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Biological and Chemical Induction of Resistance to the *Globodera tabacum solanacearum* in Oriental and Flue-Cured Tobacco (*Nicotiana tabacum* L.)

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Abstract: The effects of acibenzolar-*S*-methyl (ASM) and four combinations of plant growth-promoting rhizobacteria (PGPR) on the reproduction of a tobacco cyst nematode, *Globodera tabacum solanacearum*, and growth of *Nicotiana tabacum* (cv. K326 and Xanthi) were tested under greenhouse and field conditions. The PGPR included combinations of *Bacillus subtilis* A13 with *B. pumilis* INR7, *B. pumilis* SE34, *B. licheniformis* IN937b, or *B. amyloliquefaciens* IN937a, respectively. Among the four rhizobacterial combinations, IN937a + A13 exhibited the most consistent reduction in *G. t. solanacearum* cysts under greenhouse and field conditions. No undesirable effects of IN937a + A13 were observed on tobacco growth under greenhouse and field conditions. Use of INR7 + A13 reduced *G. t. solanacearum* reproduction on flue-cured tobacco cv. K326 but not on oriental tobacco cv. Xanthi. Application of ASM reduced final numbers of *G. t. solanacearum* cysts, but also resulted in phytotoxicity mainly under the greenhouse conditions. When oriental tobacco seedlings were pre-grown in a IN937a + A13-treated soil-less medium, a single application of ASM at 200 mg/L one week after transplanting significantly reduced *G. t. solanacearum* reproduction in the field.

Key words: biological control, induced resistance, management, oriental tobacco.

Production of flue-cured tobacco (*Nicotiana tabacum* L.) in Virginia is significantly affected by *Globodera tabacum solanacearum* (Miller and Gray, 1972) Behrens, 1975 (Miller and Gray, 1972; Johnson, 1998), the tobacco cyst nematode. Approximately 37% of Virginia's flue-cured tobacco fields are in counties commonly infested with *G. t. solanacearum* (C.S. Johnson, unpub. data). *G. t. solanacearum* is also reported to occur in North Carolina and Maryland in the USA, and in Japan, Argentina, Mexico, France, Morocco, and Spain (Johnson et al., 2005; Sumiya et al., 2002). Symptoms caused by *G. t. solanacearum* include cysts attached to the root surface, and stunting of roots and shoots. Severe infestation can cause significant crop loss by reducing leaf weight and quality (LaMondia, 1988; Johnson et al., 2005). Flue-cured tobacco yield reduction and pesticide expenses due to *G. t. solanacearum* were approximately 2 million dollars in Virginia alone during the year 2007 (C. S. Johnson, unpub. data). Current *G. t. solanacearum* management practices include nematicide application, resistant cultivars, and cultural practices. Preplant soil fumigation with expensive and toxic fumigant nematicides such as chloropicrin, metam sodium and 1,3-dichloropropene, or application of the non-fumigant nematicide, aldicarb, are recommended to tobacco growers to reduce the nematode populations to below the damage threshold (Johnson, 2008). However, most fumigant and non-fumigant nematicides pose severe health and environmental risks due to their hazardous active ingredients, and fumigant use has declined due to costs and regulatory requirements (LaMondia, 2008; U.S. Environmental Protection Agency, 2008). Cultural practices, such as crop rotation, destruction of tobacco roots after harvest, and deep plowing, help to reduce nematode inoculum level for the next cropping season (LaMondia, 2008). Economic and agronomic considerations frequently limit effective use of cultural practices and resistant cultivars in *G. t. solanacearum* management, so that tobacco producers routinely apply nematicides in *G. t. solanacearum* -infested fields. Therefore, more environmentally friendly, durable, and effective management is desirable.

Plants defend themselves against fungi, bacteria, viruses, nematodes, and insects through metabolic, biochemical, and molecular defenses, as well as physical and structural barriers (Hammerschmidt, 2007). Plant defense mechanisms can be either preformed (passive or basal) or induced (active) (Huang, 1998). Constitutive resistance includes naturally occurring secondary plant metabolites or physical barriers. In contrast, induced resistance occurs when a plant recognizes invading pathogens and initiates defense reactions (Zacheo et al., 1997). Application of biotic or abiotic elicitors triggers induced resistance in plants, and such resistance may be categorized as systemic acquired (SAR) or induced systemic (ISR) resistance (Pieterse and Van Loon, 2007). Some soil-inhabiting, non-necrotizing, and non-pathogenic bacteria indigenous to the plant rhizosphere are capable of improving plant growth and vigor (Ryu et al., 2004). Collectively referred to as plant-growth promoting rhizobacteria (PGPR), they are often comprised of gram-negative *Pseudomonas* spp. or gram-positive *Bacillus* spp. Successful root colonization by a number of PGPR strains not only improve plant growth and vigor, but also trigger host defense mechanisms (Ryu et al., 2004). Several strains of PGPR have been shown to induce systemic resistance (ISR) to foliar diseases in cucumber, tobacco, arabidopsis, radish, and

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tomato (Park and Kloepper, 2000; Van Loon et al., 1998). This systemic resistance depends on the host genotype (Van Loon et al., 1998) and does not have any known toxic side effects.

Acibenzolar-*S*-methyl (ASM), commercially available as ACTIGARD (Syngenta Crop Protection, Greensboro, NC) in the USA, is a novel SAR elicitor released in 2000 that belongs to the benzothiadiazole group (Syngenta Crop Protection, 2001). ASM is a synthetic functional analogue of salicylic acid that induces SAR within 4-5 d in many crops, including tobacco (Mandal et al., 2008). The SAR induced by ASM confers broad-spectrum resistance to fungi, bacteria, and viruses (Mandal et al., 2008; Syngenta Crop Protection, 2001). The product is registered in the United States for control of tomato spotted wilt (*Tomato spotted wilt virus*) and blue mold (*Peronospora tabacina* Adam.) of tobacco, downy mildew (*Peronospora* spp.) of leafy vegetables, and bacterial leaf spot (*Xanthomonas vesicatoria* (Doidge) Dowson) and leaf speck (*Pseudomonas syringae* pv. *Tomato* (Okabe) Young, Dye & Wilkie) of tomato (*Solanum lycopersicum* L.) (Syngenta Crop Protection, 2001). Our research objectives were to evaluate the potential influence of an SAR inducer (ASM) and four mixtures of PGPR (*Bacillus* spp.) on *G. t. solanacearum* reproduction and plant growth.

Materials and Methods

Greenhouse experiments: Greenhouse experiments were conducted in 2005 and 2006 at the Virginia Polytechnic Institute and State University in Blacksburg, VA. Independent experiments were conducted for *G. t. solanacearum*-susceptible cultivars of oriental (cv. Xanthi) and flue-cured (cv. K326) tobacco. Each experiment was repeated once. A randomized complete block experimental design was used in each experiment with six replications of the following six treatments: untreated control, ASM, and the following four mixtures of PGPR strains: combinations of *Bacillus subtilis* (Ehrenberg) Cohn A13 with *B. pumilus* Meyer and Gottheil INR-7, *B. pumilus* SE34, *B. licheniformis* (Weigmann) Chester IN937b, or *B. amyloliquifaciens* (ex Fukumoto) Priest et al. IN937a, respectively.

Seedling preparation and transplanting: Seeds of Xanthi were germinated in a soil-less plant growth medium (Carolina Choice Tobacco Mix, Carolina Soil Company, Kinston, NC, USA) and maintained by applying a 150 ppm N liquid nutrient solution prepared from a 16:5:16 NPK water-soluble fertilizer (ULTRASOL, SQM North America, Atlanta, GA, USA). Four weeks after seeding, individual equal-sized plants were transplanted into 15.2-cm-diam. clay pots filled with a mixture of 666 cm^3 steam-sterilized topsoil and 333 cm^3 PROFILE porous ceramic, greens grade, soil conditioner (Profile Products LLC, Buffalo Grove, IL, USA). Media in each pot was fertilized every 10 d with 150 ml of a 200 ppm nitrogen, 62.5 ppm phosphorus, 200 ppm potassium solution prepared using a 16:5:16 NPK water-soluble fertilizer. Five grams of slow release fertilizer (OSMOCOTE 14:14:14 NPK) was also applied to each pot just after transplanting. Plants were watered uniformly from above as needed to maintain moisture content.

Source and application methods of inducers: PGPR mixtures were obtained from Gustafson LLC and Bayer CropScience (Research Triangle Park, NC). Bacteria were formulated on a dry flake carrier to achieve a bacterial concentration of $3x10^9$cfu/g. Prior to filling pots, each of the PGPR formulations was mixed separately with a topsoil:Profile mixture at a rate of 593.5 g per cubic meter of mixture, to obtain 0.6 g of product per pot (1000 cm^3 soil) or $1.8x10^9$ cfu/pot. ASM was applied as a foliar spray of 200 mg/L water to run-off, every 10 d, for 11 weeks after transplanting, for a total of seven sprays. The average quantity of ASM applied in each of the seven sprays was 0.5, 2, 4, 4, 10, 10, and 10 mg/plant respectively, equivalent to 20.25 g a.i./1,000 plants.

Nematode inoculum preparation and infestation: Stock populations of *G. t. solanacearum* were maintained continuously on tomato (*Solanum lycopersicum* cv. Rutgers) in greenhouse culture. The *G. t. solanacearum* population was transferred to new plants approximately every 90 d. Cysts were extracted using a modified Fenwick can and crushed in a blender for one minute to release eggs (Shepherd, 1986). Contents of the blender were passed through a 150 μm sieve nested over a 25 μm sieve. Eggs retained on the 25 μm sieve were diluted to 500 eggs/ml with tap water. Ten milliliters of the nematode egg suspension, containing approximately 5,000 eggs, were added to each pot one week after transplanting. Each pot was infested by adding inoculum to three 1-cm wide by 2.5-cm deep holes spaced 3-cm apart at the base of the plant stem. Holes were filled with soil immediately after infestation and watered lightly from above.

Nematode extraction and plant morphological data: Mature cysts were extracted from a 250 cm^3 sub-sample of soil from each pot 10 wks after infestation using a modified semi-automatic elutriator and sucrose centrifugation technique (Byrd et al., 1976). Cysts from 250 cm^3 soil were counted under 40x, then crushed in a blender to release eggs, which were then stained with acid fuchsin (Byrd et al., 1983). Stained eggs were counted at 40x in two 10 ml aliquots sub-sampled from a 250 ml suspension in tap water. Reproductive index was calculated by dividing the egg population at the end of the experiment (P_f /1000 cm^3 soil) by the initial egg inoculum level (P_i = 5,000). Additionally, plant height, fresh stalk and leaf weight, fresh and dry root weight, and leaf numbers were measured, and fresh shoot to root weight ratio was calculated, at the end of the experiments. The dry root weight of each plant was obtained by drying the roots in a hot air oven for 5 d at 65°C.

Statistical analysis: Analyses of variance (PROC GLM) were conducted using SAS 9.1v software (SAS Institute, Cary, NC) to evaluate treatment effects. All nematode data were log-transformed ($\log_{10}$ x+1) prior to statistical analysis. Treatment means were compared using the Waller-Duncan *t*-test (*k*-ratio = 100).

Field experiments: Separate field experiments were conducted using *G. t. solanacearum*-susceptible oriental tobacco cv. Xanthi and flue-cured tobacco cv. K326 in 2006 and 2007 at the Southern Piedmont Agricultural Research and Extension Center, Blackstone, Virginia. The oriental tobacco experiment was conducted in both years, while the flue-cured tobacco test was conducted only in 2007. Each experiment was arranged in a randomized complete block design with four replications. Plots were 12.2 m long and 1.2 m wide, with two border rows on either side of a single data row. Plants were spaced 0.3 m apart. Based on the initial greenhouse trials, treatments included in the oriental tobacco experiments were 1) an untreated control; 2) seedlings grown in medium amended with IN937a + A13 at the rate of 593.5 g per cubic meter of mixture; 3) hand application of approximately 1 g of IN937a + A13 per plant 7 d after transplanting (DAT); 4) seedlings grown in IN937a + A13-amended medium sprayed 7 DAT with 200 mg/L ASM at the rate of 7.57 g a.i./7,000 plants; 5) 200 mg/L ASM sprayed 21 DAT at the rate of 7.55 g a.i./7,000 plants; 6) ASM sprayed 42 DAT at the rate of 7.58 g a.i./7,000 plants; and 7) 200 mg/L ASM sprayed every 10 d at the rate of 7.66 g a.i./7,000 plants on an average, from 7 DAT until harvest at 63 DAT. The three treatments in the flue-cured tobacco experiment included an untreated control; seedlings grown in IN937a + A13-amended medium; and ASM applied every 10 d, from seven DAT until harvest. Cultivation practices in both experiments followed recommendations of Virginia Cooperative Extension (Reed et al., 2007).

Nematode soil sampling, extraction, and counting: Soil samples were collected the day before transplanting and at harvest. Twenty-five soil cores, 0.30-m deep and 0.05-m wide, were collected from each data row. Core samples were bulked and stored at room temperature until processing (Byrd et al., 1983). Soil samples were processed in batches over a two-day period. Soil from each bag was first passed through a 2,800 μm sieve and a 250 cm^3 subsample was placed into a modified Fenwick can. Cysts were floated from soil in water for five minutes, washed through a 710 μm sieve, and collected on a 250 μm sieve. Material retained in the 250 μm sieve was stored in a glass vial until cysts were counted under a stereomicroscope at 40x. Cysts were subsequently crushed in a blender for one minute to release eggs, which were stained with acid-fuchsin. Two 10 ml aliquots of eggs suspended in tap water were then counted at 40x and the average recorded (Caswell et al., 1985; Byrd et al., 1983).

Nematode root sampling, staining, and counting: Root samples were collected between 14 and 18 DAT, and later at 63 DAT. On each date, two plants were randomly selected and carefully removed from the data row in each plot, cleaned with tap water, and blotted dry. After fresh whole and feeder root weights were recorded, a 1 g feeder root sub-sample was collected randomly from each plant. Feeder root subsamples were cut into sections approximately 0.5-cm long and soaked in a 1.6% sodium hypochlorite solution for five minutes for surface sterilization and to remove any soil adhering to root surfaces. Root pieces were then rinsed thoroughly with tap water on a 25 μm sieve and transferred back to 100 ml of tap water. Ten milliliters of acid-fuchsin stain was added and root pieces were microwaved for 70 sec at medium power level (Byrd et al., 1983). The stained roots were transferred to a 25 μm sieve and rinsed under tap water. Roots were microwaved for 70 sec at medium power level after being transferred to a flask and addition of 50 mL acidified glycerin. Stained roots were examined under a stereomicroscope at 40x to count *G. t. solanacearum*, which were categorized into vermiform (second-stage juveniles successfully penetrated into the root without any obvious feeding), swollen (a distinct sausage-shaped juvenile), flask-shaped or pyriform, and saccate adult females bearing eggs. Vermiform juveniles were considered "preparasitic", while swollen, pyriform, and saccate *G. t. solanacearum* were classified as "parasitic" stages.

Plant morphological data: Plant morphological data were obtained from the two plants collected on each sample date. Plant height, fresh weight of stalks, leaves, whole roots and fibrous feeder roots, and number of leaves, were recorded. Dry weight of whole roots was also recorded after drying at 60°C for seven days.

Statistical analysis: Analyses of variance (PROC GLM) were conducted using SAS 9.1v software (SAS Institute, Cary, NC) to evaluate treatment effects. All nematode data were log-transformed ($\log_{10}$ x+1) prior to statistical analysis. Combined analyses of variance were conducted and combined results are presented where interactions between treatments and experiments were not statistically significant ($P \leq 0.05$). Treatments means were compared using the Waller-Duncan *t*-test (*k*-ratio = 100).

Results

Greenhouse experiments

Effects of PGPR and ASM on nematode reproduction: ASM and IN937a + A13 significantly (*k*-ratio = 100) reduced final numbers of cysts per 250 cm^3 of soil, and per gram dry root weight, compared to the untreated control in both 2005 tests on oriental tobacco (Table 1). Final numbers of cysts per gram dry root weight were also lower for the other three PGPR combined with A13 compared to the untreated control in the fall 2005 study, but final numbers of cysts per 250 cm^3 soil were actually higher for SE34 + A13 than for the untreated control in the spring 2005 trial. Reproductive indices for SE34 + A13 and IN937b + A13 were also higher than

TABLE 1. Influence of acibenzolar-*S*-methyl (ASM) and four combinations of plant-growth promoting rhizobacteria on reproduction of *Globodera tabacum solanacearum* on oriental (cv. Xanthi) and flue-cured (cv. K326) tobacco. Results were obtained under greenhouse conditions at the Virginia Polytechnic Institute and State University in Blacksburg, VA.*

	ORIENTAL TOBACCO						FLUE-CURED TOBACCO					
	Cysts /250cm^3 soil		Cysts/g dry root weight		Reproductive index (P_f/P_i)		Cysts/250cm^3 soil		Cysts/g dry root weight		Reproductive index (P_f/P_i)	
Treatment	Spring 2005	Fall 2005	Spring 2005	Fall 2005	Spring 2005	Fall 2005	Spring 2006	Fall 2006	Spring 2006	Fall 2006	Spring 2006	Fall 2006
Untreated	32.0b	44.7a	15.4ab	30.5a	5.7c	3.3a	26.3ab	38.3a	18.8a	8.2ab	1.2ab	2.3a
SE34+A13	78.2a	22.5ab	22.7a	13.0b	18.4a	4.0a	31.3a	24.0b	12.9ab	4.2c	1.6ab	1.4bc
IN937b+A13	54.3ab	25.0ab	17.2ab	12.7b	13.8ab	3.7a	42.5a	24.5b	16.5ab	3.9c	2.9a	1.7ab
INR7+A13	48.5ab	29.0ab	14.4b	10.1b	7.9bc	2.2ab	17.5ab	21.5b	5.4b	3.6c	1.1ab	1.2bc
IN937a+A13	17.7c	15.5b	6.4c	7.0b	4.4c	2.5ab	16.8ab	23.8b	8.0ab	5.2bc	1.6ab	1.2bc
ASM	17.2c	17.0b	8.1c	9.0b	3.4c	1.2b	15.7b	23.7b	16.1ab	9.9a	0.7b	0.8c

* Data presented are non-transformed means of six replications. INR7 = *Bacillus pumilus* INR7; SE34 = *B. pumilus* SE34; IN937b = *B. licheniformis* INR937b; IN937a = *B. amyloliquifaciens* IN937a; A13 = *B. subtilis* A13. Means within a column followed by the same letter(s) are not significantly different according to the Waller-Duncan *t*-test (*k*-ratio =100).

for the untreated control in the spring 2005 experiment. No PGPR combined with A13 reduced nematode reproductive index in either trial. Only ASM reduced reproductive index significantly (*k*-ratio = 100) compared to the untreated control, and that only in the fall 2005 oriental tobacco test (Table 1).

ASM and all of the PGPR combinations significantly (*k*-ratio = 100) reduced final numbers of cysts per 250 cm^3 of soil in the fall 2006 test on flue-cured tobacco, but differences with the untreated control were not statistically significant in the spring 2006 test (Table 1). Final numbers of cysts per 250 cm^3 soil were higher for IN937b + A13 and SE34 + A13 than when ASM had been applied in the spring 2006 experiment. Mixtures of INR7, SE34, and IN937b with A13 significantly (*k*-ratio = 100) reduced cysts per gram dry root weight compared to the untreated control and ASM in the fall 2006 test (Table 1). However, only INR7 + A13 reduced cysts/gram dry root weight compared to the untreated control in the spring 2006 test (Table 1). The only significant difference (*k*-ratio = 100) in nematode reproductive index in the spring 2006 trial was between IN937b + A13 and ASM; all other differences were not statistically significant. However, ASM and all of the PGPR treatments except IN937b + A13 significantly (*k*-ratio = 100) reduced reproductive index compared to the untreated control in the fall 2006 test (Table 1).

Effects of PGPR and acibenzolar-S-methyl on tobacco growth: There were no differences (*k*-ratio =100) between the untreated control and any of the PGPR combinations with respect to plant height, fresh weight of stalks and leaves, or number of leaves, for either oriental or flue-cured tobacco (Table 2). Application of ASM was associated with reduced height (*k*-ratio =100) of flue-cured, but not oriental, tobacco (Table 2). Use of ASM also reduced fresh stalk and leaf weight

TABLE 2. Influence of acibenzolar-*S*-methyl (ASM) and plant-growth promoting rhizobacteria on oriental (cv. Xanthi) and flue-cured (cv. K326) tobacco growth under greenhouse conditions at the Virginia Polytechnic Institute and State University in Blacksburg, VA.*

	Oriental tobacco, 2005									
	Plant height (cm)		Fresh stalk and leaf weight (g)		Fresh root weight (g)		Dry root weight (g)		Leaf number	
Treatment	Spring	Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring	Fall
Untreated	73.7a	84.2a	225.5a	225.8a	34.5a	33.9a	8.4bc	6.5b	23.2a	22.8ab
SE34+A13	82.6a	83.8a	239.6a	232.5a	37.2a	33.0a	13.3a	8.0b	21.5ab	24.3a
IN937b+A13	81.7a	83.0a	238.2a	225.8a	36.9a	33.0a	11.9a	8.9ab	21.3ab	24.5a
INR7+A13	84.2a	84.2a	241.2a	225.0a	41.9a	38.3a	13.0a	12.4a	21.5ab	24.8a
IN937a+A13	85.9a	82.6a	239.2a	229.2a	38.7a	37.0a	11.0ab	8.7ab	20.7ab	25.7a
ASM	69.9a	79.6a	197.2b	190.8b	24.3b	23.9b	8.3c	8.2ab	19.8b	20.7b
	Flue-cured tobacco, 2006									
Treatment	Spring	Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring	Fall
Untreated	103.8a	99.7a	349.7a	339.2ab	39.9b	98.6a	8.5a	20.6a	21.8a	21.7a
SE34+A13	98.2ab	99.5a	363.1a	340.8ab	50.9ab	109.6a	9.9a	24.2a	20.2ab	23.3a
IN937b+A13	102.4a	99.7a	367.3a	364.3a	51.6ab	126.2a	9.9a	26.3a	20.5ab	21.8a
INR7+A13	107.2a	103.9a	388.1a	327.3ab	61.3a	122.8a	13.1a	27.8a	21.8a	21.3a
IN937a+A13	98.2ab	102.0a	315.6a	334.0ab	59.1ab	97.9a	8.8a	18.9a	19.7ab	22.0a
ASM	68.7b	84.7b	217.3b	317.8b	21.1c	65.2b	3.6b	10.8b	18.5b	23.5a

* Data presented are non-transformed means of six replications. INR7 = *Bacillus pumilus* INR7; SE34 = *B. pumilus* SE34; IN937b = *B. licheniformis* INR937b; A13 = *B. subtilis* A13; IN937a+A13 = *B. amyloliquifaciens* IN937a + *B. subtilis* A13. Means within a column followed by the same letter(s) are not significantly different according to the Waller-Duncan *t*-test (*k*-ratio =100).

compared to the untreated control in the oriental tobacco trials and in the spring 2006 flue-cured tobacco experiment, and was associated with lower fresh weight of above ground plant parts in the fall 2006 test compared to IN937b + A13. Fewer leaves were also associated with ASM versus the untreated control in the oriental tobacco experiments and in the spring 2006 trial.

Fresh root weights were similar between the PGPR treatments and the untreated control in the oriental tobacco tests and in the fall 2006 flue-cured tobacco study (Table 2). In the spring 2006 flue-cured tobacco experiment, fresh root weight was higher (*k*-ratio = 100) for INR7 + A13 than for the untreated control. All PGPR combinations except IN937a + A13 increased dry root weight of oriental tobacco versus the untreated control in the spring 2005 test, but an increase was only noted for INR7 + A13 in the fall 2005 experiment (Table 2), and dry weights of flue-cured tobacco roots were similar among PGPR treatments and the untreated control in all experiments (Table 2). ASM reduced root fresh weight of flue-cured and oriental tobacco compared to all other treatments in all experiments, but reductions in dry root weight versus the untreated control only occurred in the flue-cured tobacco tests (Table 2).

Field experiments

Effects of IN937a + A13 and ASM on G. t. solanacearum penetration, development, and reproduction: Growing seedlings in media amended with IN937a + A13 did not reduce numbers of *G. t. solanacearum* vermiform juveniles in roots 14-18d after transplanting (DAT) for either flue-cured or oriental tobacco in any of the three field experiments (Table 3). In 2007, treatment with IN937a + A13 alone reduced (*k*-ratio = 100) the number of *G. t. solanacearum* swollen juveniles in flue-cured tobacco roots and the number of adult *G. t. solanacearum* in roots of oriental tobacco, but otherwise the number of *G. t. solanacearum* juveniles in tobacco roots 14-18 DAT was similar to that in untreated roots. One ASM application 7 DAT did not reduce nematode parasitism of oriental tobacco 7-11d later in 2006 or 2007, but did reduce (*k*-ratio = 100) *G. t. solanacearum* numbers in flue-cured tobacco roots in 2007 (Table 3). However, in both field experiments, ASM application 7 DAT to transplants grown in IN937a + A13-amended media generally reduced (*k*-ratio = 100) the number of *G. t. solanacearum* juveniles of all life stages in oriental tobacco roots 14-18 DAT (Table 3).

The only treatment differences among nematode numbers 63 DAT were in the number of *G. t. solanacearum* swollen juveniles in oriental tobacco roots (Table 4). Plants originally produced in IN937a + A13-amended media contained fewer (*k*-ratio = 100) *G. t. solanacearum* swollen juveniles 63 DAT compared to untreated plants in 2007 (Table 4). In 2006, fewer (*k*-ratio = 100) *G. t. solanacearum* swollen juveniles were found in roots of plants treated with IN937a + A13 at seeding compared to those sprayed with ASM 42 DAT. In 2007, fewer (*k*-ratio = 100) *G. t. solanacearum* swollen juveniles were noted in plants treated with IN937a + A13, or sprayed with ASM 21 DAT, compared to plants treated with ASM five times on a 10d interval. No statistically significant treatment differences were observed in nematode numbers 63 DAT in flue-cured tobacco (Table 4).

Nematode reproductive index data were combined across 2006 and 2007 oriental tobacco experiments

TABLE 3. Effects of acibenzolar-*S*-methyl (ASM) and *Bacillus amyloliquefaciens* IN937a + *Bacillus subtilis* A13 (IN937a+A13) on *Globodera tabacum solanacearum* root development 14-18 d after transplanting oriental (cv. Xanthi) and flue-cured tobacco (cv. K326) in the field at the Southern Piedmont Agricultural Research and Extension Center, Blackstone, VA.*

	Oriental tobacco (cv. Xanthi):							
	G. t. solanacearum/g feeder root fresh weight							
	Vermiform		Swollen		Pyriform		Adult	
Treatment	2006	2007	2006	2007	2006	2007	2006	2007
Untreated	61.1a	24.8a	132.9a	32.3a	13.4b	5.0a	1.3a	1.3ab
IN937a+A13 at seeding (BY-S)[a]	71.3a	24.3a	121.8ab	34.8a	30.0a	7.3a	1.8a	2.0a
IN937a+A13 7d after transplanting	41.8a	19.5a	101.3ab	35.5a	13.9b	7.3a	1.1a	0.3c
BY-S+ASM 7d after transplanting[a]	28.3a	6.5b	70.3b	16.8b	4.8c	1.5b	0.0b	0.5bc
ASM 7d after transplanting	38.6a	27.8a	89.8ab	27.3a	13.3b	3.0ab	0.8ab	1.0abc
	Flue-cured tobacco (cv. K 326):							
Treatment	2006	2007	2006	2007	2006	2007	2006	2007
Untreated	*nt*	78.0a	*nt*	102.0a	*nt*	22.0a	*nt*	4.8a
IN937a+A13 at seeding	*nt*	61.8a	*nt*	70.3b	*nt*	22.5a	*nt*	2.8a
ASM 7d after transplanting	*nt*	25.5b	*nt*	29.5b	*nt*	5.0b	*nt*	1.5b

* Data presented are non-transformed means of four replications, each containing two sub-samples for a total of eight observations within each mean. Data were log-transformed [$Log_{10}(x+1)$] prior to statistical analysis. Means within a column followed by the same letter(s) are not significantly different according to the Waller-Duncan *t*-test (*k*-ratio =100).

[a] BY-S = IN937a+A13 mixture incorporated into soil-less medium at seeding.

nt = not tested.

TABLE 4. Effects of acibenzolar-*S*-methyl (ASM) and *Bacillus amyloliquefaciens* IN937a + *Bacillus subtilis* A13 (IN937a+A13) on *Globodera tabacum solanacearum* root development 63 d after transplanting and reproductive index (P_f/P_i) in oriental (cv. Xanthi) and flue-cured tobacco (cv. K326) in the field at the Southern Piedmont Agricultural Research and Extension Center, Blackstone, VA.

	Oriental tobacco (cv. Xanthi):								
	G. t. solanacearum/g feeder root fresh weight*								Reproductive index** (P_f/P_i)
	Vermiform		Swollen		Pyriform		Adult		
Treatment	2006	2007	2006	2007	2006	2007	2006	2007	2006+2007
Untreated	19.8a	1.6a	7.5ab	1.0ab	4.9a	0.5a	1.3a	0.3a	12.4a
IN937a+A13 at seeding (BY-S)[a]	3.9a	0.9a	2.1b	0.3c	2.5a	2.3a	0.7a	0.3a	6.4abc
IN937a+A13 7d after transplanting	14.8a	1.0a	6.8ab	0.4bc	2.1a	0.4a	0.5a	0.1a	15.5a
BY-S and ASM 7d after transplanting[a]	11.8a	0.4a	5.5ab	0.5bc	4.1a	1.3a	1.2a	0.1a	2.7c
ASM 21d after transplanting	8.8a	1.3a	5.0ab	0.4bc	2.8a	0.5a	0.7a	0.3a	11.7abc
ASM 42d after transplanting	14.0a	0.5a	7.9a	1.0ab	3.9a	0.6a	1.5a	0.0a	7.9abc
ASM every 10d (5 sprays)	5.2a	0.9a	2.0b	1.8a	3.3a	0.9a	1.8a	0.0a	5.4bc
	Flue-cured tobacco (cv. K 326):								
Treatment	2006	2007	2006	2007	2006	2007	2006	2007	2007**
Untreated	*nt*	35.5a	*nt*	7.8a	*nt*	2.5a	*nt*	6.0a	20.7a
IN937a+A13 at seeding	*nt*	27.5a	*nt*	8.5a	*nt*	2.8a	*nt*	2.5a	13.3a
ASM every 10d (5 sprays)	*nt*	21.0a	*nt*	7.0a	*nt*	3.8a	*nt*	3.5a	14.8a

* Data presented are non-transformed means of four replications, each containing two sub-samples, for a total of eight observations per mean. Data were log-transformed [$Log_{10}(x+1)$] prior to statistical analysis. Means within a column followed by the same letter(s) are not significantly different according to the Waller-Duncan *t*-test (*k*-ratio =100).

** Oriental tobacco experiments were conducted in 2006 and 2007; the flue-cured tobacco experiment was conducted in 2007. Data presented are non-transformed means across the four replications of the two oriental tobacco tests and of the four replications in the flue-cured tobacco trial. Data were log-transformed [$Log_{10}(x+1)$] prior to statistical analysis. Means within a column followed by the same letter(s) are not significantly different according to the Waller-Duncan *t*-test (*k*-ratio =100).

[a] BY-S = IN937a+A13 mixture incorporated into soil-less medium at seeding.

nt = not tested.

because an analysis of variance combined across field experiments in those years found no significant interactions ($P \leq 0.05$) between treatments and experiments. Spraying an average of 7.66 g a.i. ASM /7,000 plants on foliage every 10 d, or one 7.57 g a.i./7,000 plants ASM spray 7 DAT to plants grown on IN937a + A13-amended medium, significantly reduced (*k*-ratio = 100) nematode reproductive index on oriental tobacco (Table 4). Nematode reproduction was similar to the untreated control in all other treatments. Nematode reproductive index was also similar among the three treatments in the 2007 flue-cured tobacco test (Table 4).

Effects of IN937a + A13 and ASM on tobacco growth: All IN937a + A13 treatments increased (*k*-ratio =100) stalk and leaf weight in the 2006 oriental tobacco test compared to the untreated control, as did a single ASM application 42 DAT, but trends among treatments in fresh stalk and leaf weight were not significant (*k*-ratio =100) in the 2007 oriental tobacco test (Table 5). Apparent treatment differences in fresh and dry root weights were not statistically significant in 2006, but incorporating IN937a + A13 into seedling-growth media was associated (*k*-ratio =100) with increased fresh root weights of oriental tobacco in 2007, and with increased dry root weight when ASM was not also applied (Table 5). Five applications of ASM every 10 d reduced oriental tobacco dry root weight and fresh feeder root weight compared to the untreated control (Table 5). The only treatment difference in plant growth characteristics in the 2007 flue-cured tobacco field experiment was a lower (*k*-ratio =100) dry root weight (k-ratio = 100) when plants had been sprayed every 10 d with ASM compared to plants that grew in IN937a + A13-amended medium in the greenhouse prior to transplanting (Table 5).

DISCUSSION

Application of ASM or IN937a + A13 consistently reduced *G. t. solanacearum* parasitism in our greenhouse experiments, particularly on oriental tobacco, and a combination of the two treatments reduced parasitism in the field. Effects on *G. t. solanacearum* parasitism by combinations of A13 with INR7, SE34, or IN937b were less consistent. Increases in *G. t. solanacearum* reproduction were associated with the SE34 + A13 and IN937b + A13 mixtures in our greenhouse tests. Some PGPR have been shown to alter root architecture by enhancing lateral root growth (Mantelin et al., 2006), which could influence the nematode infection process, most likely by increasing the number of penetration sites available to second stage *G. t. solanacearum* juveniles. Although our results did not indicate the mechanisms involved in the suppression of nematode parasitism by ASM and IN937a + A13, but ASM, IN937a, and IN937a + A13 have been previously reported to induce resistance to various pathogens of tobacco and other crops (Cole, 1999; Park and Kloepper, 2000; Csinos et al., 2001; Johnson, 2004; Perez et al., 2003; Mandal et al, 2008; Kokalis-Burelle et al, 2002). Our results

TABLE 5. Influence of acibenzolar-*S*-methyl (ASM) and *Bacillus amyloliquefaciens* IN937a + *B. subtilis* A13 (IN937a+A13) on tobacco growth 63-70 d after transplanting in the field at the Southern Piedmont Agricultural Research and Extension Center, Blackstone, VA.*

Oriental tobacco (cv. Xanthi):								
	Fresh stalk and leaf weight (g)		Fresh root weight (g)		Dry root weight (g)		Fresh feeder root weight (g)	
Treatment	2006	2007	2006	2007	2006	2007	2006	2007
Untreated control	813.4c	705.0a	73.5a	90.0cd	25.7a	31.5bc	12.6a	17.2a
IN937a+A13 at seeding (BY-S)[a]	882.2b	863.8a	69.2a	149.0a	24.4a	49.6a	12.2a	21.2a
IN937a+A13 7d after transplanting	853.5b	758.8a	72.7a	118.7abc	23.7a	37.1b	12.6a	20.0a
BY-S and ASM 7d after transplanting[a]	1089.8ab	796.3a	73.2a	126.9ab	21.2a	35.2bc	13.9a	19.8a
ASM 21d after transplanting	807.4bc	708.1a	72.0a	103.2bcd	23.2a	29.7c	10.6a	15.4ab
ASM 42d after transplanting	1140.8a	855.0a	84.9a	00.4bcd	29.3a	29.8c	13.4a	15.9ab
ASM every 10 d (5 sprays)	955.6abc	733.8a	86.7a	83.7d	26.1a	23.5d	13.7a	11.9b
	Flue-cured tobacco (cv. K 326):							
	2006	2007	2006	2007	2006	2007	2006	2007
Untreated	*nt*	1215.1a	*nt*	119.7a	*nt*	28.9ab	*nt*	13.2a
IN937a+A13 at seeding	*nt*	1378.8a	*nt*	133.6a	*nt*	33.9a	*nt*	17.4a
ASM every 10d (5 sprays)	*nt*	1151.3a	*nt*	97.5a	*nt*	24.7b	*nt*	12.8a

* Data presented are non-transformed means of four replications (ASM – acibenzolar-*S*-methyl, DAT – Days after transplanting). Means within a column followed by the same letter(s) are not significantly different according to the Waller-Duncan *t*-test (*k*-ratio = 100).

[a] BY-S = IN937a+A13 mixture incorporated into soil-less medium at seeding.

nt = not tested.

agree with reports from other crops demonstrating possible induced resistance effects to nematodes through either SAR or ISR mechanisms (Chinnasri and Sipes, 2005; Hasky-Günther et al., 1998; Hoffmann-Hergarten et al., 1998; Kempster et al., 2001; Oka and Cohen, 2001; Reitz et al., 2000).

The application methods for ASM and IN937a + A13 were altered in our field trials to accommodate practical considerations of tobacco production, making it difficult to identify factors responsible for the differing results between greenhouse and field trials. The more promising field trial results obtained from root samples collected early in the growing season (versus harvest) suggest that some re-application of inducers may be necessary in the field to extend induced resistance throughout the entire growing season (Anderson et al., 2006; Leadbeater and Staub, 2007). In tobacco, such follow-up applications might be possible and practical because the crop is normally cultivated two to three times during the first month after transplanting (Hawks and Collins, 1983). Various formulations of bacteria or PGPR could also be tested for their suitability and performance in extending suppression of plant-parasitic nematodes throughout the growing season.

However, combining production of tobacco transplants in IN937a + A13-amended medium with a single application of ASM shortly after transplanting resulted in reduced *G. t. solanacearum* parasitism and reproduction on oriental tobacco. Although application of ASM alone every 10 d was consistently associated with reduced *G. t. solanacearum* parasitism, phytotoxicity and reductions in plant growth were observed in some circumstances. Mandal et al. (2008) noted phytotoxicity from a single application of 4.0 g ASM/7,000 plants to flue-cured tobacco seedlings. Their greenhouse research applied ASM in a foliar spray that was followed by a subsequent water spray that moved ASM to the root zone. We also observed phytotoxic symptoms in our greenhouse work, although our foliar ASM applications did not involve subsequent "wash-off" sprays that could reduce foliar absorbance of the compound, but increase the chances of possible root uptake. Our field ASM applications were not followed by wash-off sprays. In addition, the spray volume applied in our field tests, based on product label specifications, did not completely cover all foliage. A spray volume in our field experiments sufficient to provide complete coverage "to run-off", as in our greenhouse tests, would have significantly increased the amount of ASM available for foliar absorbance from each ASM application, perhaps resulting in more phytotoxic symptoms. However, as applied in the experiments reported here, a single application of ASM at 7.57 g a.i. ASM/7,000 plants, combined with PGPR, consistently reduced *G. t. solanacearum* in our field testing, without any phytotoxicity.

In summary, application of ASM reduced *G. t. solanacearum* parasitism in flue-cured and oriental tobacco, but also often induced undesirable effects on plant growth. Application of a combination of the PGPR IN937a and A13 suppressed *G. t. solanacearum* reproduction without undesirable effects on plant growth. Further research is necessary to confirm the principle mechanisms behind the observed suppression of nematode parasitism by ASM and IN937a + A13, respectively, and to improve the consistency and extent of nematode suppression. Exploring alternative application methods, such as timing, number of applications, and product formulation, may be important in identifying practical systems to optimize disease control from induced resistance.

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