Biological and Molecular Analyses of the Acibenzolar-S-Methyl-Induced Systemic Acquired Resistance in Flue-Cured Tobacco Against *Tomato spotted wilt virus*

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ABSTRACT

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Tomato spotted wilt virus (TSWV) is an economically important virus of flue-cured tobacco. Activation of systemic acquired resistance (SAR) by acibenzolar-S-methyl (ASM) in flue-cured tobacco was studied under greenhouse conditions by challenge inoculation with a severe isolate of TSWV. ASM restricted virus replication and movement, and as a result reduced systemic infection. Activation of resistance was observed within 2 days after treatment with ASM and a high level of resistance was observed at 5 days onward. Expression of the pathogenesis-related (PR) protein gene, PR-3, and different classes of PR proteins such as PR-1, PR-3, and PR-5 were detected at 2 days post-ASM treatment which inversely correlated with the reduction in the number of local lesions caused by TSWV. Tobacco plants treated with increased quantities of ASM (0.25, 0.5, 1.0, 2.0, and 4.0 g a.i./7,000 plants) showed increased levels of SAR as indicated by the reduction of both local and systemic infections by TSWV. The highest level of resistance was at 4 g a.i., but this rate of ASM also caused phytotoxicity resulting in temporary foliar spotting and stunting of plants. An inverse correlation between the TSWV reduction and phytotoxicity was observed with the increase of ASM concentration. ASM at the rate of 1 to 2 g a.i./7,000 plants activated a high level of resistance and minimized the phytotoxicity. Use of gibberellic acid in combination with ASM reduced the stunting caused by ASM. Present findings together with previous field experiments demonstrate that ASM is a potential option for management of TSWV in flue-cured tobacco.

Systemic acquired resistance (SAR) is a broad-spectrum defense system present in plants, which was realized as early as 1901 (5,11,64), and was first demonstrated against a virus by Ross (66) in tobacco against *Tobacco mosaic virus* (TMV). Subsequent studies showed the SAR response to be long lasting and effective against diverse pathogens such as bacteria, fungi, nematodes, and parasitic plants, and was considered a potential tool for crop protection (18,24,31,32,41,56,75). Activation of SAR is associated with a series of signal transduction events and the expression of the SAR gene family (24,69,76,77). The SAR genes encode various pathogenesis-related (PR) proteins, which play an active role in the resistance process against fungal pathogens (2,39,40).

Several synthetic chemicals such as β-aminobutaric acid, isonicotinic acid, benzol [1,2,3] thiadiazol-7-carbothioic acid-S-methyl ester (BTH), acibenzolar-S-methyl (ASM, a derivative of BTH), salicylic acid (SA), and phosphates have been shown to be activators of SAR response in a variety of crop–pathogen systems (7,13,18,20,23,31,33,42,49,56,67,68,72,73,75). Of all these chemicals, only ASM was commercialized as an activator for disease resistance in plants. ASM was discovered in 1989 by Ciba Geigy (Novartis) (20). It was classified and registered as a 'reduced risk compound' in 1998 in the United States (73) and marketed under the trade name Actigard in the United States (55) (Syngenta Crop Protection Inc., Greensboro, NC) and BION in Europe (Syngenta Ltd., Basel, Switzerland). ASM activates the same defense responses as the natural signal molecule SA does in the biological activation of SAR (69). ASM/BTH was shown to activate antifungal and antibacterial activity in several monocot and dicot crop species (1,4,6,8–10,14,15,21,22,25,28,29,37,38,44,45,51,61,65). BTH was shown to activate SAR response against viruses such as TMV in tobacco (20), *Turnip crinkle virus* (TCV) in *Arabidopsis* (36), and *Cucumber mosaic virus* (CMV) in tomato (3).

Tomato spotted wilt virus (TSWV), a member of the genus *Tospovirus* and family *Bunyaviridae* (19), is one of the most widely occurring and economically important plant viruses (53,54, 58,62,71). TSWV is transmitted by several species of thrips (74,80). TSWV is a serious threat to many important crops such as peanut, pepper, flue-cured tobacco, and tomato in the southern United States. In flue-cured tobacco, TSWV causes spotted wilt disease characterized by localized and systemic necrosis and stunting symptoms (17,43). In Georgia, flue-cured tobacco is seriously affected by TSWV causing stand loss in excess of 30% with an estimated loss of more than $17 million in 2004 (60). Control of TSWV in flue-cured tobacco by insecticides and cultural practices was found unsatisfactory (46,47). Resistant cultivars were considered to be the most effective approach for managing TSWV (12). However, no source of resistance against TSWV is available in flue-cured tobacco. Moreover, resistance breaking strains of TSWV were reported (26,27,35,52) which were likely due to genome reassortment (63) making breeding for

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durable TSWV resistance more challenging. The genetic diversity of TSWV isolates from flue-cured tobacco was documented (57). Abundance of thrips vectors, wide range of alternate sources of infection, and lack of host resistance make it difficult to control TSWV in flue-cured tobacco. It was shown that the treatment of flue-cured tobacco (cv. K-326) with ASM resulted in significant reduction of final incidence of spotted wilt disease (16,59). Both studies suggested that ASM alone or in combination with imidacloprid is an effective tool for management of spotted wilt in flue-cured tobacco. ASM was also shown to reduce the incidence of spotted wilt disease caused by TSWV in field-grown tomato (50) and mosaic disease caused by TMV in flue-cured tobacco (48). Although, ASM was shown to reduce spotted wilt disease in flue-cured tobacco under field conditions, the relation between ASM rate of application and development of TSWV infection, time of activation of resistance, and the mechanism of action have not been studied. We report the results of a comprehensive study on the biological and biochemical aspects of ASM-induced SAR in tobacco against a severe isolate of TSWV.

MATERIALS AND METHODS

Source of inoculum. A lettuce isolate of TSWV (GAL), collected from Colquitt County, Georgia, causing severe necrosis and mortality in flue-cured tobacco seedlings, cv. K326, was used in this study (43). The isolate was maintained on cv. K326 in the greenhouse. Fresh leaves with systemic symptoms of TSWV were harvested at 15 days postinoculation (DPI) and were used as the source of inoculum.

Plant materials. In the greenhouse, tobacco seeds of cv. K326 were sown in seed pans containing soil mix consisting of Canadian sphagnum peat moss (75 to 85%), perlite (15 to 20%), and vermiculite 5 to 10% (Berger Peat Moss-Lee Berger Itee, Saint-Modeste, Quebec, Canada). Twenty five to thirty days after seeding (DAS), seedlings were transplanted in Styrofoam flats (Speedling Inc., Sun City, FL) with 6.45 cm^2 cell. Eight to ten days after first transplanting, seedlings were again transplanted to a nine-cell flat floating on water or to pots (24.5-cm diameter).

ASM treatment. Plants of K326 at 40 to 45 DAS were sprayed with water followed by ASM (as 50% active ingredient [a.i.] in wettable powder formulation) dissolved in water (16). Applications were made with a backpack CO_2 powered sprayer fitted with three TX-12 tips and pressurized to 310 kPa. To evaluate the effect of various concentrations of ASM on TSWV infection, plants were treated with ASM at the rates of 0, 0.25, 0.5, 1.0, 2.0, and 4.0 g a.i./7,000 plants. Imidacloprid (Imidacloprid 2F, Bayer Crop Science, Research Triangle Park, NC) was sprayed at the rate of 9.94 g a.i/1,000 plants (16,59). After the application of ASM, the plants were washed by spraying with 80 ml of water per flat containing nine plants, to move the ASM into the root zone. To determine the time required for SAR activation, plants were treated with two concentrations of ASM (2 and 4 g a.i. of ASM/7,000 plants) as described above. To determine the effect of age of tobacco plants on SAR activation, seedlings (47 DAS) and older plants (77 DAS) were treated with 2 g a.i./7,000 plants. For analyzing the PR gene expression, plants were treated with only 2 g a.i. of ASM/7,000 plants. The nontreated control plants were sprayed with distilled water only. Each experiment was repeated at least twice.

Gibberellic acid (GA3) treatment. The effect of GA3 (Sigma Chemical Company, St. Louis, MO) was evaluated to determine its effect in reducing the stunting caused by ASM treatment. GA3 at the rate of 50 µg/plant was used alone and in combination with ASM (2 g a.i./7,000 plants). Plants were treated with GA3 first and then after a few hours ASM was applied. This treatment was compared with ASM alone, GA3 alone, GA3+ASM, and nontreated control and included six replicates. Nine plants (45 DAS) in a Styrofoam flat were used for each replicate in each treatment.

Challenge inoculation. ASM-treated plants were challenged by mechanical inoculation with TSWV. Inoculum was prepared by grinding systemically infected leaves in 0.1 M phosphate buffer, pH 7.0, containing 0.2% Na_2SO_3 and 0.01 M mercaptoethanol at the rate of 1:10 tissue and buffer ratio (wt/vol). Debris was removed by squeezing the extract through a layer of nonabsorbent cotton. To this extract, 2% Carborundum 320 grit and 1% Celite 545 (Fisher Scientific, Fair Lawn, NJ) were added. Inoculum was maintained on ice and applied to the two youngest, fully expanded leaves of each seedling by gently rubbing with a cotton swab dipped in the inoculum. Inoculations were done in the greenhouse after sunset at 25 to 30°C. After inoculation, plants were lightly misted with water.

Plants were inoculated 7 to 8 days posttreatment (DPT) except in the experiment to determine the time of SAR activation, where inoculation was done at 1-day intervals until 7 DPT.

Assessment of TSWV infection, phytotoxicity, and statistical analysis. After inoculation, plants were inspected daily for symptom development. The number of local lesions that developed on the two inoculated leaves of each plant was counted at 6 DPI. Local infection was confirmed by testing a portion of inoculated leaves (200 mg from each plant) by enzyme-linked immunosorbent assay (ELISA). ELISA was performed with a commercially available kit (Agdia Inc., Elkhart, IN). ELISA was conducted in 10-fold serial dilution of the sap to determine the relative levels of TSWV in the inoculated leaves of plants treated with ASM. Two samples from each treatment showing the highest and the lowest ELISA values in the previous test for examination of local infection were selected for the dilution tests. From the four trials, a total of eight plants for each concentration of ASM treatment was analyzed by ELISA. Plants were allowed to grow for a longer period of time after evaluation of local infection to study the development of systemic symptoms. Appearance of systemic symptoms was determined. The presence of TSWV was determined in roots and newly emerged leaves by ELISA. The number of leaf spots developed due to ASM treatment was counted on the treated leaves at 14 DPT. Plant height was measured from the base of the plant to the longest tip of leaf. Root length was measured carefully pulling out of the plant from the cell of the flat.

Data on local lesions, percent plants locally and systemically infected, relative levels of TSWV, and phytotoxicity were analyzed by SAS (SAS version 5, SAS Institute, Cary, NC). The local lesion data at varying concentrations of ASM were fit to the nonlinear regression model: $a \times e$ ($-bx$ rate). To analyze the effect of ASM on the local and systemic infection, multiple regression analysis was based on the following model: $y = I + aX + bX^2$, where regression coefficients are I = 105.3, a = –34.6, and b = 2.77. The association between ELISA absorbance values and ASM rate was quantified by polynomial regression (imidacloprid treatment was excluded). The model used was $y = a + bA_L + cA_Q + dT_L + eT_Q + fA_LT_L + gA_LT_Q + hA_QT_L + iA_QT_Q + e$, where y = measured data, a = intercept, $b - i$ = coefficient determined during regression, A_L = the linear effect of $\log_e$ ASM rate, A_Q = the quadratic effect of $\log_e$ ASM rate, T_L = the linear effect of $\log_{10}$ dilution level, T_Q = the quadratic effect of $\log_{10}$ dilution level, A_LT_L, A_LT_Q, A_QT_L, A_QT_Q = interaction, and e = unexplained error.

Local lesion data were subjected to analysis of variance in SAS. The experiment had a factorial design with ASM treatments and inoculation as factors. Time was fitted to a quadratic equation to relate number of lesions to DPT of ASM. A sampling unit consisted of the mean number of lesions per leaf calculated from eight plants evaluated separately.

Assay of mRNA of PR protein induced. Five leaves were collected from each treated and nontreated plant and pooled for total RNA extraction. The ASM-treated samples included were (i) treated leaves showing spots from phytotoxicity and (ii) top young leaves that emerged after ASM treatment from the same

plant as in sample 1, which were not showing any phytotoxic symptoms. The nontreated control samples included were from lower and upper leaves with the corresponding position as in case of the treated plants. Nontreated cowpea (*Vigna unguculata*) leaf tissues were included as a non-tobacco control. Total RNA was isolated using RNeasy Mini Kit (Qiagen Inc., Valencia, CA) according to manufacturer's instructions. From the total RNA, mRNA was purified by the Oligotex mRNA Kit (Qiagen Inc.). Equal amount of total mRNA (800 ng) for each sample was electrophoresed in a formaldehyde agarose gel following the protocol described by the manufacturer. The gel was blotted to a nylon membrane by the capillary transfer method (70). Biotinylated DNA probe to PR-Q gene was prepared by random priming method using north2South Biotin Random Prime Kit (Pierce, Rockford, IL). PR-Q clone was obtained from L. Friedrich (Novartis). The mRNA blot was hybridized with the nonradioactive probe to PR-Q DNA using North2South Chemiluminescent Nucleic Acid Hybridization and Detection Kit (Pierce).

Protein extraction and western blot assay for PR proteins. Five plants treated with ASM or water were collected daily until 10 DPT. On each day, 1.5 g of leaf sample was collected from five plants and stored at –80°C. The nontreated sample was collected from the water-treated plants and was collected until 3 DPT, and then was pooled as one sample. Total protein was extracted from treated or nontreated samples following the methods described by Jung et al. (30) and Ziadi et al. (81). Briefly, frozen leaf material of 1.5 g was ground in 2.5 ml of 0.5 M sodium acetate buffer, pH 5.2, containing 15 mM 2-mercaptoethanol. The extract was centrifuged at 15,000 × *g* for 15 min, and the supernatant was desalted by dialyzing for 18 to 20 h with three changes of 0.05 M sodium acetate buffer, pH 5.2.

Total protein content in the supernatant of the each sample was determined in six replicates by using the Bio-Rad Protein Assay Kit II with bovine serum albumin as standard (Bio-Rad Laboratories, Hercules, CA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done as previously described (30,34). To visualize the protein bands induced by ASM, the gel was stained with Coomassie blue. To detect PR proteins, western blot assays were performed using antisera to tobacco PR proteins PR-1(C), PR-2(2), PR-3(Q), PR-5(S), PR-6(basic), and PR-8(acidic) (antisera were provided by B. Fritig, Institute de Biologie Moleculaire des Plantes, Strassbourgh, France).

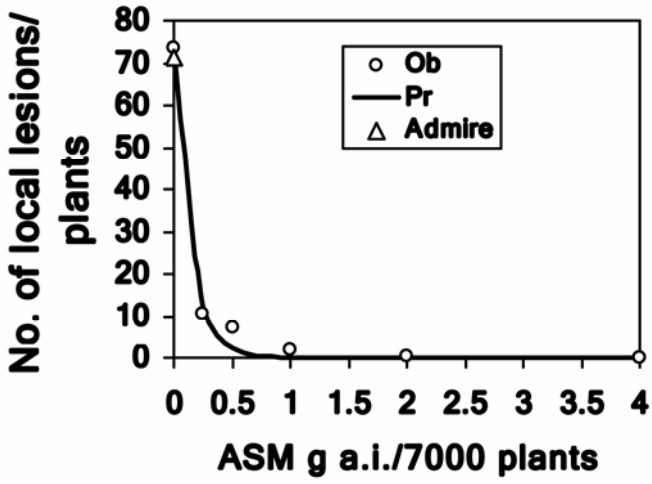

Fig. 1. Relationship of quantity of acibenzolar-S-methyl (ASM) used to treat tobacco plants of cv. K326 and the development of number of local lesions caused by *Tomato spotted wilt virus*. Plants were treated with ASM at the rate of 0, 0.25, 0.5, 1.0, 2.0, and 4.0 g/7,000 plants or imidacloprid at the rate of 39.78 g/1,000 plants. Local lesions were counted at 6 days postinoculation. Ob = observed; Pr = predicted.

RESULTS

Effect of ASM on local infection. Local lesions were observed on the inoculated leaves at 3 DPI and appeared as chlorotic rings, and by 5 DPI the lesions became necrotic. The lesions had the characteristic brown margin with a blighted center. Mechanically inoculated plants produced an average of 73.6 lesions per plant on the nontreated plants. Leaves wilted and eventually withered. At 0.25 g a.i. of ASM, only 10.6 lesions per plant developed, which was a significant reduction compared with that in the nontreated control. The mean number of local lesions declined further to 1.8 to 0.2 at 1 to 4 g a.i. of ASM (Fig. 1). The majority of the plants were free from local lesions when treated with ≥2 g a.i. of ASM. Plants treated with imidacloprid had a similar number of local lesions compared with nontreated plants. The proportions of plants with local symptoms were 96.7 and 84.4% at 0.25 and 0.5 g a.i. of ASM/7,000 plants, respectively (Fig. 2A). These two concentrations did not have any significant role in reducing the local symptoms compared with the nontreated plants. Treatment with ≥2 g a.i. of ASM reduced by 6.2 to 18.7% of plants with local symptoms (Fig. 2A). TSWV was detected in 63 to 99.7% of the inoculated leaves among plants treated with 0.25 to 4 g of ASM/7,000 plants. All plants treated with imidacloprid showed local lesions and infection by TSWV that was confirmed by ELISA.

Effect of ASM on systemic infection. When the locally infected plants were allowed to grow, systemic symptoms began developing from 6 to 10 DPI onwards. Local infection took place in all the inoculated plants by 3 to 5 DPI but systemic infection resulted in only 18.3% of plants by 30 DPI. In the ASM-treated plants, the percent symptomatic plants was reduced from 7.8 to 0.3% as the concentrations of ASM treatment were increased from 0.25 to 4 g a.i./7,000 plants (Fig. 2B) and ELISA-positive plants were reduced from 12.5 to 0.2%. At ≥1 g of ASM, plants were free from systemic symptoms. Imidacloprid treatment did not result in reduction in systemically infected plants compared with nontreated plants.

Effect of ASM on the level of viral antigen. The ELISA readings for nontreated plants and imidacloprid-treated plants were 1.4 and 1.2, respectively, at 10^{-1} dilution of sap (Fig. 3). The dilution end point of the viral antigen in these groups of plants was 10^{-4}. In the ASM-treated plants, the dilution end points of the viral antigen were 10^{-4} at 0.25 g a.i., 10^{-3} at 0.5 to 2 g a.i., and 10^{-2} at 4 g a.i. The highest absorbance values were obtained with the lowest rate of ASM, and as the rate increased the absorbance values decreased in the treated plants. Analysis of the absorbance values at 10-fold serial dilutions showed that the ASM treatment significantly reduced the level of TSWV in flue-cured tobacco plants (Fig. 3).

Phytotoxicity caused by ASM. Minute white necrotic lesions developed on the ASM-treated leaves but not on the imidacloprid- or water-treated leaves. The lesions the were caused due to ASM-induced phytotoxicity and those produced by TSWV were distinguishable by the absence of a brown necrotic zone around the blighted center of the phytotoxic lesions (Fig. 4B, which was present in the lesions caused by TSWV (Fig. 4A). The lesions per plant increased with ASM concentrations. Significantly more lesions (100/plant) developed in the ASM-treated plants at the rate of ≥1 g a.i./7,000 plants. The spots were not seen in the successive leaves that emerged after the ASM treatment. ASM treatment affected the height of the plants (Table 1). At 0.25 g of ASM, plant height was significantly higher than in control plants. At ≥1 g of ASM, a significant reduction of plant height was observed. The stunting caused by ASM was overcome by treating plants with ASM and GA3 (50 µg/plant). Root length, however, was not influenced by the ASM treatment (Table 1).

Time required for resistance activation. The numbers of local lesions in the ASM-treated and nontreated plants developed over

the time posttreatment are presented in Figure 5. One day after treatment with 4 g of ASM, 88.8 and 99 lesions developed per plant treated with ASM or water, respectively. At 2 DPT onward, there was a decline in the number of local lesions relative to control. At 5 days onward, plants produced a few lesions (Fig. 6) compared with the nontreated (87.3 to 77.1 lesions/plant) plants. Similarly, plants treated with 2 g of ASM also showed a declining trend in the number of lesions with the increase of time of treatment. At 5 DPT, plants treated with both dosages (2 or 4 g a.i.) showed fewer local lesions (4.3 at 4 g a.i and 8.1 at 2 g a.i.) compared with the nontreated (71.6 at 4 g a.i and 77.3 at 2 g a.i) plants.

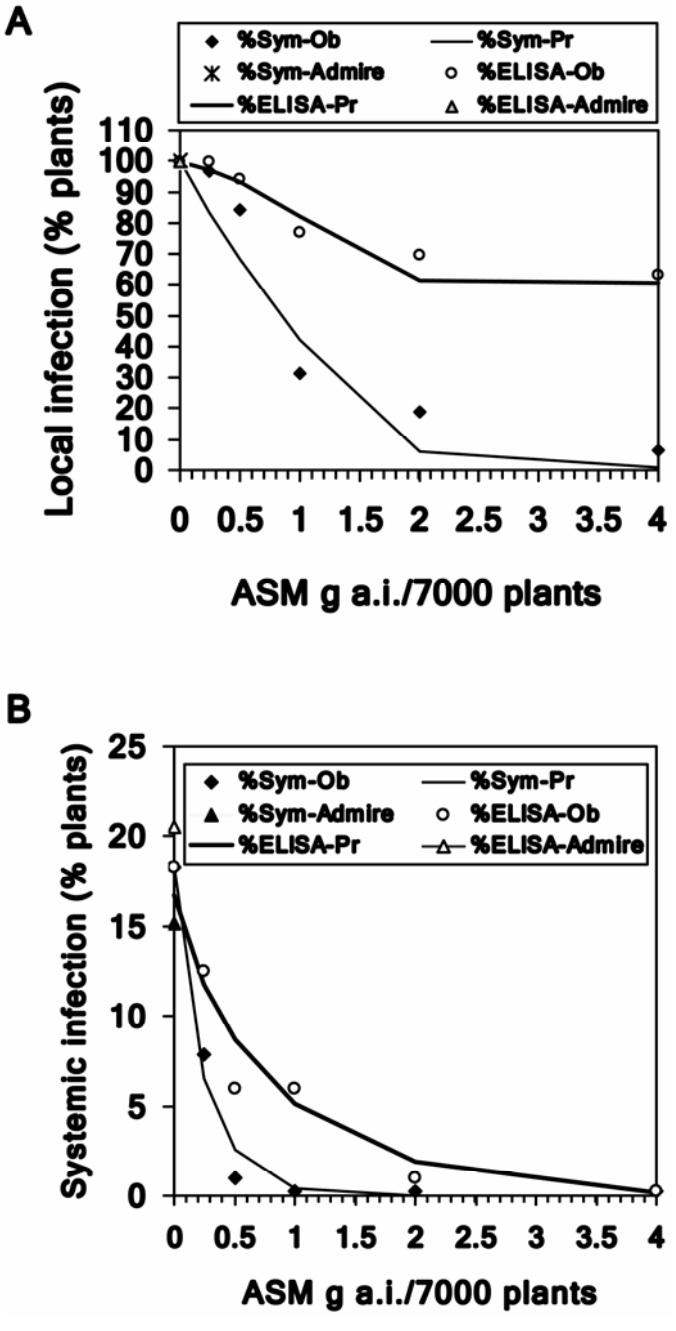

Fig. 2. Effect of concentration of acibenzolar-S-methyl (ASM) on infection of tobacco cv. K326 with *Tomato spotted wilt virus* (TSWV). **A,** Local infection and **B,** systemic infection. Plants were treated with ASM at the rate of 0, 0.25, 0.5, 1.0, 2.0, and 4.0 g a.i./7,000 plants or imidacloprid at the rate of 39.68 g/1,000 plants. Infection was judged by presence of symptoms and enzyme-linked immunosorbent assay (ELISA) following mechanical inoculation of TSWV. Sym = symptomatic plants; ELISA = ELISA-positive plants; Ob = observed; Pr = predicted.

Effect of age of plants on the activation of resistance. Inoculation of TSWV in the nontreated seedling plants resulted in 87.6 lesions per plant at 47 DAS (Table 2). All plants were locally infected and 73.3% became systemically infected as determined by ELISA. In ASM-treated seedlings, 60% of the plants were locally infected (10% symptomatic) and 6.7% of the plants were systemically infected (3.3% had systemic symptoms). In older plants (77 DAS), there were 20.3 and 3.1 local lesions/plant, 100 and 90% of plants with localized infection and 13.3 and 6.7% of plants with systemic infection in the nontreated and ASM-treated plants, respectively. The differences of these responses between the treated and nontreated older plants were statistically significant (Table 2). In the older plants, 100% of the nontreated and 63.30% of the treated plants showed local symptoms, although, none of these plants produced systemic symptoms.

Effect ASM on tobacco cultivars for resistance activation. Tobacco cvs. K326, NY21, NC71, NC72, NC297, NC2326, Cu748, and Cu752 treated with ASM showed significant reductions in number of local lesions per plant as well as incidence of systemic infection compared with the nontreated control plants (data not shown). High-level resistance was observed to be activated by ASM in cvs. NC71, NC72, NC297, and NC2326 as none of the plants were systemically infected.

Induction of PR gene and protein expression. The PR-Q gene's transcript was detected in leaves treated with ASM and in the younger leaves developed after the ASM treatment (Fig. 7A). In the nontreated tobacco and cowpea plants, accumulation of PR-Q mRNA was not detected.

SDS-PAGE gel showed higher levels of certain proteins in the ASM-treated plants compared with the nontreated plants. The most prominent protein band was seen at molecular weight (MW) of 36,000, although this band was also present in the nontreated plants. In the ASM-treated plants there were three protein bands ranging from MW of 46,000 to 45,000. An additional eight protein bands ranging between MW of 14,000 to 36,000 also were observed. Proteins of MW 29,000 and smaller may represent PR-3 and PR-5 and the 21,000 Da protein may represent PR-1. The quantity of the total proteins increased by 2.7 to 4 times in the ASM-treated samples compared with the nontreated plants over the period of time after the ASM treatment. Western blotting (Fig. 7B) with antibody raised against PR-1 showed mild accumulation of PR-1 a day after treatment with ASM. An increased amount of

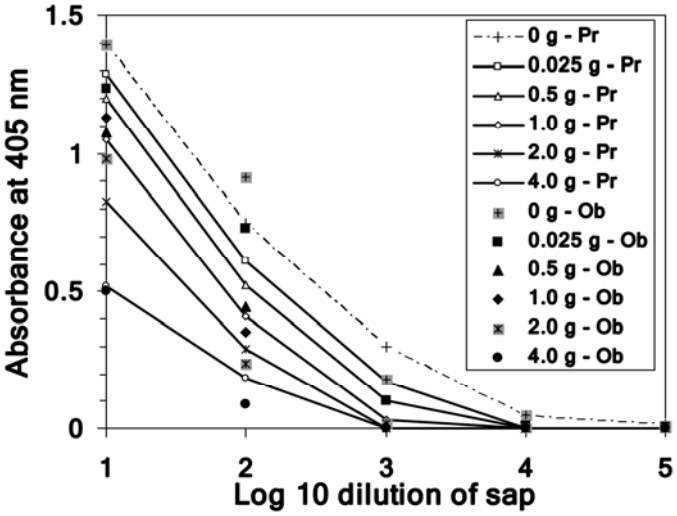

Fig. 3. Effect of different dosage of acibenzolar-S-methyl (ASM) on the relative levels of *Tomato spotted wilt virus*. Seedlings of tobacco cv. K326 were treated with 0, 0.25, 0.5, 1.0, 2.0, and 4.0 g of ASM/7,000 plants or imidacloprid at the rate of 39.7 g/1,000 plants. Regression lines representing ASM treatment are significantly different ($P < 0.05$). Pr = predicted, Ob = observed.

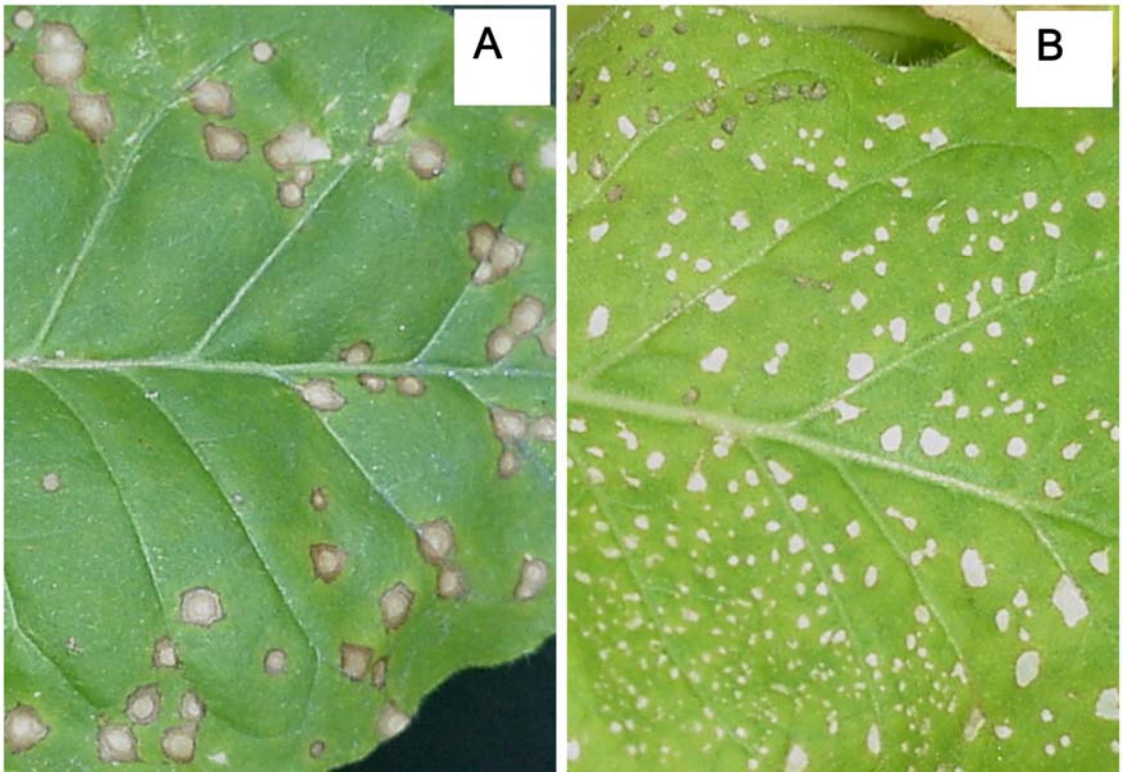

Fig. 4. Lesions caused by **A,** *Tomato spotted wilt virus* and **B,** phytotoxic leaf spots due to application of acibenzolar-S-methyl (4 g a.i./7,000 plants) developed in tobacco cv. K326.

TABLE 1. Root length and plant height of flue-cured tobacco cv. K326 in response to different concentrations of acibenzolar-S-methyl[z]

ASM g a.i./7,000 plants	Root length (cm)	Plant height (cm)
Imidacloprid	23.7 a	32.8 a
0	24.3 a	33.1 a
0.25	23.6 a	36.6 b
0.5	23.9 a	34.7 a
1.0	23.8 a	31.2 c
2.0	24.6 a	27.1 d
4.0	23.3 a	26.6 d

[z] Root length and plant height were measured 60 days posttreatment. Values in columns marked with same letter are not significantly different at $P = 0.05$ (least significant differences for root length and plant height were 5.5 and 1.6, respectively). Concentration of imidacloprid was 39.68 g a.i./7,000 plants.

PR-1 was observed from 2 days onward. PR-3 and PR-5 were also accumulated at 2 days onward after treatment with ASM. No accumulation of PR-2, PR-6, and PR-8 were observed in the ASM-treated plants (not shown).

DISCUSSION

The present study demonstrates that treatment of flue-cured tobacco with ASM activates high levels of resistance against a severe isolate of TSWV and the activation of resistance is correlated with the coordinated expression of PR proteins following treatment with ASM.

Previous studies showed that the field experiments with foliar sprays of ASM provided protection in flue-cured tobacco against spotted wilt disease (16,59). The appropriate quantity of ASM required for activation of resistance was not known and thus a higher dosage of ASM (8.4 to 67.3 g a.i/7,000 plants) was used (16,59). In this study, we utilized uniform environmental conditions and mechanical inoculation to ensure a high rate of infection which facilitated a detailed examination of the interaction of

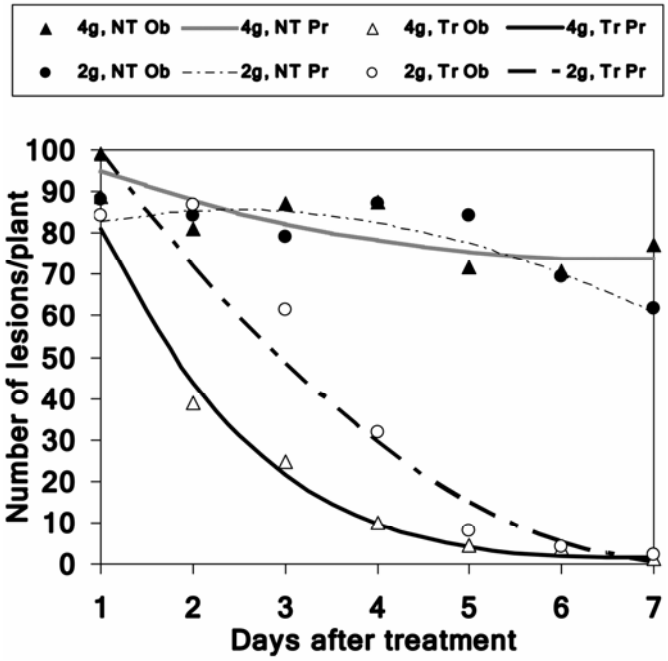

Fig. 5. Number of local lesions that developed on seedlings of tobacco cv. K326 treated with 2 or 4 g of acibenzolar-S-methyl/7,000 plants followed by inoculation with *Tomato spotted wilt virus* at 1-day interval until 7 days posttreatment. Number of lesions was counted at 6 days postinoculation. NT = nontreated; Ob = observed; Pr = predicted.

TSWV and tobacco under the ASM-induced SAR. As the dosage of ASM increased from 0.25 to 4 g a.i/7,000 plants, the number of local lesions, incidence of plants showing local and systemic infection, and the relative levels of TSWV in the treated plants decreased. At 4 g of ASM, the highest level of disease resistance was observed compared with the other lower dosage. But at this

dose plants showed the highest level of phytotoxic symptoms such as white necrotic lesions on the treated leaves and overall stunting of plants. The phytotoxicity increased with the increase of ASM dosage. However, the lesions associated with phytotoxicity do not persist in the new growth of foliage. Field experiments with ASM showed reduction of growth in flue-cured tobacco (16,48,59). Csinos et al. (16) conducted greenhouse experiments to determine the effect of ASM dosage on tobacco growth and found varying levels of growth reduction at 21 DPT. In the present study, when ASM-treated plants were allowed to grow for a longer period of time (60 days after transplanting) significant reductions in plant height were observed at rates of 1 to 4 g of ASM. Although ASM suppressed TSWV, stunting caused by ASM could be an issue. Use of ASM with GA3 showed compensation of the ASM-induced stunting. There was a dose-dependent response of ASM on anti-TSWV activity and phytotoxicity. The higher the rate, the greater the reduction of TSWV and more phytotoxicity. Therefore, it was necessary to find a compromise between an effective dose for suppressing TSWV and minimizing the phytotoxicity. A dose between 1 to 2 g of ASM showed significant disease suppression and less phytotoxicity. A dose-dependent SAR activation by ASM was shown against downy mildew pathogen (81).

ELISA revealed the presence of symptomless infections. In the field situation a similar situation was observed (16,59). Thus, ASM appears to suppress symptoms rather than prevent plants from getting infected. However, in the present study, analysis of ELISA absorbance values showed reduction of relative virus levels in ASM-treated plants suggesting a possible suppressive effect of ASM on virus replication. Reduction of CMV-Y was observed in BTH-treated tomato plants (3) and TCV was not detected in *Arabidopsis* plants treated with BTH (36).

Yield losses caused by TSWV in tobacco are due to the development of systemic symptoms. Under field conditions, local symptoms were often observed without adverse effects on plants if systemic symptoms fail to develop (A. S. Csinos, *unpublished data*). In the present study, local infection of TSWV was found in a higher percentage (63.0 to 99.8% based on the quantities of ASM) compared with those that developed systemic infection (0.3 to 12.5%). This suggests that ASM is potentially restricting TSWV movement to the site of inoculation while it cannot be ruled out that there are other factors that influence the development of systemic infection of TSWV in flue-cured tobacco.

In field trials, repeated application of ASM a month after transplanting did not reduce TSWV incidence, although ASM treatment of plants prior to transplanting showed reduction in TSWV incidence (16). This observation led us to examine the role of plant age in response to ASM treatment. ASM-treated seedlings (45 DAS) showed greater reduction of both local and systemic infection by TSWV than older plants (75 DAS), which did not show a significant reduction of TSWV compared with nontreated plants. Our results, along with the previous observations (16), indicate there is a definite age of tobacco for sensitization by ASM. SAR induction in seedlings is crucial because the plant is susceptible to TSWV at this stage and as the plant gets older, mature plant resistance leads to fewer new infections. Information on the ASM-induced SAR would help in determining the optimum time of application of ASM to ensure the highest level of SAR. The challenge inoculation of TSWV every 24 h after treatment with ASM showed activation of high levels of resistance from 5 days onward as indicated by the drastic reduction of number of local lesions caused by TSWV. This result suggests that 5 to 6 days should be allowed after the ASM treatment for inducing a high level of resistance. The duration of persistence of ASM-induced SAR was not investigated in this study. While the ASM-induced protection seen in field conditions (16,59) was in response to thrips-mediated inoculation under natural conditions, the response of tobacco to TSWV in these studies was evaluated based on mechanical inoculations. To minimize the possibility of generating defective interfering RNAs and/or attenuated strains, the number of passages of the original isolate was kept at the minimum.

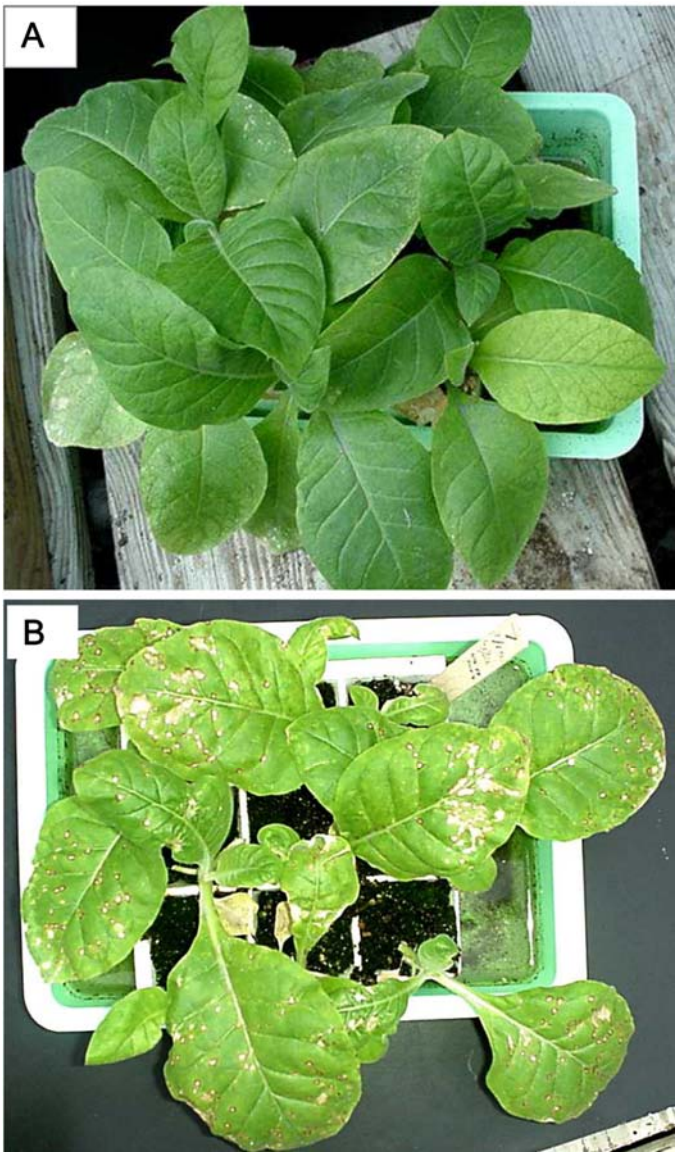

Fig. 6. Protection of tobacco plants of cv. K326 by acibenzolar-S-methyl (ASM) from infection by *Tomato spotted wilt virus* at 5 days posttreatment. Plants were treated with **A,** ASM at the rate of 4 g a.i./7,000 plants or **B,** water.

Imidacloprid was shown to be effective in suppressing spotted wilt in the field (16,59). Challenge inoculation of TSWV to the imidacloprid-treated plants did not result in suppression of local and systemic infection or the ELISA absorbance values. Under field conditions, the suppressive effect of imidacloprid may be due to its insecticidal properties.

Biological or chemical activation of SAR was correlated with systemic accumulation of PR proteins (31,36,79,81). Expression of PR gene and proteins was examined to understand the possible mechanism of action of ASM against TSWV. Total proteins in the ASM-treated plants significantly increased a day after treatment with ASM. Of the several protein bands observed in the ASM-treated plants compared with the nontreated plants, only PR-1, PR-3, and PR-5 were detected, while PR-2, PR-6, and PR-8 were not detected. Accumulation of PR proteins was weak at 1 DPT but a stronger accumulation was observed at 2 DPT. Challenge inoculation of plants at different times after ASM treatment showed

TABLE 2. Responses of seedlings and older plants treated with acibenzolar-S-methyl (ASM) to *Tomato spotted wilt virus*[y]

		Local infection			Systemic infection	
			% Plants		% Plants	
Age of plant[z]	Treatment	No. of lesions/plant	Symptomatic	ELISA positive	Symptomatic	ELISA positive
75	Treated	3.0	63.3	90.0	0.0	6.7 a
	Nontreated	20.3	100.0	100.0	0.0	13.3 a
45	Treated	0.2	10.0	60.0	3.3	6.7 a
	Nontreated	87.6	100.0	100.0	50.0	73.3 b

[y] Plants of tobacco cv. K326 were treated with 2 g of ASM/7,000 plants and mechanically inoculated at 7 days posttreatment. Local lesions were counted at 6 days postinoculation (DPI) and systemic infection was judged by testing root samples by enzyme-linked immunosorbent assay (ELISA) at 30 DPI. ASM treatment significantly reduced local and systemic infections compared with nontreated control plants only in plant age 45 days after seeding (DAS) but not in 75 DAS according to the least significant difference test at $P = 0.05$. Values in columns marked with same letter are not significantly different at $P = 0.05$.
[z] Plants were transplanted to pots 35 DAS.

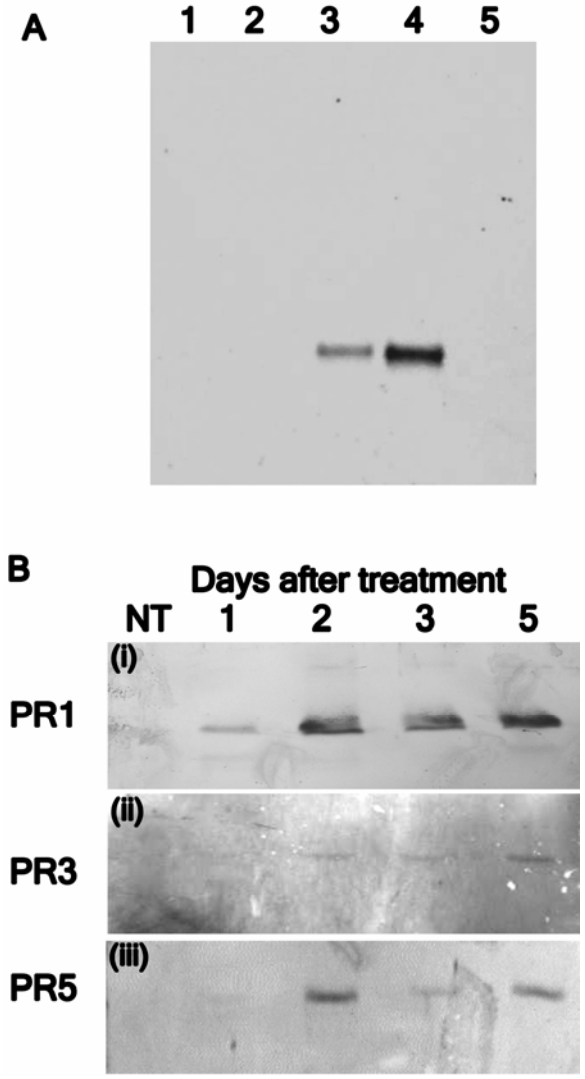

Fig. 7. Accumulation of pathogenesis-related (PR) gene and proteins following treatment with acibenzolar-S-methyl (ASM). **A,** Northern blot showing systemic expression of mRNA of PR-Q gene. Two months after seeding, tobacco cv. K326 was treated with 2 g of ASM/7,000 plants and total mRNA was isolated at 15 days posttreatment. Lane 1, lower leaves from plants treated with water; lane 2, young leaves from the same plants as in lane 1; lane 3, ASM-treated lower leaves; lane 4, young leaves from the same treated plants as in lane 3; and lane 5, cowpea leaves treated with water. The mRNA blot was hybridized with the nonradioactive probe to PR-Q cloned DNA (1,020 nucleotides). **B,** Western blots showing accumulation of PR proteins. Seedlings of tobacco cv. K326 were treated with ASM at the rate of 2 g/7,000 plants and protein was isolated at 1, 2, 3, and 5 days posttreatment. Antisera used were raised against tobacco PR proteins PR-1 (B, i), PR-3 (B, ii), and PR-5 (B, iii). NT = nontreated control (protein sample from plants treated with water).

reduced number of local lesions caused by TSWV 2 DPT. A correlation between resistance induction and PR protein induction by ASM was observed.

Ziadi et al. (81) used the same antisera, as in the present study, to analyze PR proteins induced by ASM in cabbage. It was reported that PR-2 (glucanase) was rapidly induced but PR-1 and PR-5 were induced slowly and in trace quantity. In our study, PR-1 was detected prominently and rapidly but PR-2 could not be detected. PR-3 (chitinase) was not induced by ASM in cabbage (81). However, we observed the expression of PR-Q (PR-3) mRNA and protein in the ASM-treated plants. Wendehenne et al. (78) also showed accumulation of PR-3 mRNA in BTH-treated tobacco. The spectrum of resistance activated and PR proteins induced by ASM are crop specific (36,56,73). We have observed induction of several proteins in the SDS-PAGE gel, but only three PR proteins were detected in western blots. Further investigation would help understand the role of PR proteins in ASM-induced SAR against TSWV in flue-cured tobacco.

This study suggests that there are certain critical factors that influence the efficacy of ASM in inducing SAR against TSWV in flue-cured tobacco: (i) the concentration of ASM should be at least 2 g a.i/7,000 plants, (ii) seedlings should be treated prior to their transplant in the field since little benefit was seen treating older plants, and (iii) 5 to 6 days should be allowed posttreatment for an effective induction of SAR. These three factors were validated in evaluating seven additional cultivars of flue-cured tobacco, where equally high levels of resistance were achieved by ASM treatment. TSWV continues to be an economic constraint to tobacco production in southern United States and management options are limited. The present and previous work (16,59) suggest that use of ASM to induce SAR could be used as a management strategy for reducing the impact of TSWV in flue-cured tobacco.

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