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Abiotic Compounds as Inducers of Resistance to Fusarium Wilt in Tomatoes

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Abstract

To study the effects of resistance inducers used to control fusarium wilt in tomatoes due to the fungus *Fusarium oxysporum* Schlecht f. sp. *lycopersici*, we evaluated the effects of ASM (acibenzolar-S-methyl), Agro-Mos, chitosan, Biopirol and neem oil on *F. oxysporum* f. sp. *lycopersici* mycelial growth and sporulation and systemic resistance in tomatoes. *In vitro* experiments comprised evaluations of the products' effects on the mycelial growth and sporulation of the PDA (potato dextrose agar) growth medium-cultured pathogen. *In vivo* experiments included product application to tomato plants of the Santa Cruz cultivar that were grown for 25 days on autoclaved soil, followed by determinations of disease severity and peroxidase, polyphenol oxidase and β-1,3-glucanase enzyme activity levels, which are related to the process of resistance induction. Pathogen inoculation occurred after 5 days at a concentration of 10^6 conidia/mL. We evaluated the disease according to a rating scale. Enzymatic activity was determined according to specific protocols. Neem oil controlled pathogen mycelial growth and sporulation, while ASM influenced sporulation. The products reduced the severity of wilt in the plants. We highlight neem oil, Agro-Mos and Biopirol due to their ability to induce significant peroxidase, polyphenol oxidase and β-1,3-glucanase expression, respectively.

Keywords: *Fusarium oxysporum* Schlecht f. sp. *lycopersici*, *Solanum lycopersicum* L., polyphenol oxidase, peroxidase, β-1,3-glucanase

1. Introduction

The tomato (*Solanum lycopersicum* L.) originated in the Andean area of South America and belongs to the Solanaceae family. It is widespread in Brazil and has great commercial importance for both fresh consumption and industrialization. This culture is of great economic importance to Brazil, as are other vegetable crops of the same family (Carvalho, Kist, & Treichel 2016); the tomato is the second-most widely grown vegetable crop in the country (Camargo Filho & Oliveira 2012).

Brazil stands out as a leading tomato producer with an estimated annual production of more than 4 million tons. The state of Maranhão is the sixth-most productive state in the Northeastern region and produces slightly more than 4,700 tonnes from 228 planted hectares (IBGE, 2015). All of this productive potential is subject to the occurrence of various diseases that are influenced by several factors such as climate, the planted cultivar and seed quality, among others, particularly the lack of effective measures for pathogen control.

Fusarium wilt stands out among the diseases that cause great losses in tomatoes. This disease is caused by the fungus *Fusarium oxysporum* Schlecht f. sp. *lycopersici* Snyder & Hansen and occurs in all Brazilian states, where it significantly damages crop plants and leads to the premature death or destruction of all plants (Inami et al., 2014).

Maranhão is deficient in the control of this disease, which is associated with environmental conditions favorable to the fungus, leading to constant economic unfeasibility of tomato cultivation, mainly for family farms that lack the resources and techniques relevant to better disease management. This situation increases production costs and significantly decreases the population's access to this highly consumed product.

Systemic acquired resistance (SAR) might be a viable alternative in the control of fusarium wilt in tomatoes. SAR results from the activation of the plant defense system in response to biotic and abiotic stimuli and leads to the expression of mechanisms related to the production of substances toxic to the pathogen and/or the formation of structural barriers that restrict tissue colonization (Kuć, 2001). SAR comprises a complex phenomenon that involves the activation of several processes, including hypersensitivity, structural barriers, the increased synthesis of phytoalexins and the accumulation of pathogenesis-related proteins (PRs) such as hydrolase β-1,3-glucanase, which breaks down fungal pathogen cell walls (Hammerschmidt, 1999).

In addition, the enzymatic changes can be related to physiological aspects in combination with the induction of the plant's resistance to diseases. Several reports have demonstrated a relationship between induced systemic resistance and enzymes such as peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, β-1,3-glucanase and chitinase (Van Loon, Bakker, & Pieterse, 1998; Chithrashree et al., 2011; Nascimento & Barrigossi, 2014; Melo et al., 2016).

The use of resistance inducers in plant disease control, including the application of both abiotic products and non-virulent *F. oxysporum* f. sp. *lycopersici* isolates, has shown success in the control of tomato fusarium wilt (Aimé, Cordier, Alabouvette, & Olivain, 2008; Farag Hanaa et al., 2011).

With the aim of validating alternatives to the situation in which the state of Maranhão finds itself relative to tomato production, we sought to evaluate resistance induction as a control method capable of reducing or inhibiting the *Fusarium oxysporum* f. sp. *lycopersici* population by evaluating the effects of abiotic products on the pathogen and the activities of enzymes involved in tomato defenses against this pathogen.

2. Material and Methods

2.1 Experimental Location and Procurement of the Fusarium oxysporum f. sp. lycopersici Isolate

The experiments were performed at the Laboratory of Phytopathology and in the Greenhouse at the Maranhao State University [Universidade Estadual do Maranhão (UEMA)].

We used the *F. oxysporum* f. sp. *lycopersici* isolate "FOL 88", which was donated by Embrapa Vegetables (Embrapa Hortaliças, Brasília, DF, Brazil). The isolate was transferred to Petri dishes that contained potato dextrose agar (PDA) growth medium and were later transferred to test tubes to preserve pure cultures.

Inoculations was performed on 30-day-old tomato plants of the Santa Cruz cultivar, according to the root injury method, on 1 side of the system, followed by the application of 20 mL of inoculum suspension at a concentration of 1×10^6 conidia/mL. Evaluations were performed through fusarium wilt development to confirm pathogenicity.

2.2 Effect of Abiotic Products Fusarium oxysporum f. sp. lycopersici on Mycelial Growth and Sporulation in vitro

Commercial products were used at different concentrations: ASM (acibenzolar-S-methyl) (0.005, 0.01, 0.02 and 0.04 mg/mL), chitosan (0.15, 0.2, 0.3 and 0.5 mg/mL), Biopirol (0.25, 0.5, 1.0 and 2.0 mL/mL), Agro-Mos (0.25, 0.5, 1.0 and 2.0 mL/L) and neem oil (1.00, 2.00, 3.00 and 4.00 mL/L). The products were added to melted PDA medium (approximately 45 °C) at different concentrations. Petri dishes that contained only PDA without the products served as controls.

A 5-mm diameter disc that contained *F. oxysporum* f. sp. *lycopersici* mycelia (taken from a colony with 7 days in PDA) was transferred to the center of each plate that contained inductors, and the plates were maintained at 25 ºC with a 12-hour photoperiod. Evaluations were performed daily until the control plate colonization nearly reached the full plate diameter (9.0 cm.) We performed measurements with a ruler in 2 diametrically opposed directions to take an average measurement of the colonies. The mycelial growth inhibition percentage value (GIP) was calculated as follows (Menten et al. 1976):

$$GIP = \frac{Control\ Growth - Treatment\ Growth}{Control\ Growth} \times 100 \quad (1)$$

The experimental design was completely randomized in a factorial scheme, 5 × 4 + 1 (5 treatments × 4 concentrations + 1 control), with 5 repetitions for each treatment; each repetition was a Petri dish. The control characterized the zero concentration for all treatments that were submitted to regression analysis.

The mycelial GIP was calculated for each dose against the control. Data were analyzed in an analysis of variance in addition to a regression analysis.

The sporulation inhibition test was performed at the end of the mycelial growth inhibition test to evaluate the level of sporulation on each of the used plates. Specifically, a spore suspension was prepared by adding 10 mL of distilled water to the plate and scraping the colonies with a glass slide. In this way, we determined the number of conidia/mL with a Neubauer chamber and an optical microscope. The number of conidia/cm^2 was later determined from the results of the mycelial growth test. Percentage data for sporulation inhibition were assessed by analysis of variance and Tukey's test.

2.3 Use of Inductors in Fusarium Wilt Control in Tomatoes

To test the effects of inducers on the tomato plants, we used the recommended concentrations of the commercial products as follows: ASM, 5.0 mg/ml; Agro-Mos®, 1.5 mL/L; neem oil, 2.0 mL/L; chitosan, 2.0 mg/mL and Biopirol®, 1.5 mL/L.

The Santa Cruz cultivar tomato seedlings were produced and, 15 days after sowing, the plants were transplanted into 5-L pots that contained autoclaved soil and manure at a density of 3 plants/pot.

The tested resistance inducers were applied to the plants 5 days before pathogen inoculation and 25 days after sowing by spraying the first pair of leaves.

The inoculation was performed 30 days after sowing by root injury, and 20 ml/plant of the inoculum suspension was applied to the region close to the roots at a concentration of 1×10^6 conidia/mL. The tomato plants were kept in a greenhouse until the end of the evaluation.

Disease severity assessments were performed 25 days after inoculation according to a grading scale proposed by Santos (1999); grade 1 was assigned to healthy plants, grade 2 to sick plants with mild vascular symptoms, grade 3 to plants with symptoms of leaf yellowing and vascular darkening, grade 4 to plants with severe wilt associated with vascular darkening, leaf chlorosis and necrosis and grade 5 to dead plants.

2.4 Evaluation of Biochemical Compounds Involved in the Induction Process

2.4.1 Total Protein Extraction

Leaf collection for enzyme activity preparations occurred at 5 and 10 days after pathogen inoculation, with 1 plant used per collection period.

Briefly, 1.0-g samples of leaves that corresponded to each treatment were macerated in a mortar with liquid nitrogen and 1% (v/v) polyvinylpyrrolidone (PVP), 5 mL of sodium acetate buffer (0.1 M, pH 5) and 1 mL of EDTA (1 mM). The extracts were centrifuged at 10,000 g for 10 minutes at 4 °C, and the supernatants were transferred to 2-mL Eppendorf tubes and stored at -80 °C. The enzyme extracts were used to determine the activities of peroxidases, polyphenol oxidases and β-1,3-glucanase.

A completely randomized experimental design was used to evaluate subdivided parcels with 6 (treatments) × 2 (collection period) × 7 (repetitions); each repetition was represented by 3 plants per pot. Data were submitted to an analysis of variance, and the averages were compared with Tukey's test.

2.4.2 Peroxidase Activity (E.C. 1.11.1.7)

Peroxidase activity estimations was performed according to an absorbance evaluation of the oxidation of guaiacol in the presence of hydrogen peroxide, defined as the variation in absorbance at 470 nm produced in the reaction medium per the time in minutes and milligrams of protein (Δ Abs/min mg). To develop the reactions, 50 µL of guaiacol (0.02 M), 0.5 mL of hydrogen peroxide (0.38 M) and 2.0 mL of phosphate buffer (0.2 M/pH 5.8) were transferred to a spectrophotometric cuvette. The mixture was gently stirred and used to calibrate the spectrophotometer. Next, 50 µL of the enzyme extract was added, the reaction was shaken gently, and readings were performed at a wavelength of 470 nm and at intervals of 10 seconds for a period of 1 minute.

2.4.3 Polyphenol Oxidase Activity (E.C. 1.10.3.1)

We added 100 µm of enzymatic tomato leaf extract to 2.9 mL of a solution that contained catechol (25 nM) dissolved in potassium phosphate buffer (Monosodium phosphate/Dibasic sodium phosphate) (100 mM, pH = 6.5). The absorbance was measured at 410 nm. The specific enzyme activity was defined as the variation of absorbance at 410 nm produced in the reaction medium per time in minutes and milligrams of protein (Δ Abs/min mg).

2.4.4 β-1,3-Glucanase Activity (E.C. 3.2.1.29)

The determination of β-1,3-glucanase activity was performed by measuring glucose during laminarin hydrolysis (Tuzun et al. 1989). For this reaction, 25 µL of the enzymatic extract, 200 µL of potassium acetate buffer (0.1 M, pH 4.8) and 200 µL of laminarin (15 mg/mL) were pipetted into test tubes. The reactions were incubated at 37 °C for 30 minutes, after which 1 ml of Somogyi reagent (Somogyi, 1952) and 10 ml of distilled-deionized water were added to each and the mixtures were stirred for 10 minutes. After stirring, the reactions were incubated in a water bath at 100 °C for 15 minutes and immediately cooled in an ice bath. Next, 1.0 mL of Nelson reagent (Somogyi, 1952) and 25 mL of deionized-distilled water were added to each reaction, followed by stirring for 15 minutes. The reactions were read spectrophotometrically at 760 nm, and the readings were compared with those of glucose standards. A standard curve of glucose was prepared according to the same standard addition method used for the samples, with the substitution of glucose solutions for laminarin (0-300 mg/L glucose).

2.4.5 Determination of Soluble Proteins

The soluble protein concentrations were colorimetrically determined according to the method described by Bradford (1976). Briefly, 200 µl of the enzyme extract and 2 mL of Coomassie Brilliant Blue (CBB) solution were pipetted into test tubes, shaken gently and read spectrophotometrically at a wavelength of 595 nm. The results of the enzyme extract sample readings were converted to soluble protein concentrations by comparing the readings to the efficiency curve generated from the readings of standard bovine serum albumin (BSA) solutions at concentrations of 0, 25, 50, 100, 150 and 200 mg/L.

The CBB solution was prepared by dissolving 0.10 g of CBB reagent (G-250) in 50 mL of absolute ethanol. Next, 100 ml of phosphoric acid (d = 1.71 g/mL) and 850 mL of distilled water were added.

3. Results

3.1 Effects of Abiotic Products on Fusarium oxysporum f. sp. lycopersici Mycelial Growth and Sporulation in vitro

While evaluating the effects of the abiotic products on *F. oxysporum* f. sp. *lycopersici* mycelial growth, a significant difference was observed between the treatments (Table 1). The treatments that yielded the maximum percentages relative to the control were based on chitosan, at a concentration of 0.400 mg/L and a GIP greater than 23%, and neem oil, with a concentration of 4.00 mL/L and a GIP greater than 17%, compared to the control. The other treatments showed mycelial growth inhibition values below 10%, which did not differ from the control at a 5% level of probability, according to Tukey's test.

Table 1. Evaluation of *F. oxysporum* f. sp. *lycopersici* mycelial growth inhibition after 10-day examinationin response to abiotic product application

Treatments	Colony diameter (cm)		GIP (%)
Agro-Mos® 0.25 mL/L	8.11	c**	1.62
Agro-Mos® 0.50 mL/L	7.84	bc	4.85
Agro-Mos® 1.0 mL/L	7.98	bc	3.24
Agro-Mos® 2.0 mL/L	8.38	c	-1.72
ASM 0.005 mg/mL	8.03	bc	2.57
ASM 0.01 mg/mL	8.14	c	1.23
ASM 0.02 mg/mL	8.11	c	1.60
ASM 0.04 mg/mL	8.26	c	-0.22
Biopirol® 0.25 mL/mL	8.00	bc	2.93
Biopirol® 0.50 mL/mL	7.68	bc	6.81
Biopirol® 1.0 mL/mL	7.85	bc	4.75
Biopirol® 2.0 mL/mL	7.51	abc	8.88
Chitosan 0.15 mg/mL	8.60	c	-4.35
Chitosan 0.20 mg/mL	8.22	c	0.26
Chitosan 0.30 mg/mL	8.17	c	0.87
Chitosan 0.50 mg/mL	6.30	a	23.56
Control	8.24	c	-
Neem 1.0 mL/L	7.97	bc	3.30
Neem 2.0 mL/L	7.51	abc	8.88
Neem 3.0 mL/L	7.47	abc	9.40
Neem 4.0 mL/L	6.81	a	17.39

Note. ** Averages followed by the same letter in the column do not differ in a Tukey's test at 1% ($p < 0.01$) significance level. CV (%) = 7.79.

When comparing the mycelial growth curve during the 10-day examination, it was noted that each inhibitor acted differentially as a function of time (Figure 1). All Agro-Mos® treatments maintained their growth since the first evaluation, similar to the control, at the second day after the experiment. Biopirol® and ASM treatments differed from the control on the fourth day of evaluation but equaled it throughout the evaluation. Neem oil showed a significant difference from the control beginning at the second day of evaluation for all applied concentrations. This variation extended along the measurements, and the 1.00, 2.00 and 3.00 mL/L concentrations only equaled the control on the tenth day; the highest concentration of 4.00 mL/L remained different from the control at the end of the evaluation.

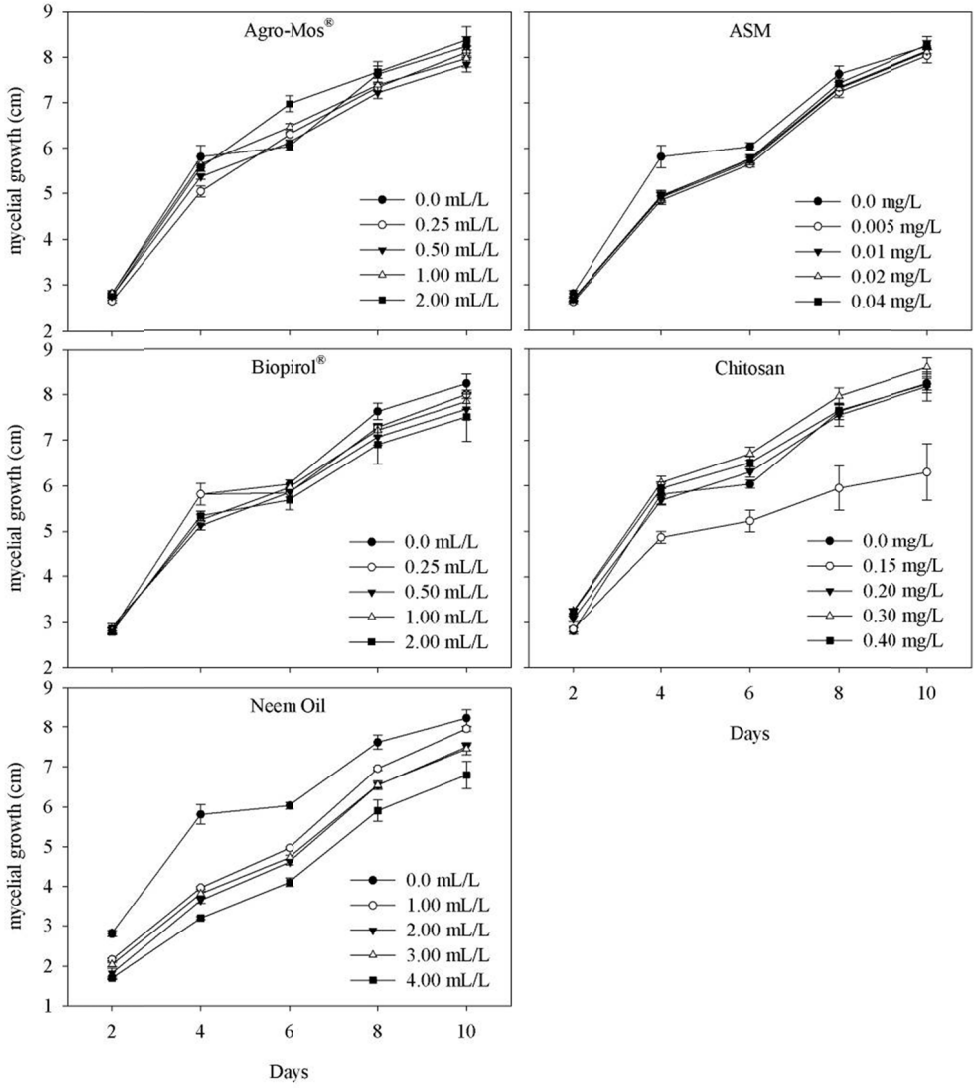

Figure 1. *Fusarium oxysporum* f. sp. *lycopersici* mycelial growth (average±SE, n = 12) as a function of the nature and concentration of the inducer over a 10-day evaluation period

Regarding the variations in the product concentrations, it is worth noting that only neem oil and Biopirol had increasing influences in the control of the pathogen mycelial growth (Figure 2), with Pearson correlation coefficients of -0.97 and -0.84, respectively, and were compared to the standard inductor ASM, which did not affect the fungus as a function of increased concentration (coefficient r = 0.42).

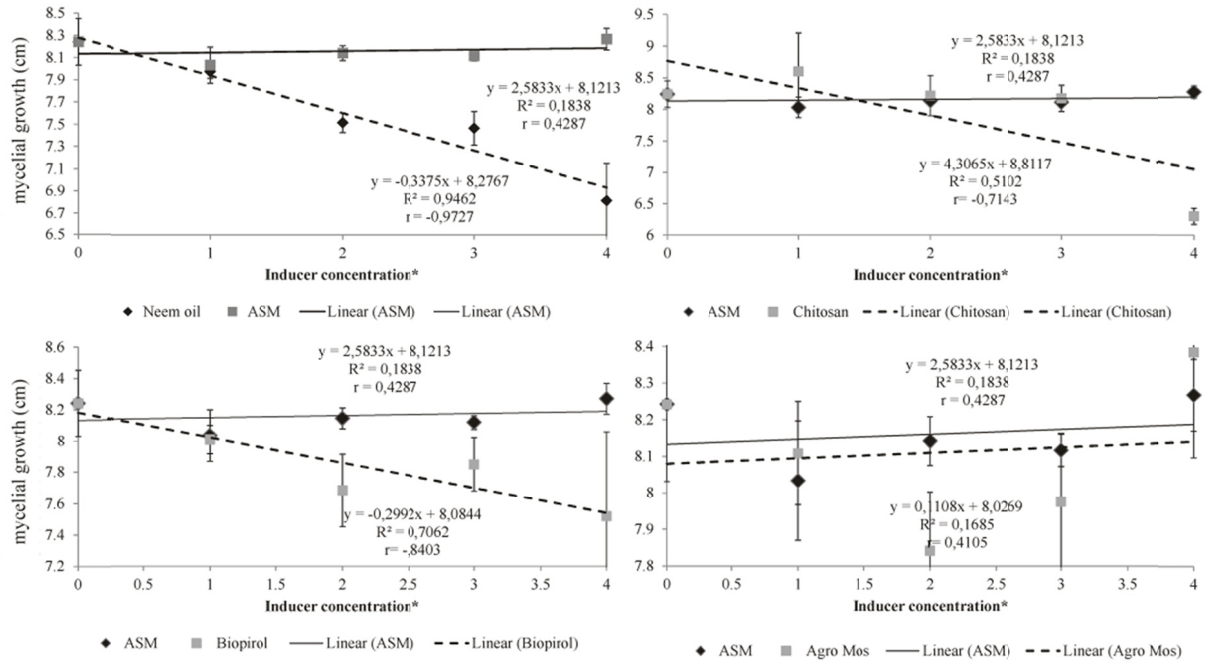

Figure 2. Linear correlation between inducer concentration and *F. oxysporum* f. sp. *lycopersici* mycelial growth (average±SE, n = 12) ($P < 0.05$). * Concentrations 0, 1, 2, 3 and 4 represent: 0.0, 0.005, 0.01, 0.02 and 0.04 mg/L ASM; 0.0, 1.00, 2.00, 3.00 and 4.00 mL/L neem oil; 0.0, 0.25, 0.5, 1.0 and 2.0 mL/L Biopirol; 0.0, 0.25, 0.5, 1.0 and 2.0 mL/L Agro-Mos®; and 0.0, 0.15, 0.2, 0.3 and 0.4 mg/L chitosan

Regarding the control of conidia production, there were significant differences between the treatments, both relative to the nature and to the concentrations of the employed products (Table 2). We highlighted the highest concentrations of neem and ASM, which had *F. oxysporum* f. sp. *lycopersici* conidia production control percentages of 88.10 and 83.17%, respectively. All concentrations of neem oil and Biopirol® differed from the control, with sporulation control percentages ranging from 51% to 88.30% and from 51.41% to 74.23%, respectively, due to increased product concentrations. Chitosan treatments did not induce significant effects when compared to the control. Agro-Mos® and ASM induced differences only at higher concentrations, with minimum concentrations of 0.5 mL/L and 0.01 mg/L, respectively.

Table 2. Evaluation of the inhibition of *F. oxysporum* f. sp. *lycopersici* sporulation in response to abiotic product application

Treatments	**Number of Conidia/mL**		**GIP (%)**
Agro-Mos® 0.25 mL/L	1.20E+06	d*	17.35
Agro-Mos® 0.50 mL/L	7.04E+05	c	51.41
Agro-Mos® 1.0 mL/L	5.16E+05	abc	64.42
Agro-Mos® 2.0 mL/L	5.38E+05	bc	62.90
ASM 0.005 mg/mL	1.22E+06	d	15.72
ASM 0.01 mg/mL	1.18E+06	d	18.83
ASM 0.02 mg/mL	5.23E+05	abc	63.93
ASM 0.04 mg/mL	2.44E+05	ab	83.17
Biopirol® 0.25 mL/mL	7.04E+05	c	51.41
Biopirol® 0.50 mL/mL	5.24E+05	bc	63.81
Biopirol® 1.0 mL/mL	4.32E+05	abc	70.20
Biopirol® 2.0 mL/mL	3.73E+05	abc	74.23
Chitosan 0.15 mg/mL	1.17E+06	d	19.37
Chitosan 0.2 mg/mL	1.20E+06	d	17.17
Chitosan 0.3 mg/mL	1.13E+06	d	22.35
Chitosan 0.5 mg/mL	1.22E+06	d	16.01
Control	1.45E+06	d	-
Neem 1.0 mL/L	7.10E+05	c	51.00
Neem 2.0 mL/L	6.52E+05	c	55.02
Neem 3.0 mL/L	6.03E+05	c	58.36
Neem 4.0 mL/L	1.70E+05	a	88.30

Note. * Averages followed by the same letter in the column do not differ in a Tukey's test at a 5% ($p < 0.05$) significance level.

Figure 3 shows that ASM and neem oil caused concentration-dependent significant reductions in pathogenic agent sporulation, with respective "r" correlations greater than -0.95 and -0.91. The other treatments showed no statistical correlations between increased concentration and sporulation.

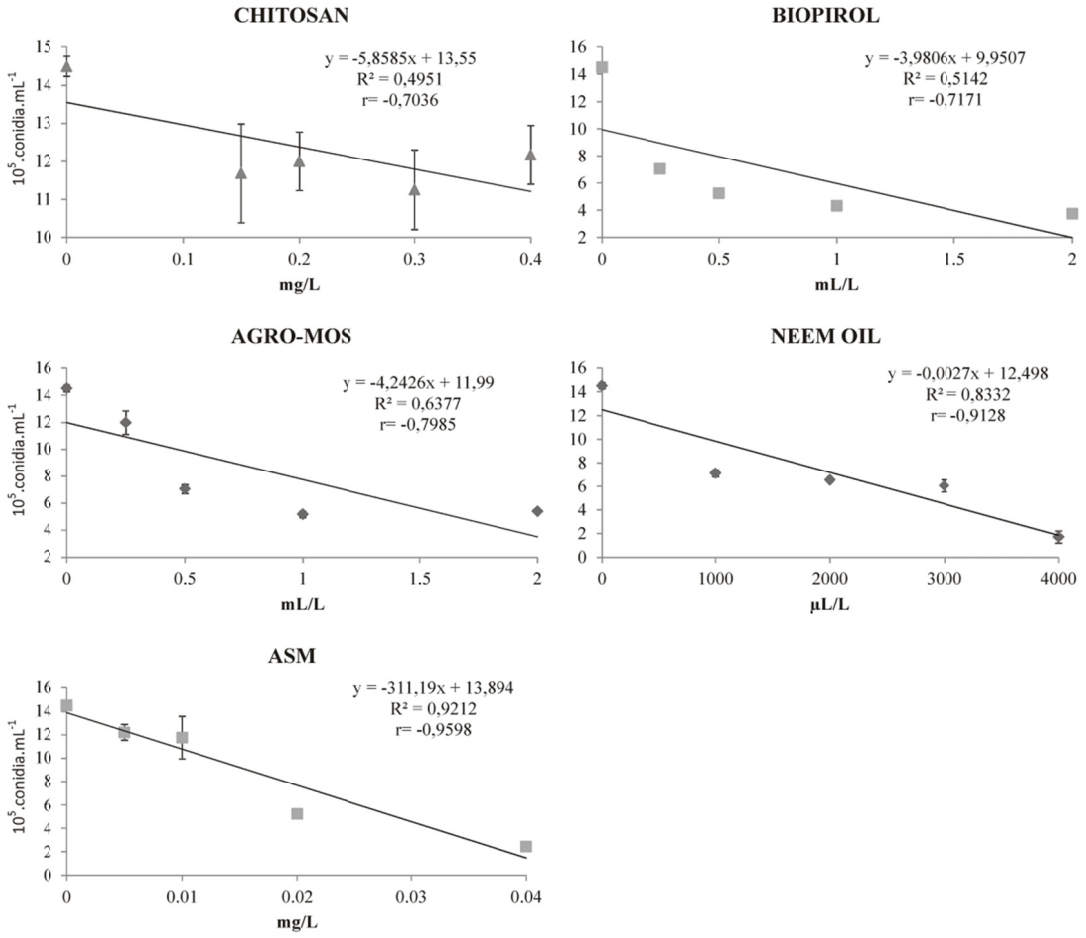

Figure 3. Linear correlation between inducer concentration and *F. oxysporum* f. sp. *lycopersici* sporulation (average±SE, n = 12) ($P < 0.05$)

3.2 Use of Inducers in the Control of Tomato Fusarium Wilt

When analyzing Table 3, we found a statistically significant difference at a 5% probability level in Tukey's test between the application of the products and the control (characterized by spraying with water). All treatments were statistically similar, with tomato fusarium wilt control percentages ranging from 38.05% to 56.05%, compared to the control.

Table 3. *In vivo F. oxysporum* f. sp. *lycopersici* control in response to abiotic product application

Treatments	Grades	Control (%)***
Control	2.39 a**	-
ASM	1.05 b	56.05
Agro-Mos®	1.19 b	50.19
Neem Oil	1.29 b	46.00
Chitosan	1.43 b	40.14
Biopirol®	1.48 b	38.05

Note. ** Averages followed by the same letter in the column do not differ in a Tukey's test at a 1% ($p < 0.01$) significance level. DMS = 0.6951 and CV (%) = 29.61%.

*** Percentage of control, considering the severity notes (Column "Grades"), obtained by the equation: Control (%) = (Control – Treatment)/Control.

3.3 Evaluation of Biochemical Compounds Involved in the Induction Process

3.3.1 Peroxidase Activity (E.C. 1.11.1.7)

Figure 4 shows that at 5 days after pathogen inoculation, only the ASM-based treatment differed significantly from the control, with a peroxidase activity level greater than 5.0 abs_{470nm}/min mg. There was an increase in the enzyme activity at 10 days, highlighting the ASM and neem oil treatments that showed increased activity, with a significant difference when compared to chitosan treatment at 10 days post-inoculation with *F. oxysporum* f. sp. *lycopersici*. Despite having the second-lowest average, the control did not differ from the other treatments.

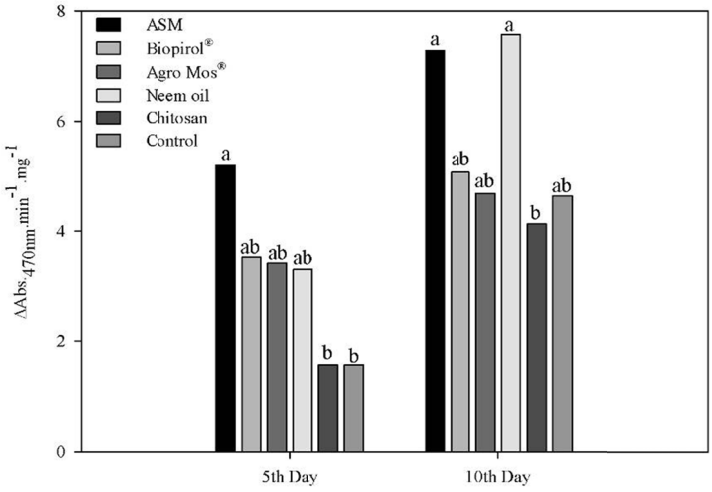

Figure 4. Hydrogen peroxide oxidation by the peroxidase enzyme in response to different resistance inducers that were applied to tomato plants in São Luís/MA. Averages followed by the same letter in the column do not differ in a Tukey's test at a 5% ($p < 0.05$) significance level

3.3.2 Polyphenol Oxidase Activity (E.C. 1.10.3.1)

All treatments had significant higher polyphenol oxidase activity than the control, except for chitosan, which equaled the control treatment during the 2 evaluations (Figure 5). We highlight the product Agro-Mos®, which had an enzymatic activity level greater than 7.5-Δabs_{410nm}/min mg at 5 days post-inoculation, which differed from the other treatments, but had a decreased in potential enzymatic activity at 10 days that equaled, on average, the Biopirol®, neem oil and ASM treatments after 10 days of evaluation.

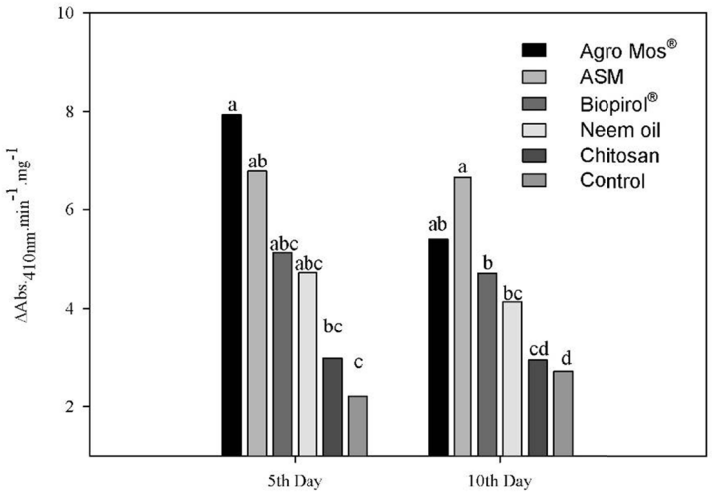

Figure 5. Abiotic product-induced polyphenol oxidase activity in tomato leaves in São Luís/MA. Means followed by the same letter in the column do not differ in a Tukey's test at a 5% ($p < 0.05$) significance level

3.3.3 β-1,3-Glucanase Activity (E.C. 3.2.1.29)

After 5 days of evaluation, only the ASM-based treatment differed significantly from the control with a specific activity greater than 1.4 Δabs_{760nm}/min mg. However, the ASM and neem oil treatments differed from the others at 10 days after post-inoculation, with enzymatic activities greater than 1.2 Δabs_{760nm}/min mg (Figure 6). Another observed feature of the above treatments is the difference in the enzymatic activity over time. Notably, for the ASM-based treatment, a decrease in the enzymatic activity occurred when the levels at 5 and 10 days post-inoculation were compared, whereas neem oil treatment increased the enzymatic activity during the same enzyme activity period.

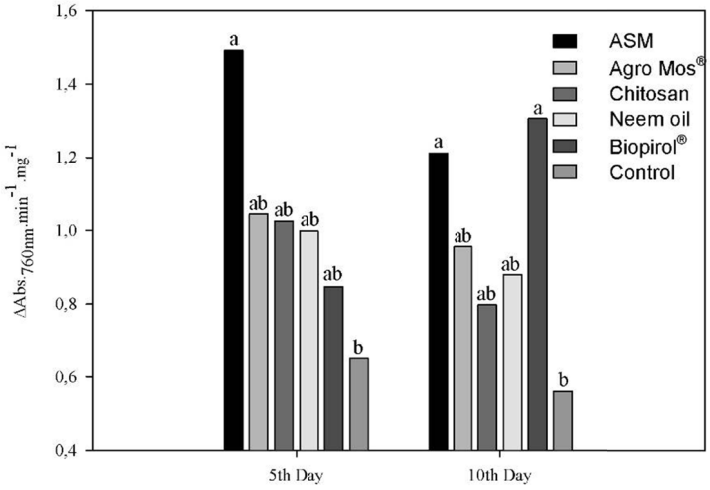

Figure 6. Abiotic product-induced β-1,3-glucanase activity in tomato leaves in São Luís/MA. Means followed by the same letter in the column do not differ in a Tukey's test at a 5% ($p < 0.05$) significance level

4. Discussion

Treatments based on chitosan and neem oil at the highest concentrations inhibited pathogen mycelial growth within the first evaluations (2 and 4 days after the beginning of the experiment; Figure 1), demonstrating the efficiency of the products in preventing the establishment of the microorganism and featuring one of the main reasons for the significant differences between the treatments and the control at the highest concentrations of neem oil and chitosan.

Cia, Benato, Pascholati, and Garcia (2010) observed that even at low concentrations (0.5% chitosan on PDA medium), chitosan controlled 100% of the mycelial growth of *Rhizopus stolonifer*. Faria,Maia,Botelho and Leite (2009) observed a 30% control rate of the fungus *Botryosphaeria* sp. with different concentrations of chitosan, reaffirming that variations in the product concentration positively affect the control of mycelial growth of the studied fungus.

The products Agro-Mos®, ASM and Biopirol® did not immediately show this effect against the pathogen, as demonstrated by the lower comparative variations between these treatments and the control throughout the experiment. Even during the early stages of growth medium colonization, the pathogen did not experience negative stimulation from the products and demonstrated normal vegetative growth. These results support the use of these substances as resistance inducers due to the absence of toxic effects of the inducing agent on the pathogen (Steiner & Schönbeck, 1995; Romeiro & Garcia, 2009).

In general, regardless of the concentrations, neem, Biopirol® and chitosan treatments presented, in ascending order, higher controlling effects on *F. oxysporum* in the *in vitro* tests (Table 1, Figure 1). Here, the qualification and quantification of the enzymes involved in resistance induction is justified because the simple spatial separation between the inducer application site (part area) and the pathogen inoculation site (root system) does not feature a precise criterion for resistance induction because the possibility of systemic translocation of the applied product as an inducer from the host application site to the pathogen infection site, where it expresses antifungal effects, remains unknown or poorly studied.

In Figure 2, it is clear that the negative correlation between varied concentrations of neem oil and Biopirol® and pathogen mycelial growth also reinforces the use of products derived from *Azadirachta indica* as fungitoxic controllers that are applied directly to disease symptoms in plants or preventively to the site of pathogen action (Diniz et al., 2006; Silva, 2010). This correlation also extends the use of Biopirol, a pyrolysis extract commonly used as a foliar fertilizer and now with a fungitoxic strand, towards mainly preventing the establishment of the pathogen or as a resistance inducer.

Even with the absence of effective pathogen control due to the fungitoxic effects of increased concentrations of ASM, Agro-Mos® and chitosan (Figure 2), we cannot discard the hypothesis that the inducers might act through different mechanisms in addition to resistance induction, such as changes in the rhizosphere quality that could encourage microbial flora at the inoculation site, even in the presence of spatial separation between induction and inoculation. The evaluation of other criteria, such as the formation and activation of enzymes related to pathogenicity, is extremely important in qualifying and/or quantifying the occurrence of resistance induction.

The results of these experiments indicate that the tested products act through different control mechanisms against *F. oxysporum* f.sp. *lycopersici* and can directly influence the area covered by the pathogen and thus limit mycelial growth (Table 1) or can exert negative effects on the ability of the fungus to increase its inoculum source through sporulation control (Table 2). However, there were significant differences in pathogen spore production for most treatments, with the exception of Chitosan, which remained similar to the control. Figure 3 showed that the variation in sporulation was only addressed by increasing the concentrations of the ASM and neem oil treatments; this result was already expected due to the referenced potential fungicide abilities of *A. indica*.

The increased fungicidal effects of the products are essential when seeking to apply products to the possible sources of inoculum to reduce the inoculum or minimize pathogen spread before the environment is contaminated while accounting for the effect of the product on the agroecosystem, as well as the use of integrated management practices. In pre-infested environments, where the goal is to control the disease by resistance induction, such products can be justified as inducers because the application occurs in the plant canopy, and even when translocation of the product to the roots occurs, the inductive effects should be more noticeable than simple sporulation inhibition. At that point, there is a combined effect of control mechanisms, either due to resistance induction or to direct control of the causal agent, in favor of better disease management.

The results obtained by Guzzo, Castro, Kida, and Martins (2001) extend beyond those obtained in this study. Although the effects of ASM have been demonstrated against *F. oxysporum* f. sp. *lycopersici* spore production, those authors observed with fluorescence microscopy that ASM, when applied *in vitro*, does not interfere with spore germination and appressorium formation in *Hemileia vastratrix* and concluded that ASM did not exert a direct antimicrobial action against the pathogens; however, ASM induced the expression of genes for resistance to the formation of compounds that would prevent or hinder the establishment or development of the pathogens.

Systemic resistance activation was observed in the in Santa Cruz tomato cultivar, and *F. oxysporum* f. sp. *lycopersici* was controlled by the application of inducers (Table 3). It was observed that even during the early stages of the disease, represented by grades 1 and 2 of Santos' scale (1999) in which plants appear to be normal or to have mild vascular symptoms, respectively, the effects of inducers on the plants could be significantly differentiated; this is best demonstrated in the evaluation of enzymes involved in the resistance induction process. The results of this experiment corroborate those reported by Cruz, Rodrigues, Coelho, and Sardinha (2011), who evaluated the effects of different inducers in tomato fusarium wilt severity reduction in greenhouse cultivation.

A priori, it can be suggested that the plant aroused or constituted sources of defense, a hallmark of induction that was described by several authors as a state of alertness, in which the plant responds with greater agility and efficiency to the establishment of the challenging pathogen. For many authors, the application of resistance inducers promotes the activation of SAR, leading to a marked reduction in disease symptoms (Kessmann et al., 1994b; Kessmann et al., 1994a; Martinez et al., 2000) because it initially occurs in the infection region to prevent or delay the entry of the pathogen (Agrios, 2005).

In an analysis of Figure 4, it was observed that resistance induction occurred in response to increasing peroxidase enzyme activation. This finding can be explained by the fact that the pathogen itself is an inductive element, and thus the recognition signals induced by contact of the plant with the organism can trigger latent defense mechanisms, even in smaller proportions.

The progressive continuity of this evaluation could validate the effective difference in enzyme activity increases with inducer application, relative to the control. More studies on the durability of this increase in enzyme activity

are required because many authors have limited their evaluations to a maximum of 7 days after pathogen inoculation (Araújo & Menezes, 2009; Mandal, Mallick, & Mitra, 2009).

This increase in enzyme activity reinforces the importance of resistance induction as a plant defense promoter over time and might suggest the hypothesis that this is a mechanism through which ASM and neem oil activate resistance in tomato plants. Peroxidase is closely related to the formation of cell wall components, wherein the increased activity promotes or predisposes wall thickening. In the first instance, cell wall strengthening occurs due to the formation of lignin, suberin and other cell wall polysaccharides, in addition to the activity of peroxidase as an antimicrobial agent that acts directly on the pathogen (Mandal, Mallick, & Mitra 2009).

According to Hiraga et al. (2001), the dehydrogenative oxidation of guaiacol results in the formation of phenoxy radicals, and the subsequent binding of unstable radicals leads to the non-enzymatic polymerization of monomers; similarly, hydroxycinnamyl alcohol and its derivatives are converted into phenoxy radicals to form lignin, and hydroxycinnamic acid is converted into suberin.

Araújo et al. (2009) observed significant differences in peroxidase activity levels in response to ASM, azoxystrobin and *Bacillus subtilis* when these were used as resistance inducers in tomato plants. Likewise, Mandal et al. (2009) demonstrated that peroxidase activity in tomato plants sharply increased in response to the application of salicylic acid, an ASM analogue, during a 168-hour (7-day) period.

Kühn (2007) observed an increase in peroxidase activity in IAC Carioca Tybatã bean plants and a reduction in the severity of the bacterial blight common in plants treated with *Bacillus cereus*.

It is noteworthy that there was a decrease in the potential of the enzyme polyphenol oxidase over time in response to most treatments (Figure 5), which allows us to infer that the action of this process is more efficient when resistance induction occurs near the onset of the disease/pathogen. As much as it is possible, under controlled conditions, to set the interval between induction with elicitors and the pathogen inoculation, under field conditions, where inoculation is natural, it is possible to initiate induction during the phase in which the tomato is more susceptible to the pathogen, with regard to the efficiency of polyphenol oxidase in disease control by directly oxidizing polyphenols into quinones, which are usually toxic to the challenging pathogen.

These results differ from those of Silva, Pascholati, and Bebendo (2007), who reported lesser effects of polyphenol oxidase in plants that were treated with inducers and inoculated with bacteria when compared to those that were treated with the water-based control and/or inoculated with the pathogen. However, these findings corroborate the results of Cavalcanti et al. (2006), who observed increasing peaks of activity for this enzyme within the first 48 hours after treatment with the inductors ASM and Ecolife® but demonstrated a decline in the enzyme efficiency from the ninth day of evaluation. Farag Hanaa et al. (2011) observed peaks for 9 polyphenol oxidase isozymes at 7 days post-inoculation with *F. oxysporum* f. sp. *lycopersici* in tomato seedlings that were treated with aqueous extracts of Neem and Melon.

Resistance induction in response to activation of the enzyme β-1,3-glucanase was shown in all treatments (Figure 6), with emphasis given to ASM and Biopirol treatments, which differed from the others at 10 days post-pathogen inoculation (Figure 6). Another observed characteristic among the described treatments is the difference in the efficiency of the enzyme over time. It is notable that for the ASM-based treatment, there was a decrease in enzymatic activity when days 5 and 10 post-inoculation were compared, whereas Biopirol treatment increased the enzymatic activity over the same period.

ASM has been widely studied as a potential inducer of systemic acquired resistance, and increases in β-1,3-glucanase activity were demonstrated in most cases (Cole, 1999; Godard et al., 1999; Resende et al., 2002; Cavalcanti et al., 2006). With the obtained results, we can infer the importance of Biopirol® as a resistance inducer and an important elicitor of β-1,3-glucanase production, an enzyme of utmost importance in plant defenses against pathogen attacks that acts directly on fungal cell wall hydrolysis. Rodrigues,Neto and Coelho (2006) found a negative correlation between the actions of inducers against enzyme activity at 5 days post-inoculation and the disease index in cowpea cultivars (*Vigna unguiculata* L.), which reinforces the importance of studies that aim to reduce disease severity through the use of resistance inducers.

5. Conclusions

This study demonstrated the possibility of tomato fusarium wilt control through an ecological management strategy and generated basic knowledge about biochemical aspects involved in tomato plant defenses. The results also indicated that the tested products acted through different mechanisms of resistance induction with regard to activated enzymes, demonstrating that the use of inducer combinations is important in increasing the extent of plant protection mechanisms.

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