is an intercellular signal mediating SAR (Orozco-Cardenas et al., 2001). H_2O_2 is an electron-accepting substrate for a variety of peroxidase-dependent reactions, thus peroxidases are normally considered as ROS-detoxifying enzymes. The decomposition of H_2O_2 by the peroxidase reaction is highly active particularly in the presence of ROS-scavenging peroxidase substrates such as flavonoids (Yamasaki et al., 1997).

Lamb and Dixon (1997) have shown that wounding and pathogen attack result in ROS accumulation locally and systemically. Plants produce ROS, particularly H_2O_2, in high amounts when exposed to stressful conditions such as pathogen invasion (Yakimova et al., 2009). Moreover, H_2O_2-scavenging enzymes such as peroxidases and catalases can be activated by both herbivore damage and pathogen challenge. Enhanced activities of such enzymes contribute to removing excess H_2O_2, which is ultimately toxic to plants (Mittler, 2002). Catalases and peroxidases can interact with ROS and thereby play a significant role in plant responses to injury or damage. ROS are produced by plant cells via the enhanced enzymatic activity of plasma-membrane-bound NADPH oxidases. Cell-wall-bound peroxidases and amine oxidases in the apoplast mediate the oxidative burst and the accumulation of H_2O_2 in plants against various

pathogens (Hammond-Kosack and Jones, 1996; Lamb and Dixon, 1997). H_2O_2 produced during this response is believed to diffuse into cells along with salicylic acid (SA) and nitric oxide (NO) (Klessig et al., 2000) to activate plant defenses, including programmed cell death (Dangl et al., 1996). The activity of ascorbate-peroxidase and catalase is suppressed during this response by the plant hormones SA and NO (Klessig et al., 2000). The production of ascorbate-peroxidase is post-transcriptionally suppressed (Mittler et al., 1998) and the production of catalase is down-regulated at the level of steady-state mRNA (Dorey et al., 1998). The plant concomitantly produces more ROS and at the same time diminishes its own capacity to scavenge H_2O_2 thereby resulting in the over-accumulation of ROS and the activation of programmed cell death (Mittler, 2002).

RATIONALE OF THE STUDY

We know that ISR can be elicited in cotton. ISR and SAR are similar in some respects, and there are many examples of plants in which both ISR and SAR can be induced, so we think SAR may be induced in cotton. We know that nematode infection can induce SAR in some plants. Therefore, we want to see if nematode infection can induce SAR in cotton. If nematode infection induces SAR in cotton, it could help explain the observed interactions when both reniform and root-knot are feeding on plants in the same field.

The overall goal of this study was to characterize SAR and its effects on nematode reproduction in cotton. We hypothesized that infection of cotton by one nematode species could induce SAR to another nematode species, and that the level of SAR might be affected by constitutive host-plant resistance to one of the nematodes. The specific objectives of this study were (i) to determine whether co-infection of cotton by *M. incognita* and *R. reniformis* affects

the population level of either nematode compared to infection by each species individually, (ii) to determine whether host-plant resistance in cotton to *M. incognita* or *R. reniformis* influences the effect of concomitant infection on nematode population levels, and iii) 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.

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CHAPTER II

INDUCTION OF SYSTEMIC ACQUIRED RESISTANCE BY *ROTYLENCHULUS RENIFORMIS* AND *MELOIDOGYNE INCOGNITA* IN COTTON FOLLOWING SEPARATE AND CONCOMITANT INOCULATIONS[1]

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Induction of Systemic Acquired Resistance by *Rotylenchulus reniformis* and *Meloidogyne incognita* in Cotton Following Separate and Concomitant Inoculations

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Abstract: Systemic acquired resistance (SAR) can be elicited by virulent and avirulent pathogenic strains and SAR against plant-parasitic nematodes has been documented. Our objective was to determine whether co-infection of cotton by *Meloidogyne incognita* and *Rotylenchulus reniformis* affects the population level of either nematode compared to infection by each species individually. Split-root trials were conducted in which plants were inoculated with i) *R. reniformis* only, ii) *M. incognita* only, iii) both *R. reniformis* and *M. incognita*, or iv) no nematodes. Half of the root system was inoculated with *R. reniformis* or *M. incognita* on day 0 and the other half with *M. incognita* or *R. reniformis* on day 0 or day 14 depending on the experiment. Experiments were conducted on cotton cultivar DP 0935 B2RF (susceptible to both nematodes), LONREN-1 (germplasm line resistant to *R. reniformis*), and M-120 RNR (germplasm line resistant to *M. incognita*), and tests were terminated 8 wk after the last inoculation. Both soil (vermiform) and roots (egg) extracted from each half of the root system to determine the total nematode population levels, and root galling was rated on a 0 to 10 scale. Mixed models analysis and comparison of least squares means indicated no differences in root galling (except on LONREN-1) or population levels when the two nematode species were introduced on the same day. When *M. incognita* was introduced 14 d after *R. reniformis*, reduction in galling (36% on DP 0935 and 33% on LONREN-1) and *M. incognita* population levels (35% on DP 0935 and 45% on LONREN-1) were significant ($P \leq 0.05$). When *R. reniformis* was inoculated 14 d after *M. incognita*, reduction in *R. reniformis* population levels (18% on DP 0935 and 26% on M-120) were significant. This study documents for the first time that infection of cotton by a nematode can elicit SAR to another nematode species.

Key words: Cotton, induced resistance, *Meloidogyne incognita*, reniform nematode, root-knot nematode, *Rotylenchulus reniformis*, split-root system, systemic acquired resistance

Meloidogyne incognita (the southern root-knot nematode) and *Rotylenchulus reniformis* (the reniform nematode) are the two predominant nematodes damaging cotton in the US (Robinson and Cook, 2001). Greenhouse studies have shown that interactions can be antagonistic for either nematode when *M. incognita* and *R. reniformis* are feeding on the same plant. In concomitant inoculations, *M. incognita* inhibited reproduction of *R. reniformis* on black gram (Mishra and Gaur, 1981). *Meloidogyne incognita* also inhibited *R. reniformis* reproduction on soybean (Singh, 1976) and sweet potato (Thomas and Clark, 1981), but *M. incognita* was not affected by *R. reniformis* in either study. In contrast, *R. reniformis* inhibited *M. incognita* on tomato (Kheir and Osman, 1977) and cowpea (Taha and Kassab, 1980). In cotton, both *M. incognita* and *R. reniformis* were capable of reducing the population density of each other when the amount of primary inoculum was higher than that of the other nematode species (Diez et al., 2003).

Interactions between *M. incognita* and *R. reniformis* in cotton fields have not been fully characterized, but it is believed that *R. reniformis* has a competitive advantage over *M. incognita* (Diez et al., 2003; Robinson, 2007). The possibility that induction of systemic resistance might be involved in the interaction between *M. incognita* and *R. reniformis* on cotton has not been studied. The induced plant is resistant to virulent pathogens and other pests as the result of enhanced expression of defense responses resulting from infection or in some cases, as the result of a chemical treatment (Van Loon et al., 1998). Induced resistance is defined as the physiological state of enhanced defense response by the plant which provides both qualitative and quantitative expression of defense mechanisms against subsequent biotic challenges (Van Loon, 1997). At least two forms of induced resistance, systemic acquired resistance (SAR) and induced systemic resistance (ISR) have been described as distinct phenomena based on the type

of inducing agents and host signaling pathways that result in resistance expression (Sticher et al., 1997; Van Loon et al., 1998). Both SAR and ISR result in similar phenotypic responses but involve different signaling mechanisms (Pieterse and Van Loon, 2004, Van Loon et al., 2006). Necrotizing pathogenic organisms can trigger SAR and nonpathogenic rhizobacteria can activate ISR (Pieterse and Van Loon, 2007). SAR results in the coordinated expression of pathogenesis-related (PR) genes (Van Loon, 1997; Hammerschmidt, 1999, 2007) that enhance the natural defense systems of plants and provide broad spectrum resistance to a range of pathogens including plant-parasitic nematodes. This process requires prior exposure of plants to a locally infecting pathogen, an avirulent form of a pathogen or some synthetic compounds (Kuc, 1982; Kessmann et al., 1994).

Induced resistance to plant-parasitic nematodes has not been as extensively studied as that to fungi and bacteria, but induced resistance (both ISR and SAR) has been documented for plant-parasitic nematodes in tomato, grape, pine, potato and soybean (Ibrahim and Lewis, 1986; Ogallo and McClure, 1995, 1996; Hasky-Gunther et al., 1998; Kosaka et al., 2001; Siddiqui and Shaukat, 2004; McKenry and Anwar, 2007; Anwar and McKenry, 2008). The goal of this study was to characterize SAR and its effects on nematode reproduction in cotton. We hypothesized that infection of cotton by one nematode species could induce SAR to another nematode species, and that the level of SAR might be affected by constitutive host-plant resistance to one of the nematodes. The specific objectives of this study were (i) to determine whether co-infection of cotton by *M. incognita* and *R. reniformis* affects the population level of either nematode compared to infection by each species individually, and (ii) to determine whether host-plant resistance in cotton to *M. incognita* or *R. reniformis* influences the effect of concomitant infection on nematode population levels.

MATERIALS AND METHODS

Experimental plants and nematode inocula: Cotton plants used in the 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 a mixture (50:50) of vermiculite and steam-sterilized soil (sand 85%, silt 11%, clay 4%) for 2 to 3 wk in 5-cm-deep, 60-cm^3, biodegradable peat pots (Jiffy-Strips, Seed and Garden LLC, Brighton, MI) with the hole in the bottom of the pot covered by a piece of plastic. For the split-root system, two square plastic pots (10 cm on each side, 950 cm^3) were taped together with a notch the same size and shape as the peat pot cut out of the adjoining sides. A peat pot with a single seedling was placed into the notch, and each pot was filled with 750 cm^3 steam-pasteurized soil. Plants were grown for two to three more weeks to allow roots to grow through the small peat pot into the two adjacent pots thereby creating a split-root system prior to nematode inoculations. Plants were watered as needed up to twice a day. Each plant was supplied with 10 g of slow release granular fertilizer (NPK-14:14:14).

Rotylenchulus reniformis and *M. incognita* were used as the nematode treatments. 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 using a mist chamber extraction technique (Viglierchio and Schmitt, 1983). Infected roots were gently washed, cut into small pieces, and placed on top of a 10-cm-deep collecting pan covered with an 18-mesh sieve and fine tissue paper. Each pan was kept inside mist chamber for 3-5 d. Mist was sprayed on the roots for 1 min at 5-min intervals. After 72 hr, juveniles were collected using 100- over 400-mesh sieves. Vermiform stages of *R. reniformis* were extracted from soil by using

gravity screening and the centrifugal sugar flotation technique (Jenkins, 1964) and collected on a 500-mesh sieve.

Experimental design and inoculation techniques: A series of split-root trials was conducted; each trial included four treatments and 10 replications in a randomized complete block design. The four treatments were single plants with a split root system inoculated with i) *R. reniformis* to one half only, ii) *M. incognita* to one half only, iii) *R. reniformis* to one half and *M. incognita* to the other half, and iv) a nontreated control. Inoculum density for nematode treatments was 7000 second stage juveniles (J2) of *M. incognita* or 7000 vermiform (mixed life stages) *R. reniformis*. Nematodes were added in three holes (3 cm deep) around the peat pot. On the susceptible DP 0935 and the reniform-resistant LONREN-1, one half of the root system of 6-week-old plants was inoculated with *R. reniformis* (inducer inoculum) and other half was inoculated with *M. incognita* (challenge inoculum) on day 0 or day 14 depending on the experiment. In similar experiments, *M. incognita* was added as the inducer inoculum and *R. reniformis* was added challenge inoculum on susceptible DP 0935 and *M. incognita*-resistant M-120 RNR.

Gall rating and final population assessment: Experiments were terminated 8 wk after inoculation with challenge inoculum, and both soil (vermiform extraction) and roots (egg extraction) from each half of the root system were processed to assess the total nematode population levels. The two halves of the split-root system were cut apart, and soil was carefully removed by hand, then roots were washed lightly to remove the remaining soil, and patted dry with a paper towel. Root-gall rating was assessed on a 0 to 10 scale based on percentage of the root system with galls (0 = no galls, 1=1-10% galls, 2 = 11-20% galls, 3 = 21-30% galls, etc.). The fresh weight was recorded from each half of the root system for root-weight analysis

between halves of the root system within a treatment and for total root weight among the treatments. Eggs were extracted from each half of the root system by immersing roots into 20% sodium hypochlorite (NaOCl) solution and immediately shaking for 4 min on a mechanical shaker (Hussey and Barker, 1973). Vermiform stages were extracted using gravity screening and centrifugal sugar flotation (Jenkins, 1964). Eggs were collected using nested 100-over 500-mesh sieves, and vermiform stages were collected on a 400-mesh sieve. Each experiment with a single cotton genotype was conducted twice as described above.

Data analysis: Data were analyzed using the mixed models (GLIMMIX) procedure of SAS (version 9.2, SAS Institute Inc., Cary, NC). Final populations (eggs + vermiform) were transformed using the function $\log_{10}(X + 1)$ to correct heterogeneity of variances and non-normality prior to analysis. Treatment replications within a trial and repetitions of the trials were considered as random effects. Treatment means were separated by comparison of least squares means ($P \leq 0.05$) using the lines and PDIFF options in SAS.

RESULTS

Effect of R. reniformis on reproduction of M. incognita on susceptible DP 0935: When *R. reniformis* and *M. incognita* were introduced onto DP 0935 on the same day, root galling was not affected by *R. reniformis*, but the root gall-index was significantly greater on plants inoculated with *M. incognita* alone compared to plants inoculated with *M. incognita* 2 wk after inoculation with *R. reniformis* (Table 2.1). Populations of *M. incognita* and *R. reniformis* did not differ between plants inoculated with both species and plants inoculated with only one species when both nematode species were added on the same day. However, following inoculation with *R. reniformis* 2 wk earlier, the reproduction of *M. incognita* was reduced by 35% (Table 2.1).

Effect of M. incognita on reproduction of R. reniformis on susceptible DP 0935: Results showed that following prior inoculations with *M. incognita*, the reproduction of *R. reniformis* was reduced ($P \leq 0.0001$). Even though *M. incognita* was introduced 2 wk before *R. reniformis*, the gall index and population levels were also reduced ($P \leq 0.0069$) compared to *M. incognita* alone (Table 2.1).

Effect of host-plant resistance on the reproduction of challenge inoculum: In the reniform-resistant LONREN-1, root galling was reduced regardless of whether *M. incognita* was applied at the same time as *R. reniformis* or 2 wk later (Table 2.2). There was no significant reduction of *M. incognita* reproduction when the two species were applied at the same time, but the reproduction of *M. incognita* was reduced by 45% when it was applied 2 wk after *R. reniformis* (Table 2.2). Similarly, in root-knot-resistant M-120 RNR, the reproduction of *R. reniformis* was unchanged when it was added the same day as *M. incognita*, but *R. reniformis* levels were significantly lower ($P = 0.0028$) when *M. incognita* was added 2 wk before the challenge inoculation with *R. reniformis* (Table 2.3).

In each trial, root weight from the two halves of the split-root system within each treatment was compared, and no differences were observed. The total fresh root weight (sum of the two halves) per plant was compared among the four treatments, and no significant differences in root weight were observed among the four treatments (data not shown).

DISCUSSION

In nature, the effects of nematode species interactions are mostly antagonistic among species with similar feeding habits mainly due to the competition for space and food (Eisenback, 1985). The competitive (suppressive) interactions between *R. reniformis* and *M. incognita* have

been documented based on population dynamics and attributed to their competition for feeding sites (Thomas and Clark, 1983a, 1983b; Diez et al., 2003). The results from the split-root experiments show that prior infection of susceptible DP 0935 cotton plants with *R. reniformis* significantly suppressed the ability of *M. incognita* to cause galls and reproduce compared to single species inoculations. Because the nematode species were physically separated, this effect could not be due to competition for feeding sites. Our results clearly document a systemic resistance response that we believe to be systemic acquired resistance (SAR). Similarly, when *M. incognita* was added 2 wk before *R. reniformis,* it induced a similar systemic resistance response against *R. reniformis*.

In SAR, active defenses are triggered by a primary infection with certain pathogens or chemical treatments that result in resistance to secondary infections (Wubben et al., 2007). Although the downstream components are similar to induced systemic resistance (ISR) mechanisms, the upstream components differ, mainly involving the salicylic acid (SA) pathway for SAR and the jasmonic acid (JA) or ethylene (Et) pathways for ISR (Pieterse and Van Loon, 2007). Systemic acquired resistance is also involved in the production of pathogenesis-related proteins (Van Loon, 1997). This active resistance mechanism is also characterized by the production of peroxidases, and by the lignin formation and the cell wall modifications (Cohn and Gisi, 1994; Cohen et al., 1999). Systemic acquired resistance induced by virulent or avirulent nematode populations against virulent nematode populations has not been studied as extensively as it has been for bacteria, viruses and fungi (Pieterse and Van Loon, 2007), but similar biochemical pathways are believed to be triggered against plant-parasitic nematodes (Kogan and Paxton, 1983; Zacheo and Bleve-Zacheo, 1995).

Previous reports have documented the ability of nematodes to induce SAR in plants. Centennial soybean, which is normally susceptible to *M. arenaria*, expressed increased resistance to this nematode after prior inoculation with *M. incognita* (Ibrahim and Lewis, 1986). In tomato and pyrethrum, SAR to the root-knot nematode *M. hapla* was observed following prior inoculation with naturally incompatible species of *M. incognita* or *M. javanica* (Ogallo and McClure, 1995). In a split-root assay, SAR against *M. hapla* was obtained on tomato by pre-inoculation with an avirulent strain of *M. incognita* (Ogallo and McClure, 1996). In pine, prior inoculation with an avirulent strain of *Bursaphelenchus xylophilus* induced SAR to a virulent strain of *B. xylophilus* (Kosaka et al., 2001). In split-root experiments, McKenry and Anwar (2007) reported that an avirulent population of *M. incognita* induced SAR to a virulent population of *M. arenaria* in Harmony grape rootstock. In tomato, challenge inoculations with a virulent population of *M. incognita* to half of the root system 7 d after inoculating the other half with an avirulent population of the same species suppressed reproduction of the virulent population (Anwar and McKenry, 2008). Our study also documents the ability of one nematode species to induce SAR to another species, but ours is the first report of SAR against a nematode in cotton, and it is also the first report of SAR induced by *R. reniformis*.

Little information is available on genotype-specific variation in the level of SAR expression. However, cultivars with constitutive host-plant resistance to the inducer species can exhibit SAR; therefore we also included genotypes with resistance to either *R. reniformis* or *M. incognita*. When *R. reniformis* was added 2 wk before *M. incognita,* reproduction of *M. incognita* was reduced by 45% on the reniform-resistant LONREN-1; and the level of suppression on the susceptible DP 0935 was 35%. Additionally, galling was reduced on LONREN-1 even when *M. incognita* and *R. reniformis* were introduced at the same time, but this

was not observed in susceptible DP 0935. Similarly in root-knot-resistant M-120 RNR, *M. incognita* induced SAR to *R. reniformis* and suppressed reproduction by 26%, whereas suppression on DP 0935 was 18%. Although we cannot directly compare the level of SAR between resistant and susceptible genotypes because they were not in the same experiment, there was a trend for a greater level of SAR in resistant genotypes.

We observed SAR in susceptible cotton as well as cotton with resistance to *R. reniformis* or *M. incognita*. Host genotypes have been shown to influence the expression of induced resistance (Walters and Fountaine, 2009). In cucumber, INA (2,6-dichloroisonicotinic acid) induced SAR to the powdery mildew fungus (*Sphaerotheca fuliginea*) and was shown to be cultivar dependent, with the highest levels of SAR expressed in moderately resistant cultivars (Hijwegen and Verhaar, 1994). In soybean, SAR induced by treatment with BTH (benzothiadiazole) or INA reduced the levels of *Sclerotinia sclerotiorum*, and the levels of reduction were greatest in susceptible cultivars (Dann et al., 1998). In contrast, BTH provided control of blue mold (*Peronospora hyocyami* f. sp. *tabacina*) in resistant tobacco plants but not in susceptible tobacco cultivars (Perez et al., 2003). Recently, tomato genotypes treated with BABA (β-aminobutyric acid) expressed significant variability in SAR expression against *Phytophthora infestans*. The level of SAR was not always associated with level of constitutive resistance of the tomato cultivars, but SAR level was influenced notably by pathogen isolates (Sharma et al., 2010). These studies indicate that the level of SAR generally varies among plant genotypes.

In our experiments, the induction of resistance was observed when the inducer inoculum was added 14 d before the challenge inoculum, and that is consistent with previous reports that there is a time delay in the expression of resistance in SAR following infection by the inducing

agent. The delay in induction of systemic resistance is due to the time required for post-infection accumulation of antimicrobial substances. Post-infection accumulation of peroxidase enzymes in tomato plants resistant to *M. incognita* reached maximum levels 10 d after inoculation with an avirulent *M. incognita* population (Zacheo et al., 1983).

It is not known whether other plant-parasitic nematodes, including species that are much less damaging than *M. incognita* and *R. reniformis*, can induce SAR in cotton. It also is not known what level of inducer inoculum is needed to elicit SAR in cotton, if that level varies among nematode species, or how long the induced resistance persists. Further studies will be needed to better understand nematode induced SAR in cotton. But this study documents for the first time that infection of cotton by a nematode can elicit SAR to another nematode species. This post-infection induction of resistance may have a significant direct effect on nematode population dynamics (Ogallo and McClure, 1996) and may help explain results that otherwise might be attributed to nematode competition for feeding sites. Unexpectedly, we found a significant reduction in galling and reproduction of *M. incognita* when it was the inducer inoculum, and that inhibition may contribute to a competitive advantage of *R. reniformis* over *M. incognita*.

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TABLES

Table 2.1. Root-gall ratings and final population levels of *Meloidogyne incognita* (*Mi*) and *Rotylenchulus reniformis* (*Rr*) following single and co-inoculations on susceptible cotton DP 0935[a].

Treatments	Gall index[b]	Final population level[c]	
		Mi	*Rr*
M. incognita and *R. reniformis* inoculated at same day			
Rr + *Mi*	4.50 a[d]	44,995 a	29,536 a
Mi only	4.65 a	51,855 a	0 b
Rr only	0.00 b	0 b	31,465 a
Control	0.00 b	0 b	0 b
M. incognita inoculated 14 days after *R. reniformis*			
Rr + *Mi*	3.40 b	28,843 b	23,366 a
Mi only	5.35 a	44,598 a	0 b
Rr only	0.00 c	0 c	32,973 a
Control	0.00 c	0 c	0 b
R. reniformis inoculated 14 days after *M. incognita*			
Rr + *Mi*	3.90 b	61,980 b	53,251 b
Mi only	4.45 a	73,483 a	0 c
Rr only	0.00 c	0 c	64,866 a
Control	0.00 c	0 c	0 c

[a] LS means of 20 replicates (data were pooled from trials I and II; each trial consisted 10 replicates).

[b] Root-gall index was assessed based on percentage of root system with galls on a 0 to 10 scale.

[c] Final population consisted of total eggs plus vermiform (statistical analysis was performed on $\log_{10}(x+1)$ transformed nematode populations).

[d] Means in each column followed by the same letters are not significantly different according to comparison of least squares means ($P \leq 0.05$).

Table 2.2. Root-gall ratings and final population levels of *Meloidogyne incognita* (*Mi*) and *Rotylenchulus reniformis* (*Rr*) following single and co-inoculations on reniform-resistant cotton LONREN-1[a].

Treatments	Gall index[b]	Final population level[c]	
		Mi	*Rr*
M. incognita and *R. reniformis* inoculated at same day			
Rr + *Mi*	2.9 b[d]	25,721 a	2,010 a
Mi only	3.5 a	29,890 a	0 b
Rr only	0.0 c	0 b	1,658 a
Control	0.0 c	0 b	0 b
M. incognita inoculated 14 days after *R. reniformis*			
Rr + *Mi*	3.30 b	20,843 b	1,000 a
Mi only	4.95 a	37,730 a	0 b
Rr only	0.00 c	0 c	1,154 a
Control	0.00 c	0 c	0 b

[a] LS means of 20 replicates (data were pooled from trials I and II; each trial consisted 10 replicates).

[b] Root-gall index was assessed based on percentage of root system with galls on a 0 to 10 scale.

[c] Final population consisted of total eggs plus vermiform (statistical analysis was performed on $\log_{10}(x+1)$ transformed nematode populations).

[d] Means in each column followed by the same letters are not significantly different according to comparison of least squares means ($P \leq 0.05$).

Table 2.3. Root-gall ratings and final population levels of *Meloidogyne incognita* (*Mi*) and *Rotylenchulus reniformis* (*Rr*) following single and co-inoculations on root-knot- resistant cotton M-120 RNR[a].

Treatments	Gall index[b]	Final population level[c]	
		Mi	*Rr*
M. incognita and *R. reniformis* inoculated at same day			
Rr + *Mi*	1.2 a[d]	585 b	48,066 a
Mi only	1.4 a	1,162 a	0 b
Rr only	0.0 b	0 c	48,007 a
Control	0.0 b	0 c	0 b
R. reniformis inoculated 14 days after *M. incognita*			
Rr + *Mi*	1.6 a	2,390 a	34,408 b
Mi only	1.7 a	2,700 a	0 c
Rr only	0.0 b	0 b	46,610 a
Control	0.0 b	0 b	0 c

[a] LS means of 20 replicates (data were pooled from trials I and II; each trial consisted 10 replicates).

[b] Root-gall index was assessed based on percentage of root system with galls on a 0 to 10 scale.

[c] Final population consisted of total eggs plus vermiform (statistical analysis was performed on $\log_{10}(x+1)$ transformed nematode populations).

[d] Means in each column followed by the same letters are not significantly different according to comparison of least squares means ($P \leq 0.05$).

CHAPTER III

INFLUENCE OF INFECTION OF COTTON BY *ROTYLENCHULUS RENIFORMIS* AND *MELOIDOGYNE INCOGNITA* ON THE PRODUCTION OF ENZYMES INVOLVED IN SYSTEMIC ACQUIRED RESISTANCE[1]

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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

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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 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):

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):

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):

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

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. 3.1). 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. 3.1). 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. 3.1).

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. 3.2). 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. 3.2). 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. 3.2).

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. 3.3). 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. 3.3). 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. 3.3).

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).

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 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 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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FIGURES

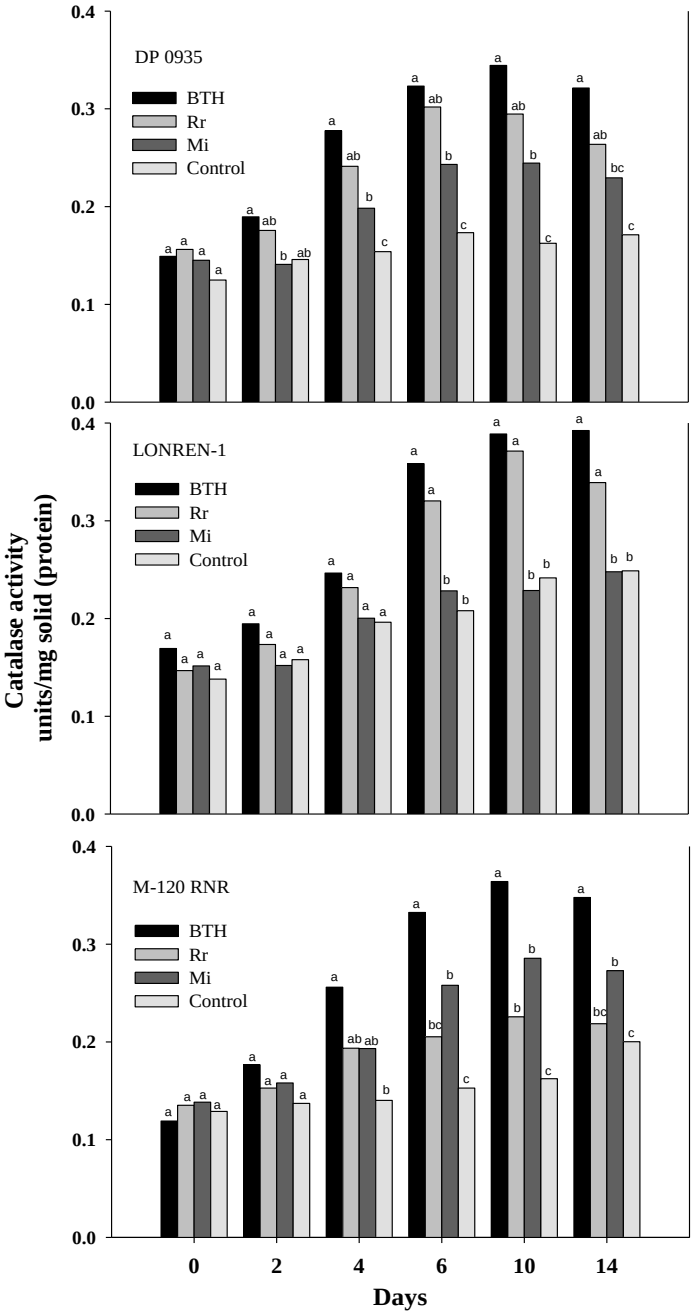

Figure 3.1. Effect of BTH, *Rotylenchulus reniformis* (Rr), and *Meloidogyne incognita* (Mi) on the activity of catalase in the leaves of susceptible DP 0935, Rr-resistant LONREN-1, and Mi-resistant M-120 RNR cotton. Means are pooled from two trials, and bars within a genotype on the same day with the same letter are not significantly different according to a comparison of least squares means ($P \leq 0.05$).

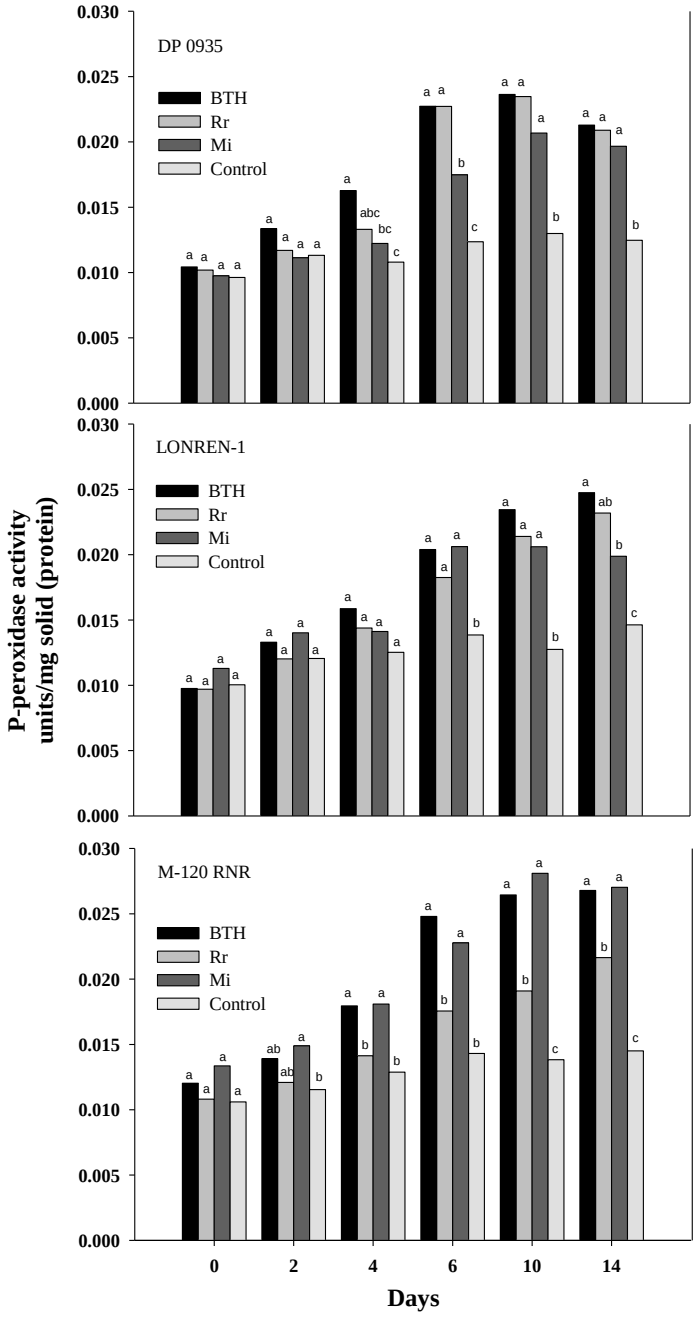

Figure 3.2. Effect of BTH, *Rotylenchulus reniformis* (Rr), and *Meloidogyne incognita* (Mi) on the activity of P-peroxidase in the leaves of susceptible DP 0935, Rr-resistant LONREN-1, and Mi-resistant M-120 RNR cotton. Means are pooled from two trials, and bars within a genotype on the same day with the same letter are not significantly different according to a comparison of least squares means ($P \leq 0.05$).

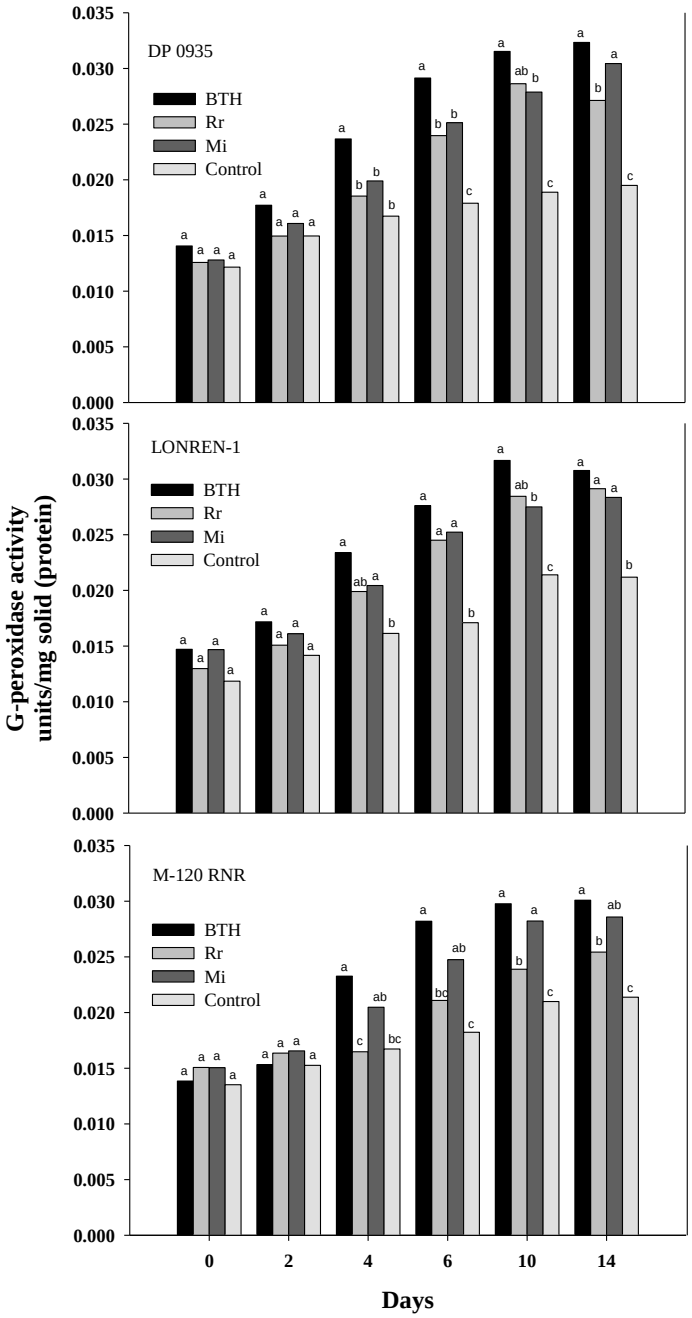

Figure 3.3. Effect of BTH, *Rotylenchulus reniformis* (Rr), and *Meloidogyne incognita* (Mi) on the activity of G-peroxidase in the leaves of susceptible DP 0935, Rr-resistant LONREN-1, and Mi-resistant M-120 RNR cotton. Means are pooled from two trials, and bars within a genotype on the same day with the same letter are not significantly different according to a comparison of least squares means ($P \leq 0.05$).

CHAPTER IV

CONCLUSIONS

The research conducted for this thesis included two different but related greenhouse studies. In the first study on different cotton genotypes, co-infection by *Meloidogyne incognita* and *Rotylenchulus reniformis* were evaluated to determine if the population level of either nematode was affected compared to infection by each species individually. Results from this study have shown that the reniform-resistant LONREN-1, or the root-knot-resistant M-120 RNR when *R. reniformis* and *M. incognita* were inoculated at the same time when induced resistance was not elicited on the susceptible cultivar Deltapine DP 0935. Induced resistance was elicited on Deltapine DP 0935and LONREN-1 when *M. incognita* was inoculated two weeks after *R. reniformis*; and induced resistance was also elicited when *M. incognita* was added 14 d before by *R. reniformis* on Deltapine DP 0935 and M-120 RNR. It is believed that the induced resistance was systemic acquired resistance (SAR). Although we cannot directly compare the level of SAR between resistant and susceptible genotypes because they were not in the same experiment, there was a trend for a greater level of SAR in resistant genotypes. The interaction also suggests that the competitive advantage of *R. reniformis* over *M. incognita* in cotton fields may be due to induce resistance. Our study clearly documents the ability of one nematode species to induce systemic acquired resistance to another species, it is the first report of SAR against a nematode in cotton, and it is also the first report of SAR induced by *R. reniformis*.

In the second study, infection of cotton by *R. reniformis* or *M. incognita* increased the activity of three enzymes involved in SAR: P-peroxidase, G-peroxidase, and catalase in a manner similar to BTH, a functional analog of salicylic acid. In most cases the effect was significant 6 d after treatment application. This study provided biochemical evidence that supports the conclusion in the first study that infection by *R. reniformis* or *M. incognita* elicited a SAR response in cotton.

Although this study demonstrated that one nematode species can elicit SAR in cotton to another species, additional research is needed to fully characterize SAR in cotton. It is not known whether other plant-parasitic nematode species, including species that are much less damaging than *M. incognita* and *R. reniformis*, can elicit SAR in cotton. It also is not known what level of inducer inoculum is needed to elicit SAR in cotton, if that level varies among nematode species, or how long the induced resistance persists. Further studies will be needed to better understand nematode-induced SAR in cotton. 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.