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Enzymatic Activity Induced by Acibenzolar-S-methyl for Control of *Pratylenchus brachyurus* in Maize

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Abstract

Despite investigations of new nematode control methods, little is known about the action mode of resistance induced involving control of *Pratylenchus brachyurus*. Thus, this work aimed to evaluate the activity of defense-related enzymes in the control of *P. brachyurus* in maize treated with acibenzolar-S-methyl (ASM). For this, maize seedlings were submitted to four treatments: plants inoculated with *P. brachyurus* and treated with ASM (IT), inoculated and not treated (INT), not inoculated and treated (NIT) and not inoculated and not treated (NINT). Ten days after germination, the plants were sprayed with ASM and inoculated (800 specimens plant^{-1}). Each five and two days after treatment (DAT), in two different experiments, the plants were collected for analysis of the enzymatic activity of POX, PPO, PAL and GLU in leaf and root tissues. At 30 and 60 days, the nematological parameters were evaluated. We observed higher activity of all enzymes in the maize roots, especially at five and six DAT in plants treated with the product. In the aerial part, plants treated with ASM also presented greater activity of the enzymes, except PAL. ASM reduced the nematode population in maize roots compared to the control plants.

Keywords: Alternative control; Resistance induction; Root lesions nematodes; Defense related proteins; *Zea mays*

Introduction

Resistance induction is triggered by an external signal (elicitor), activating secondary messengers and promoting the expression of specific genes responsible for the production of proteins that determine resistance in plants [1]. Among the main proteins related to the process of resistance induction, phenylalanine ammonia-lyase (PAL), peroxidase (POX), polyphenol oxidase (PPO) and β-1.3-glucanase (GLU) are those most commonly associated with the increased resistance of plants to nematodes [2-4].

Each enzyme plays a different role in the process of inducing resistance. PAL is responsible for catalyzing the first reaction in the phenylpropanoid pathway, converting L-phenylalanine to cinnamic acid. The activity of this enzyme has been reported in the lignification process, providing precursors of hydroxycinnamic acid and increasing the synthesis of phenolic species required in the defense process [5]. PPO is associated with catalyzing o-hydroxylation of monophenols to o-diphenols and the oxidation of o-diphenols to o-quinones, compounds that have high toxicity to microorganisms [6]. POX promotes the catalysis of phenolic compounds' oxidation through the phenylpropanoid pathway by the reduction of hydrogen peroxide (H_2O_2), being functional in a wide range of physiologic processes related to stress, promoting lignification and suberization [7]. Finally, GLU is responsible for the hydrolysis of β-1.3-glucan polymers, which can act as elicitors of the defense response in the host [8,9].

Acibenzolar-S-methyl is one of the main elicitors studied in the control of plant nematodes, being an analogue to salicylic acid (AS), related to the activation of mechanisms, similar to that observed in systemic acquired resistance (SAR), promoting the expression of pathogenesis related proteins [10,11]. Studies involving application of ASM to control sedentary nematodes have correlated reduction in the nematode population with the increase of hydrogen peroxide production and the activity of peroxidase and β-1.3-glucanase [12-14]. In the specific case of *Pratylenchus brachyurus* (Godfrey) Filipjev and Schuurmans Stekhoven, reduction was observed in the final nematode population in maize at 60 days after treatment [15,16].

Despite the potential control of this pathogen by ASM application due to its ability to induce defense related proteins, there are still no studies about the activity of the main defense proteins for *P. brachyurus* control. Thus, the present study analyzed the enzymatic activity of guaiacol peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase and β-1,3-glucanase in maize treated with ASM as well as their interaction in the presence of *P. brachyurus* nematodes.

Materials and Methods

The experiments were carried out in a greenhouse at the geographic coordinates 23°47'34.5" S and 53°15'22.1" W, in two different periods, from February 15 to April 20, 2016 (experiment 1) and from October 5 to November 13, 2016 (experiment 2). The experiments were composed of four treatments: maize plants inoculated with *P. brachyurus* and treated with ASM (IT), non-inoculated plants treated with ASM (NIT), inoculated plants not treated (INT) and non-inoculated and non-treated plants (NINT). For this purpose, two seeds of maize cv. AL. Bandeirantes were sown in each pot containing 700 mL of substrate composed of soil and sand mixture 2:1 (v:v), previously autoclaved (120°C 2 h^{-1}).

After germination, thinning was performed maintaining one seedling per pot. Ten days after germination, each maize seedling was inoculated with 4 mL of solution containing 800 *P. brachyurus* specimens. The inoculum was obtained from a pure nematode population that was multiplied in roots of soybean plants in a greenhouse and extracted according to the method proposed by Coolen and D'Herde [17]. Plants were inoculated and treated with ASM on the same day. For the treatment with ASM, the plants were sprayed on the aerial part until surface runoff with a commercial product (Bion 500®, Syngenta) at the dose of 0.5 gL^{-1} when they were in stage V1 (one fully expanded leaf). Non-treated plants were sprayed with water.

In experiment 1, samples were collected at three different times, 5, 10, and 15 days after treatment (DAT), while in experiment 2 they

were collected at six different intervals, 2, 4, 6, 8, 10 and 12 DAT. For this purpose, five plants of each treatment were removed, and the root systems were washed for soil removal. Then approximately 0.5 g of leaf tissue (third fully expanded leaf) and 0.5 g of root tissue (middle portion of root system) were collected, and placed in labeled aluminum foil envelopes and kept on ice for later freezing (-20°C) and extraction.

For the enzymatic analysis, the samples were macerated in a mortar with liquid nitrogen plus 1% (v/v) of polyvinylpyrrolidone (PVP) and 4 mL of 50 mM potassium phosphate buffer at pH 7.0, containing 0.1 mM EDTA. Extracts were centrifuged at 21,155g (14,500 rpm) for 30 minutes at 4°C and the supernatant, called enzymatic extract, was transferred to 1.5 mL microtubes and stored in a freezer (-20°C) for posterior protein quantification and assessment of enzymatic activity. The absorbance reading in analyzing the proteins and activity of each enzyme was carried out using a UV 5200S spectrophotometer (Global Trade Technology).

The Bradford test was used for quantification of total proteins present in the samples [18]. The protein concentration was expressed in mg mL^{-1} sample and was determined using the standard concentration curve obtained from bovine serum albumin. Guaiacol peroxidase (POX; EC 1.11.1.7) activity was expressed in $_{abs}$ 470 nm min^{-1} mg^{-1} protein [19] while the polyphenol oxidase (PPO; EC 1.10.3.2) activity was expressed in $_{abs}$ 420 nm min^{-1} mg^{-1} protein [20].

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) was measured by the absorbance difference between sample and control (without L-phenylalanine addition) which was plotted on the standard curve of trans-cinnamic acid and expressed in mg trans-cinnamic acid h^{-1} mg^{-1} protein [21]. In turn, β-1.3-glucanases activity (GLU; EC 3.2.1.6) was determined by the colorimetric quantification of reducing sugars released from laminarin [22,23]. The amount of sugars was determined using the standard glucose curve and the values were expressed in mg glucose h^{-1} mg^{-1} protein.

Five plants of the inoculated treatment were maintaining until 30 and 60 days after inoculation (experiment 1) and 30 days after inoculation (experiment 2) to evaluate the nematological parameters. The roots of these plants were carefully washed, placed on adsorbent paper to remove the excess water and weighed. These tissues were submitted to the extraction process according to the method previously mentioned and then the total number of nematode specimens was quantified with a Peters counting chamber and optical microscope.

In both experiments a completely randomized design was used with five replicates for each treatment. The data obtained were transformed into and submitted to analysis of variance (ANOVA). The final population was compared by the t-test (LSD) at 5% probability when the ANOVA result was significant. Protein content and enzyme activity were determined in duplicate for each replicate. The mean of each enzyme was compared by the Tukey test at 5% probability and analyzed in a 4 x 3 factorial arrangement (treatment x time) in experiment 1 and 4 x 6 in experiment 2. When significant interactions were present, there was a comparison of the levels of a factor within the fixed levels of another factor. All statistical analyses were performed using the Sisvar program [24].

Results

We observed an interaction between the factors treatment and time in experiment 1 for POX activity in the aerial part (Table 1). There was an increase of POX activity in the IT plants at 5 DAT compared to the other treatments. At 10 DAT, plants in the IT and NIT groups were statistically the same but both presented higher values than inoculated (INT) and non-inoculated (NINT) controls, while at 15 DAT higher POX activity was verified in the NIT plants followed by IT and inoculated control (INT) groups, the last one being equal to plants in the non-inoculated control group (NINT). Regarding the time factor within treatments, there was no statistical difference among the times studied in the IT group, while the NIT plants showed higher activity at 15 DAT and in both control groups (NIT and NINT), higher activities were observed at 10 and 15 DAT (Table 1).

Treatments	Days after treatment			Total mean	
	5	10	15		
Peroxidase ($_{abs}$ 470 nm min^{-1} mg^{-1} protein)					
IT	38.66 a A	44.73 a A	39.95 b A	41.07	
INT	13.42 c B	14.31 b B	33.94 bc A	19.57	
NIT	25.85 b B	49.72 a A	60.79 a A	44.14	
NINT	13.15 c B	22.20 b A	22.97 c A	19.16	
Total mean	21.6	30.9	38.24	CV(%)	12
Polyphenol oxidase ($_{abs}$ 420 nm min^{-1} mg^{-1} protein)					
IT	1.29 ns	1.39 ns	1.53 ns	1.40 a	
INT	0.77	0.71	1.23	0.89 b	
NIT	1.68	1.32	2.13	1.69 a	
NINT	0.83	0.5	0.81	0.70 b	
Total mean	1.1113 B	0.9413 B	1.3858 A	CV(%)	13.6
Phenylalanine ammonia-lyase (mg trans-cinnamic acid h^{-1} mg^{-1} protein)					
IT	0.043 b A	0.061 a A	0.068 a A	0.057	
INT	0.025 c C	0.053 a B	0.086 a A	0.051	
NIT	0.088 a A	0.062 a AB	0.059 a B	0.069	
NINT	0.045 b A	0.023 b B	0.036 b AB	0.034	
Total mean	0.048	0.0488	0.0607	CV(%)	14

Table 1: Specific activity of the peroxidase, polyphenol oxidase and phenylalanine ammonia-lyase enzyme in leafs of maize plants inoculated or non-inoculated with 800 *P. brachyurus* specimens and treated or non-treated with acibenzolar-S-methyl, analyzed in experiment 1.

Means followed by the same lower case letter in the column and capital letter in line do not differ significantly by Tukey test at 5% of probability; IT= inoculated plants with *P. brachyurus* and treated with ASM; INT= inoculated plants with *P. brachyurus* and non-treated with ASM; NIT= non-inoculated with *P. brachyurus* and treated with ASM; NINT= non-inoculated with *P. brachyurus* and non-treated with ASM; ns= not significant; CV= coefficient of variance.

There was no interaction between the factors for PPO activity and it was only significant when analyzed separately, in which higher activity of this enzyme was verified in the plants sprayed with ASM (IT and NINT) compared to the controls (INT and NINT), while for the time factor, higher activity was observed at 15 DAT (Table 1). Similar to the observation for POX enzyme in leaf tissues, there was also interaction between the factors in PAL enzyme activity. Analyzing the treatment factors within times, higher activity was verified at 5 DAT for NIT followed by IT and NINT. At 10 and 15 DAT, lower PAL activity was observed for the non-inoculated control group (NINT) compared to the other treatments. Regarding the analysis of the time factor within treatments, there was no statistical difference among the times studied for the IT plants. The NIT plants showed higher activity at 5 and 10 DAT. In the inoculated control group (INT), higher activity was observed at 15 DAT while in the non-inoculated control (NINT) this was verified at 5 and 15 DAT.

In maize roots, interaction between the factors to PPO and PAL activity was observed while POX activity was only significant for the treatment factor in experiment 1 (Table 2). Regarding this last factor, higher POX activity was found for the IT plants followed by NIT when in comparison with both controls (INT and NINT).

Treatments	Days after treatment			Total mean	
	5	10	15		
Peroxidase ($_{abs}$ 470 nm min^{-1} mg^{-1} protein)					
IT	483.46 ns	389.70 ns	492.31 ns	453.93 a	
INT	93.69	142.62	124.2	119.29 c	
NIT	298.91	332.46	302.49	310.92 b	
NINT	116.01	148.67	109.87	124.29 c	
Total mean	222.95 ns	241.10 ns	234.14 ns	CV(%)	11.12
Polyphenol oxidase ($_{abs}$ 420 nm min^{-1} mg^{-1} protein)					
IT	1.29 ns	1.39 ns	1.53 ns	1.40 a	
INT	0.77	0.71	1.23	0.89 b	
NIT	1.68	1.32	2.13	1.69 a	
NINT	0.83	0.5	0.81	0.70 b	
Total mean	2.38	2.0232	4.0534	CV(%)	25.64
Phenylalanine ammonia-lyase (mg trans-cinnamic acid h^{-1} mg^{-1} protein)					
IT	0.847 a A	0.427 a B	1.145 a A	0.776	
INT	0.237 b A	0.219 b A	0.159 c A	0.204	
NIT	0.269 b B	0.454 a A	0.593 b A	0.428	
NINT	0.205 b A	0.290 ab A	0.266 c A	0.252	
Total mean	0.353	0.341	0.474	CV(%)	16.01

Table 2: Specific activity of the peroxidase, polyphenol oxidase and phenylalanine ammonia-lyase enzyme in roots of maize plants inoculated or non-inoculated with 800 *P. brachyurus* specimens and treated or non-treated with acibenzolar-S-methyl, analyzed in experiment 1.

Means followed by the same lower case letter in the column and capital letter in line do not differ significantly by Tukey test at 5% of probability; IT= inoculated plants with *P. brachyurus* and treated with ASM; INT= inoculated plants with *P. brachyurus* and non-treated with ASM; NIT= non-inoculated with *P. brachyurus* and treated with ASM; NINT= non-inoculated with *P. brachyurus* and non-treated with ASM; ns= not significant; CV= coefficient of variance.

Concerning PPO activity in roots, there was higher activity in IT plants at 5 and 15 DAT than in the control groups (INT and NINT) while for the NIT plants, higher activity was found than in the NINT plants at 15 DAT. Analyzing the time factor within treatments, higher activity of PPO was observed in the IT group at 5 and 15 DAT, while in the NIT group this occurred at 15 DAT (Table 2). The PAL activity was higher in the IT plants than in the controls (INT and NINT), independent of time, while the NIT plants showed higher activity at 10 and 15 DAT compared to the controls. In relation to the time factor within treatments, the IT plants showed higher PAL activity at 5 and 15 DAT while in the NIT group this occurred at 10 and 15 DAT. There was no significance of this parameter in both controls.

There was significant interaction of factors in experiment 2 regardless of the enzyme analyzed in leaf tissue (Table 3). Higher activity of POX was observed in plants sprayed with ASM (IT and NIT) at all times compared to both controls (INT and NINT), except at 2 DAT, when IT and NIT did not differ from INT. Analyzing the time factor within treatments, higher activity peaks were observed at 6 and 8 DAT in the IT plants, at 10 and 12 DAT in the INT group, and at 12 DAT in the NIT and NINT plants.

Treatment	Days after treatment						Total mean
	2	4	6	8	10	12	
Peroxidase ($_{abs}$ 470 nm min^{-1} mg^{-1} protein)							
IT	10.59 a D	10.43 a D	16.21 a AB	12.06 a CD	14.15 a BC	18.47 a A	13.50

INT	11.24 a B	5.91 b B	5.67 b B	5.67 b B	11.12 b A	13.90 b A	8.00	
NIT	9.81 a C	10.08 a C	13.85 a B	13.85 a B	12.48 ab BC	20.26 a A	13.25	
NINT	6.23 b B	6.61 b B	5.18 b B	5.18 b B	6.87 c B	11.64 b A	7.12	
Total mean	7.89	8.13	9.62	9.97	10.97	15.88	CV(%)	7.58
Polyphenol oxidase ($_{abs}$ 420 nm min^{-1} mg^{-1} protein)								
IT	0.544 a C	0.574 a BC	0.787 a B	0.679 a BC	0.670 ab BC	1.185 a A	0.726	
INT	0.470 ab BC	0.404 a BC	0.482 b BC	0.323 b C	0.584 b AB	0.721 b A	0.490	
NIT	0.570 a C	0.543 a C	0.595 ab C	0.650 a BC	0.865 a AB	1.100 a A	0.708	
NINT	0.362 b AB	0.244 b B	0.315 c AB	0.299 b B	0.391 c AB	0.469 c A	0.343	
Total mean	0.483	0.430	0.531	0.471	0.615	0.842	CV(%)	10.04
Phenylalanine ammonia-lyase (mg trans-cinnamic acid h^{-1} mg^{-1} protein)								
IT	0.018 ab A	0.005 b B	0.016 a A	0.006 b B	0.008 b AB	0.009 b AB	0.010	
INT	0.009 bc C	0.036 a A	0.007 bc C	0.015 a BC	0.023 a AB	0.027 a AB	0.018	
NIT	0.028 a AB	0.029 a AB	0.011 ab C	0.021 a BC	0.026 a AB	0.040 a A	0.025	
NINT	0.006 c AB	0.003 b B	0.004 c B	0.004 b B	0.005 b B	0.013 b A	0.005	
Total mean	0.014	0.015	0.009	0.010	0.014	0.021	CV(%)	20.31
β-1.3-Glucanase (mg glucose h-1 mg-1 protein)								
IT	0.055 b B	0.056 a B	0.084 a A	0.053 a B	0.034 a C	0.084 a A	0,060	
INT	0.037 c AB	0.021 c C	0.052 b A	0.027 b BC	0.022 a C	0.039 b AB	0.032	
NIT	0.074 a A	0.033 b BC	0.044 bc B	0.039 ab BC	0.027 a C	0.045 b B	0.043	
NINT	0.050 bc AB	0.029 bc C	0.034 c BC	0.033 b C	0.026 a C	0.052 b A	0.037	
Total mean	0.053	0.034	0.052	0.037	0.027	0.053	CV(%)	10.6

Table 3: Specific activity of the peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase and β-1,3-glucanase enzyme in leafs of maize plants inoculated or non-inoculated with 800 *P. brachyurus* specimens and treated or non-treated with acibenzolar-S-methyl, analyzed in experiment 2.

Means followed by the same lower case letter in the column and capital letter in line do not differ significantly by Tukey test at 5% of probability; IT= inoculated plants with *P. brachyurus* and treated with ASM; INT= inoculated plants with *P. brachyurus* and non-treated with ASM; NIT= non-inoculated with *P. brachyurus* and treated with ASM; NINT= non-inoculated with *P. brachyurus* and non-treated with ASM; CV= coefficient of variance.

Similar to the reaction to POX, plants sprayed with ASM (IT and NIT) showed higher PPO activity than the non-inoculated control (NINT), independent of the time studied (Table 3). In relation to the inoculated control group (INT), the IT and NIT groups showed greater PPO activity compared to it at 8 and 12 DAT, while the NIT group did not differ from the INT at 6 DAT, and the IT group did not differ from the INT at 10 DAT. With regard to the time factor within treatments, higher peaks of PPO activity were verified at 12 DAT in the IT plants, at 10 and 12 DAT in INT and NIT plants, while in the NINT group the peaks were observed at 2, 6, 10 and 12 DAT.

PAL activity was higher in the INT and NIT plants than in the IT and NINT at 4, 8, 10 and 12 DAT (Table 3). At 2 and 6 DAT, IT and NIT showed higher PAL activity compared to the NINT group. With respect to the analysis of time within treatments, peaks of activity in the IT group were observed at 2, 4, 10 and 12 DAT, in the INT plants at 4, 10 and 12, in the NIT group at 2, 4, 10 and 12, and in the NINT plants at 2 and 12 DAT. The GLU activity was higher in the IT group at 4, 6, 8 and 12 DAT compared to the controls (INT and NINT), while at 2 DAT only the NIT plants showed higher activity compared to the controls. Higher activity peaks for GLU were observed in the IT plants at 6 and 12 DAT, the INT group at 1, 6 and 12 DAT, the NIT group at 2 DAT and finally at 2 and 12 DAT in the NINT plants.

In experiment 2, the activities of these four enzymes in maize roots were similar to the results observed in leaf tissue, showing significant interaction between the time and treatment factors (Table 4). For POX activity, higher values were observed in the IT plants in all times evaluated except at 4 DAT, compared to the controls (INT and NINT). However, the NIT plants showed higher activity compared to both

controls at 2, 10 and 12 DAT. Regarding the time factor within treatments, higher activity peaks were found in the IT group at 2, 10 and 12 DAT, the INT at 2, 6, 10 and 12 DAT, and the NIT at 10 and 12 DAT, while in the NINT group, lower activity was observed at 4 DAT. For PPO activity, the IT group showed higher activity compared to the non-inoculated control (NINT) at all times except at 4 DAT, and higher values than both controls at 10 and 12 DAT. The NIT group showed higher PPO activity compared to non-inoculated control (NINT) at 8 and 12 DAT. Higher activity peaks were observed in the IT plants at 2, 10 and 12 DAT, the INT at 6, 8 and 12 DAT, the NIT at 12 DAT and the NINT plants at 2, 4, 10 and 12 DAT.

Treatment	Days after treatment						Total mean	
	2	4	6	8	10	12		
Peroxidase ($_{abs}$ 470 nm min^{-1} mg^{-1} protein)								
IT	225.6 a A	108.3 a D	163.3 a BC	136.4 a CD	206.4 a AB	172.6 a ABC	166.3	
INT	82.9 c AB	55.2 b B	107.5 bc A	73.4 b AB	91.0 b AB	112.2 b AB	85.9	
NIT	134.6 b C	140.6 a BC	145.6 ab BC	102.8 ab C	215.7 a A	194.1 a AB	153.3	
NINT	67.7 c B	108.5 a A	78.5 c AB	71.4 b AB	74.4 b AB	90.8 b AB	81.3	
Total mean	120.8	100.5	121.4	94.3	139	139.2	CV(%)	10.68
Polyphenol oxidase ($_{abs}$ 420 nm min^{-1} mg^{-1} protein)								
IT	3.226 a AB	1.086 b C	2.474 a B	2.370 a B	4.458 a A	4.749 a A	2.913	
INT	0.931 a C	1.063 b C	2.313 a A	2.038 a AB	1.196 b BC	2.688 b A	1.637	
NIT	2.367 ab B	2.608 a B	1.880 ab B	1.816 a B	1.687 b B	4.213 a A	2.363	
NINT	1.443 bc AB	1.881 ab A	1.287 b AB	0.702 b B	1.005 b AB	1.895 b A	1.332	
Total mean	1.891 B	1.600 B	1.959 B	1.659 B	1.895 B	3.283 A	CV(%)	15.16
Phenylalanine ammonia-lyase (mg trans-cinnamic acid h^{-1} mg^{-1} protein)								
IT	0.017 a C	0.007 a C	0.049 a B	0.045 ab B	0.097 a A	0.045 a B	0.038	
INT	0.003 b C	0.003 a C	0.009 b BC	0.054 a A	0.019 c B	0.019 b B	0.014	
NIT	0.014 a C	0.004 a D	0.014 b CD	0.027 b BC	0.055 b A	0.032 abB	0.021	
NINT	0.011 a B	0.002 a C	0.018 b AB	0.035 ab A	0.031 c A	0.024 b AB	0.018	
Total mean	0.010 D	0.004 E	0.020 C	0.039 A	0.046 A	0.029 B	CV(%)	19.67
β-1.3-Glucanase (mg glucose h-1 mg-1 protein)								
IT	0.387 a AB	0.232 a C	0.308 a BC	0.387 a AB	0.490 a A	0.360 b ABC	0.356	
INT	0.152 b BC	0.118 b C	0.170 b BC	0.219 bc AB	0.096 c C	0.319 b ABC	0.172	
NIT	0.191 b C	0.240 a BC	0.368 a B	0.288 ab BC	0.188 b C	0.733 a A	0.313	
NINT	0.191 c A	0.135 b A	0.108 b A	0.175 c A	0.173 bc A	0.197 c A	0.161	
Total mean	0.222	0.177	0.226	0.261	0.216	0.38	CV(%)	13.19

Table 4: Specific activity of the peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase and β-1,3-glucanase enzyme in roots of maize plants inoculated or non-inoculated with 800 *P. brachyurus* specimens and treated or non-treated with acibenzolar-S-methyl, analyzed in experiment 2.

Means followed by the same lower case letter in the column and capital letter in line do not differ significantly by Tukey test at 5% of probability; IT= inoculated plants with *P. brachyurus* and treated with ASM; INT= inoculated plants with *P. brachyurus* and non-treated with ASM; NIT= non-inoculated with *P. brachyurus* and treated with ASM; NINT= non-inoculated with *P. brachyurus* and non-treated with ASM; CV= coefficient of variance.

For PAL activity, higher values were observed in the IT group at 6, 10 and 12 DAT compared to both controls (INT and NINT), while the NIT plants showed superior results than controls at 10 DAT (Table 4). With respect to the peaks of activity at time intervals, higher values

were observed in the IT and NIT groups at 10 DAT and in the INT group at 8 DAT, while NINT plants showed elevated peaks at 6, 8 and 10 DAT. Concerning the GLU activity, the IT group showed higher values at all times compared to controls (INT and NINT), except at 12 DAT, while in the NIT group, better results were observed at 4, 6, and 12 DAT compared to both controls. Higher activity peaks were verified in the IT group at 2, 8, 10 and 12 DAT, the INT at 8 and 12 DAT and the NIT at 12 DAT, while for the NINT plants there was no difference among the time intervals studied.

ASM application also provided reductions of 70.8 and 59.2% in the final population of nematodes root^{-1} system in the experiment 1 at 30 and 60 DAT, respectively, and reduction of 65.4% at 30 DAT in the experiment 2, in relation to the plants without ASM application (Table 5).

Treatments	Nematode g^{-1} of root	Reduction (%)
Experiment 1-30 days after treatment		
Inoculated and treated (IT)	306 b	70.8
Inoculated and non-treated (INT)	594 a	-
CV (%)	11.26	
Experiment 1-60 days after treatment		
Inoculated and treated (IT)	901 b	59.1
Inoculated and non-treated (INT)	2450 a	-
CV (%)	23.82	
Experiment 2-30 days after treatment		
Inoculated and treated (IT)	310 b	65.4
Inoculated and non-treated (INT)	676 a	-
CV (%)	11.48	

Table 5: *Pratylenchus brachyurus* number per root gram (final population) and reduction percentage compared to control in maize evaluated at 30 and 60 days (experiment 1) and at 30 days after treatment (experiment 2) with acibenzolar-S-methyl.

Means followed by the same letter in the column, within each evaluation time, do not differ significantly by the t test (LSD) at 5% of probability; CV= coefficient of variation.

Discussion

The nematode control results were probably directly or indirectly related to activity of enzymes linked to the plant resistance to pathogens. We observed an increase in the mean activity of POX and PPO in the plants treated with ASM (IT or NIT) in comparison with the controls (INT or NINT) in both experiments and tissues studied, showing the systemic character of action of this product. Moreover, the increase of enzymatic activity promoted by ASM has been observed in cotton plants, with higher activity of POX and catalase enzymes [13], and in tomato plants, in which ASM promoted the expression of genes responsible for encoding the anionic enzyme POX in the root system [14].

There are reports that peroxidase can promote an increase of cell wall resistance, acting in the formation of insoluble networks of extensins and covalent linkages between feruloyl residues, and also that it participates in polymerization of hydroxyamyl alcohol into lignin [25-28]. Extensins are glycoproteins rich in hydroxyproline secreted in the apoplasts as soluble monomers that are oxidized by peroxidases anchored to pectin, promoting cross-linking of theses monomers and creating an insoluble, dense and solid network in the plant cell wall, limiting pathogen colonization [7,29,30]. Extensin accumulation has been observed and correlated with the occurrence of mechanical damage, stress and disease resistance in plants [31,32].

Furthermore, some studies have shown an increase in gene expression responsible for encoding different types of extensins during the interaction of plants and sedentary nematodes, since they induce the formation of nourishing cells, using these proteins in cell expansion and multiplication and consequently formation of feeding sites [33,34]. However, an increase in the intercellular concentration of extensins in the cortex and pericycle cells neighboring the feeding sites has also been observed. This may promote an increase in the cell wall resistance, restricting the size of the feeding sites, indicating a possible defense mechanism of the plant triggered by the nematode or even by the formation of these sites [35]. Due to the migratory character of the genus *Pratylenchus*, possibly the action of this protein is not restricted to cell expansion and multiplication of feeding sites, but also promotes an increase in cell wall resistance.

Similarly, in the presence of peroxidase and hydrogen peroxide, the feruloyl residues can bind to each other, allowing the formation of crosslinks between the polysaccharides that were esterified with these residues [26,36,37]. The formation of feruloyl residues is the result of ferulic acid binding to cell wall polysaccharides, more specifically, to arabinopyranose residues of arabinoxylan hemicellulose by ester-type linkages [29]. Ferulic acid is synthesized in plants through caffeic acid, an intermediate compound of the phenylpropanoid pathway, by the action of the enzyme catechol-O-methyltransferase [38]. An in vitro study with a suspension of non-lignified maize cells submitted to different mixtures of cell wall degradative enzymes showed that the crosslinks of diferulates (feruloyl residues) reduced the accessibility of hydrolytic enzymes to the polysaccharides present in the cell wall [39]. Thus, a higher number of crosslinks with esterified diferulates promotes an increase in the cell wall stiffness and makes enzymatic degradation more difficult [40].

Therefore, it is assumed that the presence of linkages between ferulic acids in cell walls may hamper the activity of cell wall degradation enzymes secreted by the nematode, such as xylanases, polygalacturonases, pectate lyase and arabinases [41,42]. Studies of cultivars of *Musa* sp. resistant and susceptable to *Radopholus similis* Cobb or *Pratylenchus coffeae* Goodey showed the presence of ferulic acid in the parenchyma cell walls in resistant cultivars and higher proportion of ferulic acid and feruloylated arabinoses compared to susceptible cultivars [41,43]. In addition to the structural importance, a direct effect of ferulic acid on nematodes was also observed, causing a strong reduction of mobility and toxic effect (LC50 of 120 µg ml^{-1}) on *Meloidogyne incognita* (Kofoid and White) Chitwood and *R. similis* [44].

The third possible action of peroxidase in the cell wall reinforcement process is its capacity to promote the oxidative dehydrogenation of monolignols in the plant [45]. After the transport of monolignols to the cell wall, they undergo dehydrogenation/oxidation by laccase and/or peroxidase, allowing the cross-linking of

these polymers and promoting lignin polymerization, forming a three-dimensional network [46,47]. Cell wall stiffening is in most cases the result of cross-linkage by the hydrogen peroxide-dependent peroxidase enzyme, which can be stimulated by the damage caused by the nematode, plant-pathogen interaction or even by resistance induction [14,26,48].

The increase in lignification can act as a structural protection mechanism, promoting cell wall stiffening and preventing the access of degradative enzymes to the cell wall polysaccharides produced by the nematodes [25, 39, 49]. A similar reaction was observed in genetically resistant plants, like in a study with hybrids of *Musa* sp. resistant to *R. similis*, in which higher activity of peroxidase and polyphenol oxidase was observed as well as the higher lignin accumulation in roots compared to susceptible hybrids [48].

In addition to the reactions discussed, PPO is responsible for catalyzing o-hydroxylation of monophenols to o-diphenols and for oxidation of ortho-diphenols to ortho-quinones, compounds with higher toxicity to microorganisms, in the presence of oxygen [6,50]. Some authors believe that this enzyme, together with POX, may be involved in the lignification process though the polymerization of lignin molecules [51, 52]. Quinones produced by PPO oxidation can also undergo cross-linkage with other phenolic compounds and form a physical barrier. They can act with the nucleophilic side chains of certain amino acids and proteins, reducing the availability of these proteins as well as the nutritional quality of plant tissues to herbivores [53,54].

However, it is important to highlight that in this study, the PPO activity in the roots of the IT plants did not differ statistically among the treatments at 10 DAT in experiment 1, while in experiment 2 there was a statistical difference of this treatment at 10 and 12 DAT compared to both controls. In this case, new analyses are needed to better define the timing of maximum PPO activation.

On the other hand, the PAL activity in the aerial part presented variable values. However, when we analyzed the PAL activity in the root system of the treated and inoculated plants (IT), they higher enzymatic activity was observed in both experiments. Phenylalanine ammonia-lyase is responsible for catalyzing the first reaction in the phenylpropanoid pathway, converting L-phenylalanine into cinnamic acid (shikimic acid pathway) and is also responsible for the production of lignin precursors (monolignols) and phenolic species required in the process of plant defense. These can act as signaling compounds and precursors in phytoalexin production [5].

Preliminary evidence of the involvement of increased PAL activity in nematode control was obtained through comparative studies between plants of the same species (cultivars) resistant and susceptible to some species of this pathogen. When analyzing the expression of the genes responsible for encoding PAL, an increase was observed in the expression of the *ZmPAL4* gene in roots of maize hybrids resistant to *M. incognita*, and absence in the susceptible hybrids [55]. In studies with cultivars resistant to migratory nematodes, an increase in PPO, POX and PAL activities in roots of resistant banana plants was observed to *P. coffeae* and *R. similis*. In the last one, greater production of phenols and lignin was observed compared to susceptible cultivars [48,56,57].

Higher activity of the β-1.3-glucanase was observed in the treatments with ASM application, starting at 4 DAT. Similarly, ASM applied via leaf spraying promoted reduction in the egg numbers of *Meloidogyne* spp. in grape vines and also was associated with an increase in the activity of this enzyme from 7 to 28 days after application of the product [12]. The role of this enzyme in nematode control is still not well understood, but it is believed that it acts on the glycoprotein and carbohydrate complexes present on the outer surface of the nematode cuticle, possibly serving as a substrate for glucanase and glycosidase activity, thus promoting the release of exoelicitors [58,59]. A similar process occurs in the action of this enzyme on fungi, because it participates in the cell wall hydrolysis of the hyphae cells, releasing exoglucans, which act as elicitors, strengthening the defense responses synergistically with endoelicitors derived from cell wall degradation [60,61].

In alfalfa infected by *P. penetrans* (Cobb) Chitwood and Oteifa, higher transcription of mRNAs related to the encoding of β-1.3-glucanase was observed in resistant cultivars compared to susceptible ones, as well as an increase in the induction of transcriptomes related to the phenylpropanoid pathway and responsible for encoding PAL [62]. In addition, higher activity of β-1.3-glucanase in roots and leaves of sugarcane resistant to *Pratylenchus zeae* Graham was observed compared to the susceptible cultivar [59].

ASM was efficient in reducing the number of nematodes root^{-1} gram of *P. brachyurus*, in both experiments, showing reductions of 59.2, 70.8 and 59.2% at 30 and 60 DAT (experiment 1) and at 30 DAT (experiment 2), respectively, compared to the control without application of ASM. The efficacy of this molecule in reducing this nematode in maize was previously observed when ASM was sprayed on the aerial part at 7 and 1 days before inoculation and 7 days after inoculation with *P. brachyurus* [15]. Furthermore, ASM also reduced this nematode when it was applied via seed treatment and sprayed on the aerial part 15 days after germination in previously infested soil [16]. ASM also showed promising results for control of this and others nematodes in soybean plants [10,63,64], as well as in others plant-nematode interactions [14,65,66].

Thus, ASM was able to induce an increase in the activity of defense-related enzymes in maize, enzymes similarly observed in other plant-nematode interactions submitted to the induction process as well as in plants with gene expression of resistance. Possibly the reduction of the *P. brachyurus* reproduction is related to the increase of cell wall reinforcement, hindering the nematodes' feeding and movement in the root system of maize.

Conclusion

Higher activity of the POX, PPO and GLU enzymes was observed in the aerial part of ASM-treated plants, while for PAL, the inoculated control presented similar values to the treatments that received the product. In the root system, an increase in the activity of POX, PPO, PAL and GLU was observed in the plants treated with ASM, starting at 5 and 6 days after application and remaining higher until 15 and 12 days. In addition, ASM also reduced the nematode population in maize roots at 30 and 60 days after treatment.

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