Crop Protection 30 (2011) 1340–1345

Contents lists available at ScienceDirect

Crop Protection

journal homepage: www.elsevier.com/locate/cropro

Management of bacterial wilt in tomatoes with thymol and acibenzolar-*S*-methyl

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ARTICLE INFO

Article history:
Received 26 January 2011
Received in revised form
19 May 2011
Accepted 22 May 2011

Keywords:
Ralstonia solanacearum
Induced resistance
Methyl bromide alternative
Integrated disease management

ABSTRACT

The combination of thymol, a monoterpene phenol compound originating from thyme, and acibenzolar-*S*-methyl (ASM; Actigard 50 WG), a systemic acquired resistance (SAR) inducer, was applied to tomato plants in field conditions to evaluate the effectiveness of both chemicals to control bacterial wilt. Thymol was applied as a soil fumigant at 9.43 kg per ha 24 h after soil infestation and seven days before transplanting. ASM was applied as a foliar spray at 3.59–8.98 ml per ha, once in the greenhouse and five times in the field. The field was inoculated by applying 50 ml of pathogen suspension (10^7 cfu/ml) into each transplanting hole eight days prior to transplanting. The experiment was performed in 2006 and repeated in 2008 at the North Florida Research and Education Center in Quincy, FL. In 2006, the combination of ASM and thymol significantly reduced disease in the bacterial wilt tolerant genotype 7514 compared to thymol alone. In 2008, the combination of ASM and thymol significantly reduced disease and increased yield compared to the control, whereas ASM or thymol alone did not significantly reduce disease or increase yield compared to the control. This is the first report of the use of both thymol and ASM to control bacterial wilt on moderately resistant tomato cultivars. Based on this study, control of the pathogen can be achieved by using both chemicals and moderately resistant cultivars.

1. Introduction

Bacterial wilt, caused by the soilborne pathogen *Ralstonia solanacearum* (Smith) Yabuuchi, occurs worldwide in tropical and subtropical regions of the world (Yabuuchi et al., 1995). The bacterium can cause disease symptoms in over 200 different plant species (Buddenhagen and Kelman, 1964; Hayward, 1991). In the southeastern United States, economic losses for important solanaceous crops including tomato, tobacco and eggplant can be attributed to bacterial wilt. The bacterium enters the plant through the root and colonizes the vascular tissue in the stem. In field conditions, signs of the disease usually appear in mature tomato plants. Leaves will wilt during the day and recover at night or during the early hours of the morning. If the weather is favorable, with high humidity and high temperatures, the disease can cause complete wilting of the plant and eventually death. In the advanced stages of wilt, the leaves of wilted plants remain green and the vascular tissue usually turns a brownish yellow. In the field, the disease occurs mostly in areas where water accumulates; however, plants showing signs of the disease can be found sporadically throughout. Plants affected by *R. solanacearum* can also be stunted, due to the lack of water and poor uptake of nutrients.

Current integrated management strategies include the use of resistant cultivars, pathogen-free transplants and crop rotation with non-host cover crops (Pradhanang et al., 2005). However, these strategies have proven to be limited due to the complex nature of soilborne pathogens. Resistant cultivars have been developed for fresh market production in the U.S.; however, the growers have only adopted moderately resistant cultivars (Scott et al., 1995). Resistant and moderately resistant cultivars are limited in terms of location, climate and resistance to strains of the pathogen (Saddler, 2004). Transplants limit the spread of the bacterium, yet due to it being a soilborne pathogen, most plants in the field can be infected. Cover crops or crop rotation can be difficult due to the diverse host range of *R. solanacearum* strains, and the fact that the pathogen is able to survive or colonize various weeds that surround the field (Hayward, 1991). With the limited control measures and the gravity of bacterial wilt on important economical crops, investigating other methods for controlling the disease has become critical.

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0261-2194/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.cropro.2011.05.019

Plants are able to activate a protective mechanism after contact by a pathogen, these plant metabolites, or by a diverse group of structurally unrelated organic and inorganic compounds. This phenomenon has been dubbed as systemic acquired resistance (SAR) (Kuc, 2001). SAR inducers are ideal for controlling diseases because they trigger a response that may protect the plant from fungal, bacterial and viral pathogens, if the product is applied at the correct time. Acibenzolar-*S*-methyl (ASM; Actigard 50 WG, Syngenta, Basel, Switzerland) is a chemical compound that triggers SAR when applied to plants (Oostendrop et al., 2001). ASM has been used to reduce the incidence of fire blight in pear and apple, bacterial spot and speck in tomato and pepper, and common bunt in wheat seedlings (Louws et al., 2001; Norelli et al., 2003; Obradovic et al., 2005; Lu et al., 2006). Previously it was reported that ASM enhanced host resistance in moderately resistant tomato cultivars against bacterial wilt (Pradhanang et al., 2005).

Thymol (2-isopropyl-5-methylphenol) is a monoterpene phenol derivative of thyme (Aeschbach et al., 1994). Essential oils have been used in the past for flavoring and preserving food, for their antioxidant power and for their antimicrobial activity (Scheie, 1989; Lambert et al., 2001; Rojano et al., 2008). Both medical and food sciences have shown that thymol is able to inhibit both Gram-positive and Gram-negative bacteria (Evans and Martin, 2000; Lambert et al., 2001; Walsh et al., 2003; Cailet and Lacroix, 2006; Shapira and Mimran, 2007). Previously thymol applied as a biofumigant was reported to effectively control bacterial wilt. Thymol applications in the field on susceptible tomato cultivars were able to reduce the incidence of bacterial wilt and increase yield (Ji et al., 2005).

In previous studies bacterial wilt was reduced by applying ASM in combination with moderately resistant tomato cultivars (Pradhanang et al., 2005), or by applying thymol and using susceptible tomato plants (Ji et al., 2005). In this study, we wanted to determine if using a combination of thymol, ASM and moderately resistant plants would elevate the level of efficacy in controlling bacterial wilt. This would be the first time that the two products had been applied together on moderately resistant tomato cultivars in a field trial. It was unknown if the chemicals would work synergistically or would have little to no effect in enhancing disease control. Success with both of the chemicals in controlling the disease would provide another tool in a small arsenal to control bacterial wilt.

2. Materials and methods

2.1. Bacterial culture and inoculum preparation

Ralstonia solanacearum strain RS5 isolated from tomato in Quincy, Florida, was used in this study (Pradhanang and Momol, 2001). Pathogenicity was determined by performing Koch's postulates by inoculating tomato plants and re-isolating RS5. Bacteria were plated on modified semi-selective agar, SMSA (Englebrecht, 1994), and casamino acid peptone glucose agar, CPG (Schaad et al., 2001). Plates were stored at 28 °C. The inoculum contained bacteria grown on CPG for 24 h and suspended in sterile deionized water. The bacterial suspension was adjusted to 10^7 CFU/ml using sterile deionized water. Inoculum concentration was estimated using a spectrophotometer (Sigma-Aldrich Co., Milwaukee, WI) at 600 nm. The actual bacterial concentration (cfu/ml) was determined by performing 10-fold dilutions of the inoculum suspension and plating on CPG. Where each tomato plant was to be transplanted, 15 cm holes were created in the soil and 50 ml of the bacterial suspension was poured into each hole (Ji et al., 2005). The field was infested 10 day prior to transplantation, in which time the field was treated with thymol and plants were treated with 1 application of ASM. The holes were covered with tape prior to the thymol fumigation.

2.2. Application of thymol and ASM

Thymol was applied as a soil fumigant 24 h after the field was infested. The field was aerated 7 days post thymol application, and transplanting occurred 3 days after field aeration. Thymol was applied at 9.42 kg per ha, in a solution consisting of water, 70% ethanol and detergent. ASM was applied as a foliar spray at a volume of 10 ml of ASM solution (25 μg/ml) per plant. The ASM solution was applied 6 times: 1 week before the seedlings were transplanted, 1 day after transplanting, followed by 2 treatments that were applied once a week and then 2 treatments that were applied biweekly.

2.3. Tomato plants and experimental design

In the 2006 trial, tomato cultivars 'Phoenix', 'FL7514' and 'BHN669' were used in the field experiment, the first being susceptible and the last two moderately resistant to bacterial wilt. For the 2008 trial, only 'Phoenix' and 'FL7514' were used. Tomato plants were grown in Terra-Lite agricultural mix (Scott Sierra Horticultural Products Co., Marysville, OH) in expanded polystyrene flats with 3.5 × 3.5 cm cells. For each experiment, 5-week-old tomato seedlings were transplanted 1 week after the thymol application.

The experiments for both years were conducted in experimental fields at the University of Florida North Florida Research and Education Center located in Quincy. Previously, the fields were used for growing tomatoes. The beds were fumigated with methyl bromide (67%) and chloropicrin (33%) at a broadcast equivalent rate of 392 kg a.i./ha for control of weeds and other soilborne pathogens, fertilized with 218-31-181 kg/ha of N–P–K and covered with polyethylene mulch 1 week prior to infestation of the field. The plots consisted of four rows, 5 m long with the raised beds, 10 cm high by 91 cm wide and centered 1.8 m apart. Tomato plants were treated with standard foliar sprays for insecticides and fungicides at weekly intervals until harvest. Over time the plants were tied and staked. A randomized complete block design was used including 6 blocks for each cultivar and treatment in 2006 and 4 blocks in 2008. Each block constituted a replication. Each block was 10–12 m long with 14 tomato seedlings transplanted per block in 2006 and 18 in 2008. Thus each treatment consisted of 84 plants per cultivar in 2006 and 72 plants per cultivar in 2008. In the 2006 experiment, each block of plants received one of the following treatments: thymol, the combination of thymol and ASM or neither thymol nor ASM, which was the untreated control (UTC). The treatments for the 2008 experiment consisted of thymol, ASM, both thymol and ASM or the UTC. In between each block was a 2 m buffer where no tomato seedlings were planted.

2.4. Disease and yield assessment and statistical analysis

Completely wilted tomato plants were removed from the field weekly and a few of the plants were tested for the presence of the bacterium. The confirmation of *R. solanacearum* was performed by a bacterial ooze test and either isolation on SMSA and confirmation by gas chromatographic profiling of whole-cell fatty acid methyl esters (FAME) (MIDI, Newark, DE), as described previously (Stead, 1992; Pradhanang et al., 2003), or by using *R. solanacearum* specific immunoassay strips (Agdia, Inc., Elkhart, IN). RS5 was used as positive control for each test. In both 2006 and 2008 completely wilted plants were counted weekly after transplanting. Bacterial wilt incidence was recorded at weekly intervals and was quantified as the percentage of plants wilted. Percentage of plants wilted was calculated by dividing the number of completely wilted plants by total number of transplanted plants. Two harvests were conducted for both trials. The total marketable and unmarketable yield was determined according to the USDA standards by using a fruit and vegetable processing machine (Model No. 1650 Roller, TEW Manufacturing Corp., Penfield, NY). Marketable fruit size was categorized as extra large, large and medium (USDA, 1976; Stavisky et al., 2002). The average fruit size and average fruit number was calculated for each size, cultivar and treatment. The variance of the treatments' effects on bacterial wilt incidence and tomato yield was analyzed by using a general linear model (GLM) conducted in Statistical Analysis System version 9.1 (SAS Institute, Cary, NC). To determine the significance of interaction of the treatments, the differences between means of the disease incidence and yield were contrasted using least significant difference (lsd) test. The results were tested for normality.

In the 2008 experiment, a week after transplanting, Hurricane Fay descended on the Florida panhandle and did not move for 72 h. During that time the experimental station received 45 cm of rain. In a normal year for the month of August the station receives on average 19 cm of rain. The water collected at the north end of the field and many of the plants were submerged. Some replications of the trial were destroyed, but data were collected from at least 2 of the 4 replications.

3. Results

3.1. Field experiment 2006

Typical bacterial wilt symptoms were observed as early as one week post transplanting. Wilted plants were sampled for *R. solanacearum* by performing a bacterial ooze test, FAME, or using the immunoassay strips. All the plants that were sampled tested positive for the presence of the bacterium. In all the replications the susceptible cultivar 'Phoenix' was affected the most by the pathogen; by the end of the experiment the 'Phoenix' plants in the UTC produced the least amount of fruit compared to the two resistant cultivars, 'BHN669' and 'FL7514' (Fig. 1). 'Phoenix' plants that received the thymol or thymol and ASM treatments had over a 200-fold increase of fruit production, and a 3-fold decrease of plants wilting for thymol and an almost 5-fold for thymol and ASM compared to the UTC. By the end of the experiment 94% of the UTC 'Phoenix' plants were completely wilted, while 30% of the thymol treated plants wilted and 19% of the thymol and ASM plants wilted (Table 1). The plants treated with thymol and ASM had over a10-fold increase in marketable fruit yield and at least a 5-fold disease incidence reduction for all three cultivars. A significant statistical difference was also observed when 'FL5714' was treated with thymol and ASM compared to thymol alone (Table 1). In addition all three cultivars treated with thymol or with thymol and ASM were statistically lower than the UTC when comparing disease incidence (Table 1).

3.2. Field experiment 2008

Typical bacterial wilt symptoms were observed as early as week 1, and all wilted plants tested were positive for *R. solanacearum*. In this experiment the 'Phoenix' cultivar survived better than FL7514, the moderately resistant cultivar, which might be due to the amount of rain received from the hurricane. Regardless of the differences between the two cultivars, the thymol, ASM and thymol and ASM treated plants resulted in a greater yield and had fewer plants wilt than the untreated controls for both cultivars (Fig. 2). Even in unfavorable weather conditions, significant statistical differences were observed in the yield for the susceptible plants and both the

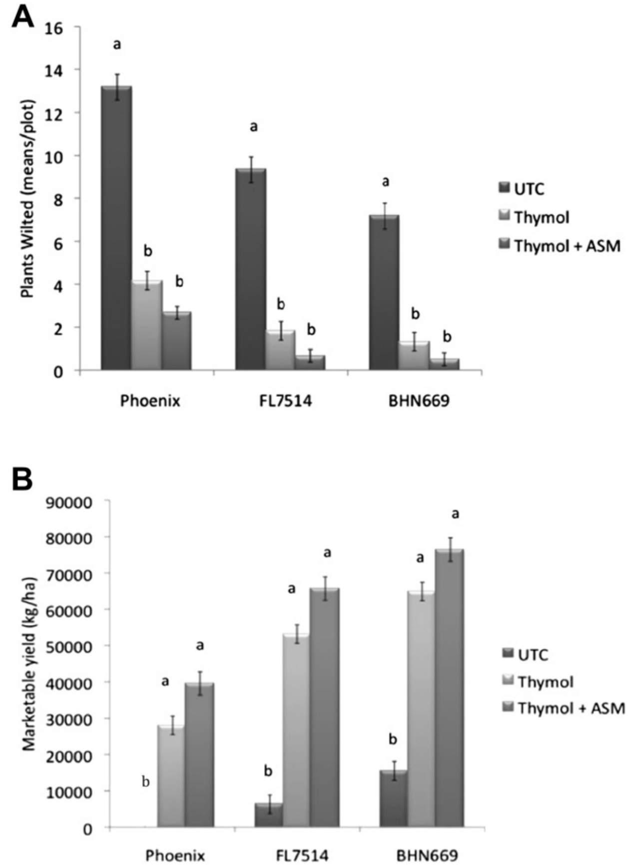

Fig. 1. The effect of thymol and the combination of thymol and ASM on the number of plants wilted (Graph A) and marketable fruit yield (Graph B) when applied to susceptible and moderately resistant tomato cultivars in a bacterial wilt field experiment (fall 2006, Quincy, FL). Means and SE (standard error of the mean) of 6 replications and 16 plants per plot. Same letter indicates no significant difference according to Duncan's multiple range test at $P = 0.05$. Untreated control (UTC).

disease incidence and yield for the moderately resistant plants treated with thymol and ASM when contrasted with the UTC (Table 2). Where thymol or ASM was applied alone there were no statistically significant differences when compared with the UTC for either cultivar. The difference in bacterial wilt incidence as determined by standard error between thymol and ASM and the UTC, thymol or ASM treatments for 'FL7514' was significant (Fig. 2).

4. Discussion

Previously, it was determined that thymol alone and ASM alone were able to decrease disease incidence and increase fruit yield (Ji et al., 2005; Pradhanang et al., 2005). This study was the first time the application of thymol and ASM were used together in field conditions to control bacterial wilt on moderately resistant tomato cultivars. We report that the use of both products will not have a negative effect on tomato production. The combination of both products numerically increased the fruit yield and decreased the disease incidence for the susceptible cultivar. For both years, the combination of thymol and ASM was statistically different from the UTC and thymol alone, when comparing the effect the treatments had on incidence of disease for moderately resistant cultivars. In the 2006 trial, resistant plants treated with the combination of both chemicals had statistically lower incidence of disease when compared to the plants treated with thymol alone. Although a few replications from the second year were damaged, the plants that

Table 1
The effect of soil fumigation with thymol, foliar application of ASM and the combination of thymol and ASM on disease incidence and marketable fruit yield for tomato plants in a bacterial wilt field experiment (fall 2006, Quincy, FL).

Cultivar[a]	Treatment[b]		Disease incidence (%)[c]			Marketable yield (kg/ha)[c]	
Phoenix	UTC		94.3			ID[d]	
	Thymol		30.0			28,100.9	
	Thymol + ASM		19.3			39,569.4	
BH669	UTC		51.4			15,519.3	
	Thymol		9.3			64,915.4	
	Thymol + ASM		3.6			76,452.0	
FL7514	UTC		66.4			6294.6	
	Thymol		12.9			53,225.7	
	Thymol + ASM		5.0			65,699.6	
	Contrast[e]	df	F	$P > F$	df	F	$P > F$
Phoenix	Thymol vs. UTC	1	177.8	0.0001	1	49.1	0.0001
	Thymol + ASM vs. UTC	1	204.6	0.0001	1	26.7	0.0004
	Thymol + ASM vs Thymol	1	2.8	0.1256	1	1.8	0.2130
BH669	Thymol vs. UTC	1	46.1	0.0001	1	38.6	0.0001
	Thymol + ASM vs UTC	1	65.6	0.0001	1	73.7	0.0001
	Thymol + ASM vs Thymol	1	2.1	0.1556	1	2.0	0.1895
FL7514	Thymol vs. UTC	1	69.8	0.0001	1	31.0	0.0002
	Thymol + ASM vs. UTC	1	91.4	0.0001	1	48.2	0.0001
	Thymol + ASM vs Thymol	1	6.6	0.0277	1	1.8	0.2066

[a] BHN669 and FL7514 are moderately resistant cultivars, and Phoenix is a susceptible cultivar to bacterial wilt.
[b] Thymol was applied once before transplanting. ASM was applied by foliar spray 6 times: once before transplanting and 5 times afterward.
[c] Disease incidence was the final percentage of wilted plants. Disease incidence and yield values were means from six replications.
[d] Insignificant data.
[e] Contrast determined by using a GLM (general linear model) and the means of disease incidence and yield treatments for each cultivar were compared using least significant difference (lsd).

Table 2
The effect of soil fumigation with thymol, foliar application of ASM, and the combination thymol and ASM on tomato plants in bacterial wilt field experiment on disease incidence of the tomato plants and marketable fruit yield in 2008 (fall, Quincy, FL).

Cultivar[a]	Treatment[b]		Disease incidence (%)[c]			Marketable yield (kg/ha)[c]	
Phoenix	UTC		38.9			3061.0	
	Thymol		37.2			5316.9	
	ASM		23.9			6553.8	
	Thymol + ASM		26.7			9440.4	
FL7514	UTC		51.7			1494.3	
	Thymol		40.6			2804.5	
	ASM		38.9			5018.7	
	Thymol + ASM		21.1			5727.8	
	Contrast[d]	df	F	$P > F$	df	F	$P > F$
Phoenix	Thymol vs. UTC	1	0.3	0.6519	1	1.4	0.3251
	ASM vs. UTC	1	2.1	0.2241	1	5.5	0.0785
	Thymol + ASM vs. UTC	1	1.3	0.3018	1	7.1	0.0376
	Thymol + ASM vs. Thymol	1	0.6	0.4923	1	1.2	0.3242
	Thymol + ASM vs. ASM	1	0.1	0.8498	1	0.1	0.7678
	Thymol vs. ASM	1	0.1	0.7406	1	0.1	0.8338
FL7514	Thymol vs. UTC	1	0.6	0.4609	1	1.0	0.3671
	ASM vs. UTC	1	1.6	0.2571	1	10.47	0.0178
	Thymol + ASM vs. UTC	1	15.5	0.0077	1	6.0	0.0499
	Thymol + ASM vs. Thymol	1	2.8	0.1556	1	0.6	0.4910
	Thymol + ASM vs. ASM	1	4.4	0.0805	1	0.2	0.6972
	Thymol vs. ASM	1	0.1	0.9001	1	0.35	0.5783

[a] BHN669 and FL7514 are moderately resistant cultivars, and Phoenix is a susceptible cultivar to bacterial wilt.
[b] Thymol was applied once before transplanting. ASM was applied by foliar spray 6 times: once before transplanting and 5 times afterward.
[c] Disease incidence was the final percentage of wilted plants. Disease incidence and yield values were means from four replications.
[d] Contrast determined by using a GLM (general linear model) and the means of disease incidence and yield treatments for each cultivar were compared using least significant difference (lsd).

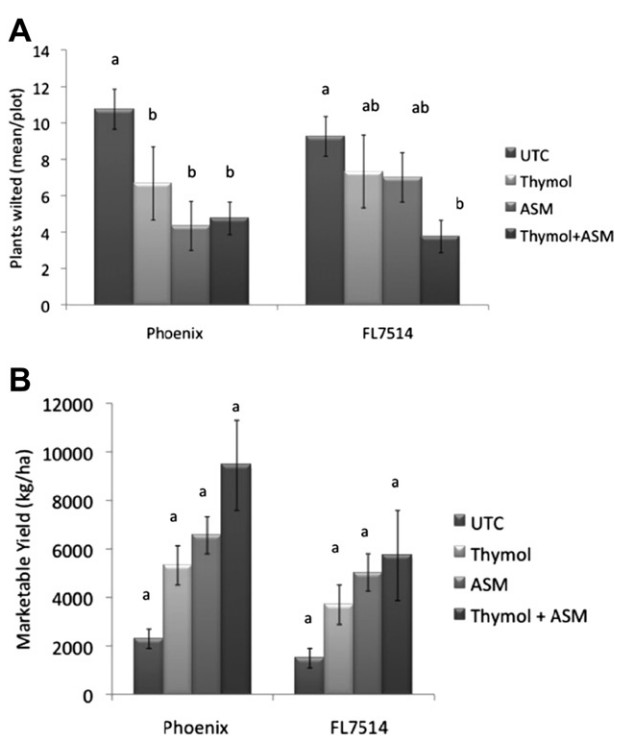

Fig. 2. The effect of thymol, ASM, and the combination of thymol and ASM on the number of plants wilted (Graph A) and marketable fruit yield (Graph B) when applied to susceptible and moderately resistant tomato cultivars in a bacterial wilt field experiment (fall 2008, Quincy, FL). Means and SE (standard error of the mean) of 4 replications and 18 plants per plot. Same letter indicates no significant difference according to Duncan's multiple range test at $P = 0.05$. Untre.

were treated with both chemicals were statistically different from the UTC when comparing fruit yield for both cultivars and disease incidence from the moderately resistant plants. The plants that were treated with thymol or ASM alone were not statistically different from the UTC. It was noted that in the 2008 trial the susceptible cultivar had a greater fruit yield than the resistant cultivar, which is contrary to the 2006 data and the literature. This abnormality could be due to the amount of rain the area received and the general fitness of the cultivars.

In both trials, the moderately resistant plants that received thymol, ASM and the combination of both chemicals had increased fruit yield and lowered disease incidence when compared to the UTC. Previously it was observed that ASM had a greater effect when applied to moderately resistant plants than susceptible plants when comparing the disease incidence to the UTC (Pradhanang et al., 2005), thus indicating that ASM could increase host resistance to bacterial wilt. It was also reported that susceptible tomato cultivars treated with ASM were resistant to the pathogen only when the soil was infested with bacterial populations of 10^5–10^6 cfu/ml. Wilt symptoms were observed when the bacterial concentration was10^7 cfu/ml or greater (Anith et al., 2004; Pradhanang et al., 2005).

Controlling bacterial wilt in field conditions has been studied for decades (Kelman, 1953), and to date no single strategy proven to effectively reduce the incidence of disease or severity of bacterial wilt exists (Denny, 2006). Factors such as the pathogen's ability to colonize alternative hosts (Hong et al., 2008), the longevity of the bacterium in fallow soil and water (Hayward, 1991) and its ability to persist in infested plant debris (Granada and Sequeira, 1983), have made it difficult to control the disease once it has become established in the field.

Good cultural practices, also referred to as Integrated Disease Management (IDM), encompass multiple strategies for controlling the disease. Included in IDM is avoiding planting in pathogen-infested soil with pathogen-free crops, irrigating with pathogen-free water and using proper sanitation practices with tools, which are all important means to exclude or reduce the pathogen (Anith et al., 2004; Denny, 2006; Hong et al., 2008; Champoiseau et al., 2009). Complete resistance to *R. solanacearum* is only found in groundnut; however, moderately resistant tomato cultivars are available to growers. Yet, these tomato cultivars are limited to geographical location (Denny, 2006).

With the decreased use of methyl bromide, alternatives to control soil pathogens have been increasingly studied (Noling and Becker, 1994; Martin, 2003; Santos et al., 2006). Thymol has proven to be effective in controlling pests such as fungi, nematodes, insects and bacteria (Lee et al., 1997; Delespaul et al., 2000; Ji et al., 2005; Šegvić Klarić et al., 2006). ASM has also shown activity against several diseases caused by soilborne fungi, nematodes and bacteria (Benhamou and Bélanger, 1998; Chinnasri et al., 2003; Pradhanang et al., 2005). Further studies to determine the effectiveness of thymol alone, in a non-pretreated field, could aid in determining its use as an alternative to methyl bromide.

Again, it is recommended to use moderately resistant cultivars to lower disease incidence and for maximum yield. We showed that if a grower were to use both chemicals, neither would be detrimental to yield production. As shown in the before mentioned studies, both products are effective at decreasing the incidence of different plant diseases. Thus, the combination of both products could offer wider protection. Further studies are needed to determine the minimum inhibitory concentration (MIC) in field conditions for the most effective and economic benefit for growers. In conjunction with determining the MIC, further studies are needed to determine the effect that the combination of thymol and ASM would have on other plant pathogens or on multiple diseases. Further research would also need to be conducted to determine the plant's responses to the chemicals. Other research suggests grafting could be a new method for controlling the disease (Rivard and Louws, 2008).

Acknowledgements

This research was supported in part by grants from USDA, SARE (Momol, Jones, Ji, Olson, 2006–2009) and from NIFA Special Grant (Momol, Jones, and Olson, NIFA 2006-34135-17484).

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