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INDUCTION OF RESISTANCE TO *Xanthomonas campestris* pv. *viticola* IN GRAPEVINE PLANTS[1]

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ABSTRACT – The objective of this work was to evaluate the effect of *Saccharomyces cerevisiae* (SC), Acibenzolar-S-Methyl (ASM), organic acids and polyphenols (OAP) and potassium silicate (SiK) on protecting grapevine plants (cv. Redglobe) from *Xanthomonas campestris* pv. *viticola*. Four application rates for each product (SC at 2.0, 2.50, 3.0, 3.50 and 4.0 mL 100 L^{-1}; ASM and OAP at 2.50, 3.00, 3.50, 4.50 and 6.00 mL 100 L^{-1} and SiK at 5.00, 6.50, 7.50 and 8.50 mL 100 L^{-1}) in different application times (0, 5, 10, and 15 days before inoculation), and the enzymatic activity of peroxidases, phenylalanine ammonia-lyase and β-1,3 glucanases were evaluated. Plants were inoculated with a bacterial suspension of 5 x 108 CFU mL^{-1} by rubbing with gauze. The epidemiological variables incidence (INC), severity (SEV) and area under the disease progress curve (AUDPC) were assessed. The treatment with ASM had the lowest averages of INC (38%) and SEV (1.52%) from 3 g 100 L^{-1}. The application of 4.5 mL 100 L^{-1} of OAP reduced the plant disease in 52% of INC and 2.45% of SEV. SiK and SC presented no significant reduction in these variables compared to control. The ASM applied 15 days before the inoculation (DBI) reduced the disease in 91.31% and the APO in 73.34%, while SC and SiK reduced the disease in 67.49 and 60.11%, respectively, with applications at 5 DBI. There was no increase of peroxidase activity in any of the treatments. There was a significant increase in activity of β-1,3 glucanases and phenylalanine ammonia-lyase in plants treated with ASM at 15 DBI, indicating an influence on the induction of resistance of plants to this disease.
Index terms: Bacterial canker, phenylalanine ammonia-lyase, β-1,3 glucanase.

INDUÇÃO DE RESISTÊNCIA EM MUDAS DE VIDEIRA A *Xanthomonas campestris* pv. *viticola*

RESUMO - Este trabalho objetivou avaliar o efeito de: *Saccharomyces cerevisiae* (SC), Acibenzonlar-S-Methyl (ASM), ácidos orgânicos e polifenóis (AOP) e silicato de potássio (SiK) na proteção de mudas de videira cv. Redglobe à *Xanthomonas campestris* pv. *viticola*. Foram avaliados para cada produto quatro doses: SC 2,0; 2,50; 3,0; 3,50 e 4,0 mL 100 L^{-1}, ASM e AOP 2,50; 3,00; 3,50; 4,50 e 6,00 mL 100 L^{-1} e SiK 5,00, 6,50, 7,50 e 8,50 mL 100 L^{-1}) em diferentes períodos de aplicação: 0, 5, 10, e 15 dias antes da inoculação bem como, a atividade enzimática de peroxidase, fenilalanina amônia-liase e β-1,3 glucanases. As plantas foram inoculadas com suspensão bacteriana a 5 x 10^8 UFC. mL^{-1} pelo método da fricção com gaze. As variáveis epidemiológicas da doença avaliadas foram: incidência (INC), severidade (SEV) e área abaixo da curva progresso e severidade da doença (AUDPC). As menores médias de INC e SEV foram auferidas pelo ASM com 38% e 1,52%, respectivamente, a partir da aplicação com 3g 100 L^{-1}. A aplicação de 4,5mL 100 L^{-1} de AOP reduziu a doença com plantas com 52% de INC e 2,45 de SEV. SiK e SC não apresentaram redução significativa nestas variáveis com relação a testemunha. Pulverização com ASM 15 dias antes da inoculação (DAI) reduziu a doença em 91,31% e AOP em 73,34%, enquanto que SC e SiK reduziram a doença em 67,49 e 60,11% respectivamente com pulverização aos 5 (DAI). Em nenhum dos tratamentos houve incremento da atividade de peroxidase. Houve significativa elevação da atividade de β-1,3 glucanases e fenilalanina amônia-liase em plantas pulverizadas com ASM aos 15 dias (DAI), indicando haver influência na indução de resistência das plantas a doença.
Termos para indexação: Cancro bacteriano, fenilalanina amônia-liase, β-1,3 glucanases.

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INTRODUCTION

Grapevine bacterial canker, caused by *Xanthomonas campestris* pv. *viticola* (Nayudu) Dye (Xcv), is currently considered a bacterial disease of greater impact on grape production in the Northeast of Brazil, especially in the states of Bahia, Ceará and Pernambuco, where it is considered a threat to the economy by the plant health legislation (A2) (RODRIGUES NETO, et al., 2011; NAUE et al., 2014). The phytobacterial diseases caused by this genus cause physiological damage to host plants, since its pathogenicity involves an elaborate secretion system with injection of over 25 effector proteins with deleterious enzymatic functions to the plant cells (KAY e BONAS, 2009). The disease is systemic and easily disseminated, making its control more difficult. The management of bacterial canker is currently carried out through cultural practices, use of tolerant varieties and application of copper fungicides, which are used due to the lack of specific bactericides registered in the Ministry of Agriculture, Livestock and Food Supply (MAPA) for the management of this disease (MARQUES et al., 2009). However, the use of copper fungicides has no effective control, since isolates from India and Brazil, found in the region of Petrolina PE and Juazeiro BA, presented significant levels of tolerance to cupric substances (MARQUES et al., 2009). The development of new alternatives to became part of the integrated management is needed. The induced systemic resistance (ISR) by chemical or biological elicitors has the advantage of enabling the cultivation of susceptible varieties, thus, causing less impact to the environment and containing no toxic effects on the pathogen, preventing the appearance of isolates tolerant to treatment with inducers (SILVA et al., 2007; BORGES et al., 2013). Chemicals such as Acibenzolar-S-Methyl (ASM), analogue of salicylic acid, organic acids and polyphenols (OAP), compounds derived from microorganisms such as phosphorylated mannan-oligosaccharides from the cell wall of *Saccharomyces cerevisiae* Meyen (SC) and potassium silicate (SiK) can trigger the production of signals to various reactions against pathogens, by chemical or structural responses that prevent or delay the entry or colonization of a microorganism in the plant (COSTA et al., 2010). In this context, the objective of this work was to evaluate the effects of application of ASM, OAP, SC and SiK in grapevine plants (cv. Redglobe) in the management of bacterial canker.

MATERIAL AND METHODS

The experiments were performed in the Phytopathology Laboratory of the Embrapa Semi-Arid, Petrolina PE, and in a greenhouse of the Bahia State University (UNEB), Department of Technology and Social Sciences, Juazeiro BA, with controlled environment (temperature of 30°C and relative humidity of 80%). Enzymatic assays were performed at the Plant Biochemistry Laboratory of the São Francisco Valley Federal University (UNIVASF), Juazeiro BA.

Preparation and inoculation of grapevine cuttings

Healthy grapevine plants (cv. Redglobe) produced from cuttings that were collected from mother plants of the Labrunier Farm in Petrolina PE (09º19'697"S e 40º22'416"W) were used. Cuttings with length of 30 cm were rooted in a substrate containing soil and sand (3:1 v:v) and maintained in a greenhouse for 90 days. The isolate *Xcv3* were used, which were from a commercial vineyard in Petrolina PE and had symptoms of bacterial canker. They were molecularly identified by Polymerase Chain Reaction (PCR) with specific primers (Xcv1F/Xcv3R). The pathogen was cultivated in a NYDA medium, consisting of: nutrient agar (23 g) yeast extract (5 g) dextrose (10 g) and 1.0 L of distilled water and incubated at 28°C for 48 hours. The preparation of the bacterial suspension was performed with sterile distilled water (SDW) and the concentration adjusted to $5x10^8$ UFC mL^{-1} in photocolorimeter (Analyser 500 M, Brazil), according to a previously defined equation. The plants were inoculated by rubbing the leaf surface with gauze (immersing the gauze in the bacterial suspension and then rubbing it over five previously identified leaves per plant) (NASCIMENTO et al., 2005).

Effect of the resistance inducers at different rates.

Grapevine plants (Redglobe) with 90 days of cultivation were sprayed, seven days before inoculation (DBI), with: *Saccharomyces cerevisiae* (SC), (Agromós, Alltech) at 2.00, 2.50, 3.00, 3.50 and 4.00 mL 100 L^{-1}; Acibenzolar-S-Methyl (ASM) (Bion Syngenta) and organic acids and polyphenols (OAP) (Bioace, Ecocert Brazil) at 2.50, 3.00, 3.50, 4.50 and 6.00 mL 100 L^{-1}; and potassium silicate SiK (Sili-K, Unaprosil Industry and Commerce Ltd.) at rates of 5.50, 6.50, 7.50, 8.50 and 10.00 ml 100 L^{-1}. Each treatment was applied in the surface of all leaves to the point of dripping (250 mL) using a hand sprayer.

A completely randomized experimental design was used, with five replications, each represented by a plant. The experiment consisted of 22 treatments, four products with five concentrations each and two controls (absolute, with plants without inoculation, and relative, with plants inoculated and treated with SDW). Five inoculated leaves of each plant were evaluated. The epidemiological variables assessed were incidence (INC), represented by the total percentage of plants with symptoms; severity (SEV), estimated by assessments every seven days for 42 days with a diagrammatic scale ranging from 2 to 91% of leaf area with symptoms (NASCIMENTO et al., 2005); and area under the disease progress curve (AUDPC), calculated by the equation: AUDPC = $\sum(y_i+y_{i+1})/2.d_{ti}$, where y_i and y_{i+1} are the severity values found in two consecutive evaluations and d_{ti} is the interval between evaluations (SHANER & FINNEY, 1977). Data were subjected to regression analysis using the software ASSISTAT 7.5, and the absolute control compared to the overall average of the treatments.

Effect of inducers in the different application times

Four application times (0, 5, 10 and 15 DBI) were assessed. The treatments were applied as described above with ASM 3.0 g 100 L^{-1}, OAP 4.50 mL 100 L^{-1}, SC 2.50 mL 100 L^{-1} and SiK 6.50 mL 100 L^{-1}. A completely randomized experimental design was used in a differentiated double factorial scheme (4x4+1+2) consisting of four resistance inducers, four application times, an additional treatment represented by applications of copper oxychloride (1.75 g L^{-1}), an inoculated control (with SDW only), and an absolute control (without inoculation). Five replicates were used for each treatment, each replication represented by a plant. Data were subjected to analysis of variance using the Dunnett test ($p \leq 0.05$) in the ASSISTAT 7.5 software. Evaluations of the disease epidemiology variables were performed according to the methodology described for testing the rates of the treatments.

Characterization of the biochemical mechanisms involved in defense responses

Plant tissue samples were collected for assessments of enzyme activity peaks in three stages (0 h, 48 h and 96 h after inoculation), retrieving one leaf from each treatment and replication, which were packed in plastic bags, identified, frozen and stored in a freezer at -16ºC to assess the peroxidase, phenylalanine ammonia-lyase and β-1,3 glucanases. Each sample was weighed and crushed in a mortar under ice cubes, according to the methodology described by Guimarães et al. (2010), for preparation of enzymatic extracts. In order to evaluate the phenylalanine ammonia-lyase in the enzyme extract, phenylalanine was used as substrate, causing reactions that form trans-cinnamic acid (TAIZ; ZEIGER, 2004). The trans-cinnamic acid was evaluated according to the methodology described by Umesha (2006). The peroxidase activity was evaluated through the oxidation of guaiacol in tetraguaicol in the presence of hydrogen peroxide (ZERAIK et al., 2008). The β-1,3 glucanase activity in the enzyme extract was evaluated according to the methodology described by Guimarães et al. (2010). Readings were performed in spectrophotometer at 480 nm and compared with glucose patterns. The glucose standard curve used was calculated at concentrations of 0, 5, 10, 20, 40, 80, 160 μg mL^{-1}. A completely randomized experimental design was used, in a factorial scheme 4x3, consisted of four application times and three times of four replications. All experiments were repeated twice. ANOVA was performed and the original means were compared by the Dunnett test ($p<0.05$). The statistical program ASSISTAT-7.5/2007 was used in all experiments.

RESULTS AND DISCUSSION

Effect of resistance inducers at different rates

The values of the epidemiological variables presented significant differences ($p \leq 0.05$) between treatments with inducers and the control after 42 days of inoculation (Figure 1 and 2). Regarding the assessed inducers, no phytotoxicity effects were found with any of the rates used. The greatest protective effect on plants was found with ASM, which presented the lowest rates of incidence and severity of bacterial canker from the minimum rate (3.0 g 100 L^{-1}). The difference in the effect of the ASM rates applied was significant for the variables incidence (INC) and index of petiole with canker (IPC), differing from the control when the product was applied at rates of 4.50 and 6.00 g 100 L^{-1}. Other inducers presented no significant differences in the variables of the disease, except the compound with organic acids and polyphenols (OAP) at a rate of 4.50 mL 100 L^{-1}, which provided a reduction of 35.64% in the SEV and 65.52% in AUDPC. The ASM effect on bacterial diseases has been studied by other authors in different pathosystems. Barret et al. (2010) found a significant reduction (67%) in the severity of bacterial wilt caused by *Ralstonia solanacearum* Smith, with rates 0.625 e 2.5 g 100

L^{-1} compared to an inoculated control. Similar results were found by Cavalcanti et al. (2006) with applications of ASM in tomato plants inoculated with *Xanthomonas axonopodis* pv. *vesicatoria* Vauterin, which increased the protection against bacterial wilt in 47.7% compared to inoculated plants and application with water only. The reduction in the values of the epidemiologic variables of bacterial canker found in the present experiment may be related to the induction of resistance activated by application of Acibenzolar-S-Metil, which is a molecule chemically similar to salicylic acid, an important indicator of plant protection against various diseases (SILVA et al., 2008).

Effect of resistance inducers in different application times.

The SEV and AUDPC variables had a significant interaction (Dunnett test; $p \leq 0.05$). The lowest percentages of severity of bacterial canker were found in plants that were applied with ASM 15 days before inoculation, presenting 11.50% of SEV and 18% of AUDPC. The results (Table 1) indicated that the other products also presented significant differences and interactions when compared with plants treated with the control treatments (copper oxychloride and inoculated control). SC had the lowest percentages of SEV at 10 (14.25%) and 15 (16.75%) days before inoculation. The OAP and potassium silicate (SiK) treatments significantly reduced the SEV (15.17% and 15.33%, respectively) when applied five days before inoculation, losing its effect with the increase in application interval. The smallest reduction of AUDPC with OAP and SiK was 30.13% and 40.60%, respectively, with applications at 10 days before inoculation. These results confirm the need of a time interval between treatment with the inducer and the subsequent inoculation of the plant (PEREIRA (2008). However, this period significantly differs between plants. Herbaceous species such as tomato and cucumber have effective defense responses that are activated in a short period of time, ranging from three to seven days when induced by applying chemicals or inoculation with non-virulent organisms (BENHAMOU e BELANGÉR, 1998; CAVALCANTI et al., 2006). There are few works with induction of defense responses in grapevine and they have variations in the results. Owen et al. (1998) evaluated the effect of ASM in inducing resistance to *Meloidogyne javanica* Treub Chitwood e *M.arenaria* Neal Chitwood in grapevine and found that the ASM applied by foliar spraying at seven days before inoculation, promoted reductions of 40 to 80% of disease symptoms compared to the control. However, the data of the present study showed that the time required for reduce the disease indexes were higher (10 to 15 days), for the studied pathosystem.

Characterization of the biochemical mechanisms involved in the defense response

Ninety-six hours after inoculation, the activity of β-1,3 glucanases and phenylalanine ammonia-lyase (PAL) in plants treated with ASM were significantly higher than the control and other treatments. The β-1,3 glucanase activity tripled in plants inoculated 15 days before with 0.25 μg mL^{-1} of glucose. The activation peak of the PAL enzyme was found in plants treated on the day of inoculation, producing 5 Abs $(g.p.f)^{-1}$. However, treatments at 5 and 10 days before inoculation were significantly higher compared to the inoculated control (Figures 4 and 5). The expression of these enzymes in plants treated with ASM was confirmed in apple (BRISSET et al., 2007), coffee (NOJOSA et al., 2009) and cocoa (COSTA et al., 2010). The β-1,3 glucanases may have direct action against the pathogen by cell wall degradation, preventing the establishment of the pathogen in the plant. In this process, polymers of n-acetilglucosamina and β-1,3 glucan, from the degraded cell wall of fungi or bacteria by these enzymes can act as elicitors and activate other defense mechanisms (COSTA et al., 2010). The PAL activates the route of phenylpropanoids, potentiating mechanisms involved in the synthesis of phenolic compounds such as phytoalexins and lignin, which grant the cell wall a greater resistance to pathogens (TAIZ e ZEIGER, 2004). Therefore, the reduction in the values of the epidemiological variables of bacterial canker found in the present study is probably related to the increase in the enzymatic activity of the β-1,3 glucanases and phenylalanine ammonia-lyase by ASM. Field works should be performed to confirm the ASM efficiency, which, based on this study, may be incorporated as a resistance inducer to the integrated management of grapevine bacterial canker. No increase in peroxidase activity was found in any of the evaluated times after inoculation, with no significant differences between the control and the plants treated with ASM in the different application times. This response may be related to two possible scenarios: first to the time intervals evaluated for the production of this enzyme, that may have been shorter, i.e., the peroxidase activity may have been initiated in a period longer than 96 hours, since in woody plants such as grapevines, as the example of cocoa, the peroxidase activity is initiated after 9 to 12 days after inoculation with *Verticillium dahliae* Kleb (PEREIRA et al., 2008); and second to the

susceptibility of the cultivar studied, since plants from the cultivar Redglobe may not have enough genes and time for peroxidase production, since the increase of peroxidase is more significant after inoculation with the pathogen in resistant varieties, as many studies have shown. Anjana et al. (2008) found larger peroxidase activity in resistant than susceptible genotypes of sunflower plants, two hours after inoculated with *Alternaria helianthi* (Hansff) Tubaki & Nishih. Other pathosystems such as common bean and *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (Hedges) (SOARES et al., 2004), cucumber and *Cladosporium cucumerinum* Ell. & Arth (MARINGONI, 2002) and eucalyptus and *Puccinia psidii* G. Winter (BOAVA et al., 2010), presented positive relationships between the peroxidase activity and the genetic resistance of plants. However, further studies on enzyme activity and host compatibility in grapevine varieties for the studied pathosystem are needed

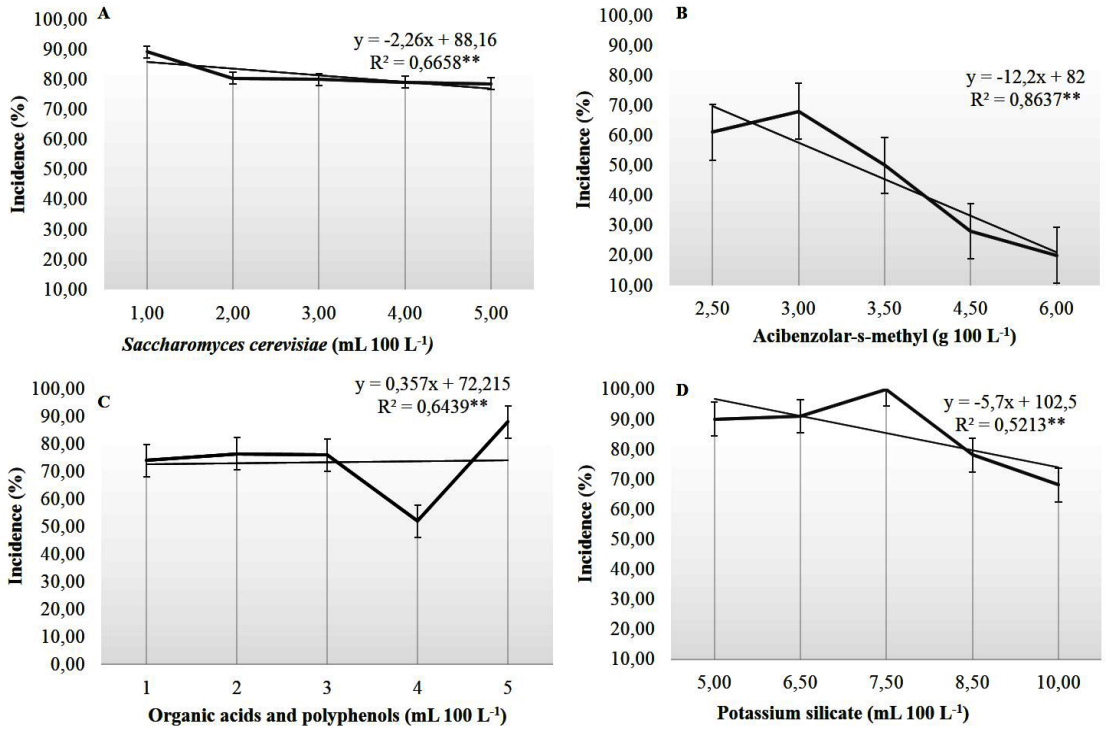

FIGURE 1-Percentage of incidence of bacterial canker 42 days after inoculation with *Xanthomonas campestris* pv. *viticola* in grapevine plants (cv. Redglobe) treated with different rates of: *Saccharomyces cerevisiae* (SC) Acibenzolar-S-Methyl (ASM), organic acids and polyphenols (OAP) and potassium Silicate (SiK), and the fitted regression equations.

**Regression models significant at 1% of probability by F test.

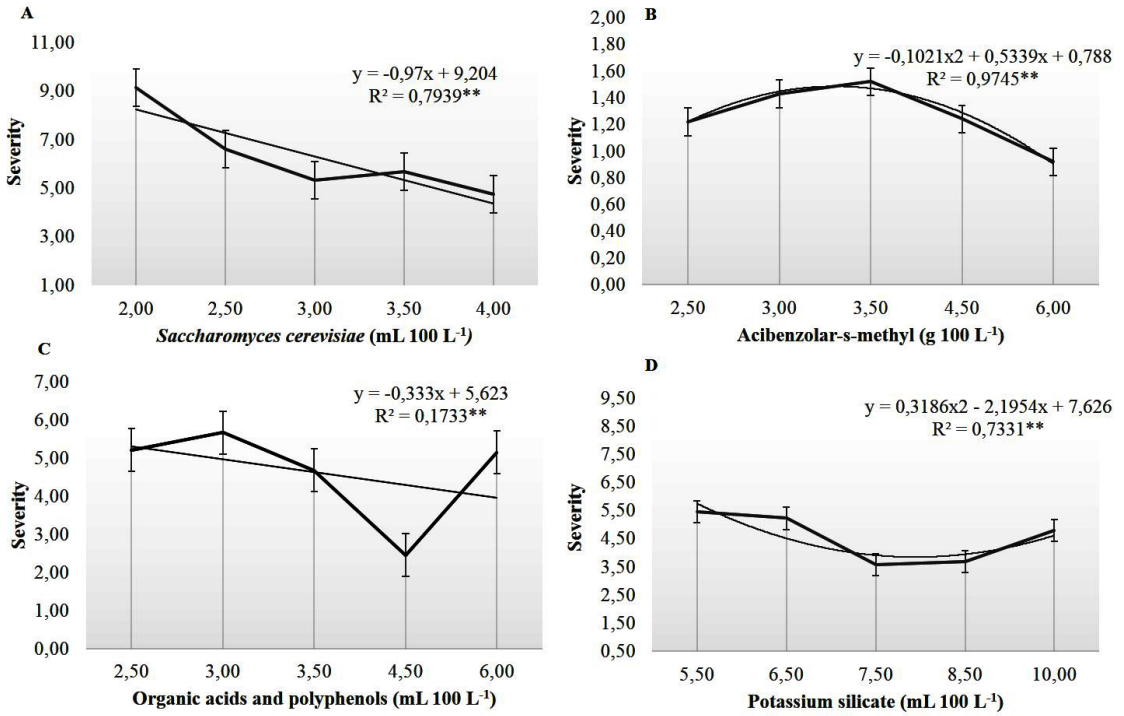

FIGURE 2 - Severity of bacterial canker in grapevine plants (cv. Redglobe) treated with different rates of: *Saccharomyces cerevisiae* (SC) Acibenzolar-S-Methyl (ASM), organic acids and polyphenols (OAP) and potassium Silicate (SiK), and the fitted regression equations.

**Regression models significant at 1% of probability by F test.

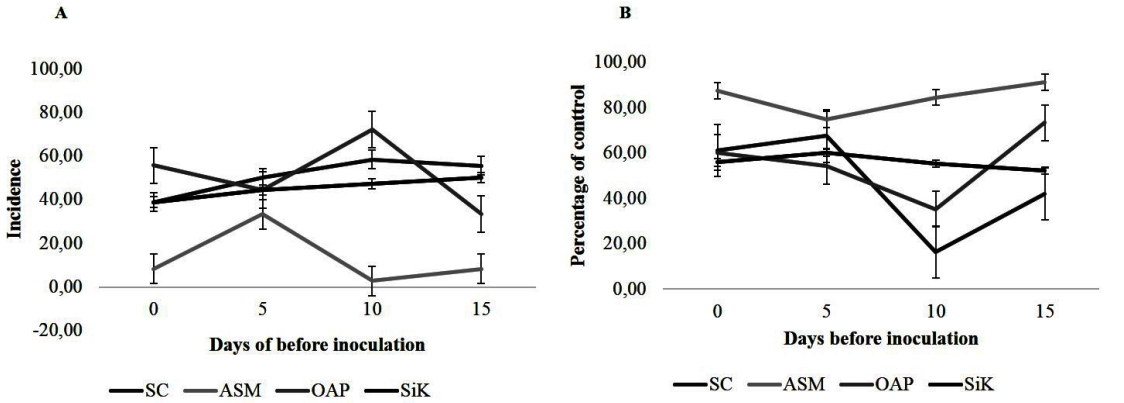

FIGURE 3- Incidence and percentage of control of bacterial canker in grapevine plants (cv. Redglobe), applied with: *Saccharomyces cerevisiae* (SC), Acibenzolar-S-Methyl (ASM), organic acids and polyphenols (OAP) and potassium Silicate (SiK) at 0, 5, 10 and 15 days before inoculation. Averages were subjected to the Tukey test at 0.05% probability.

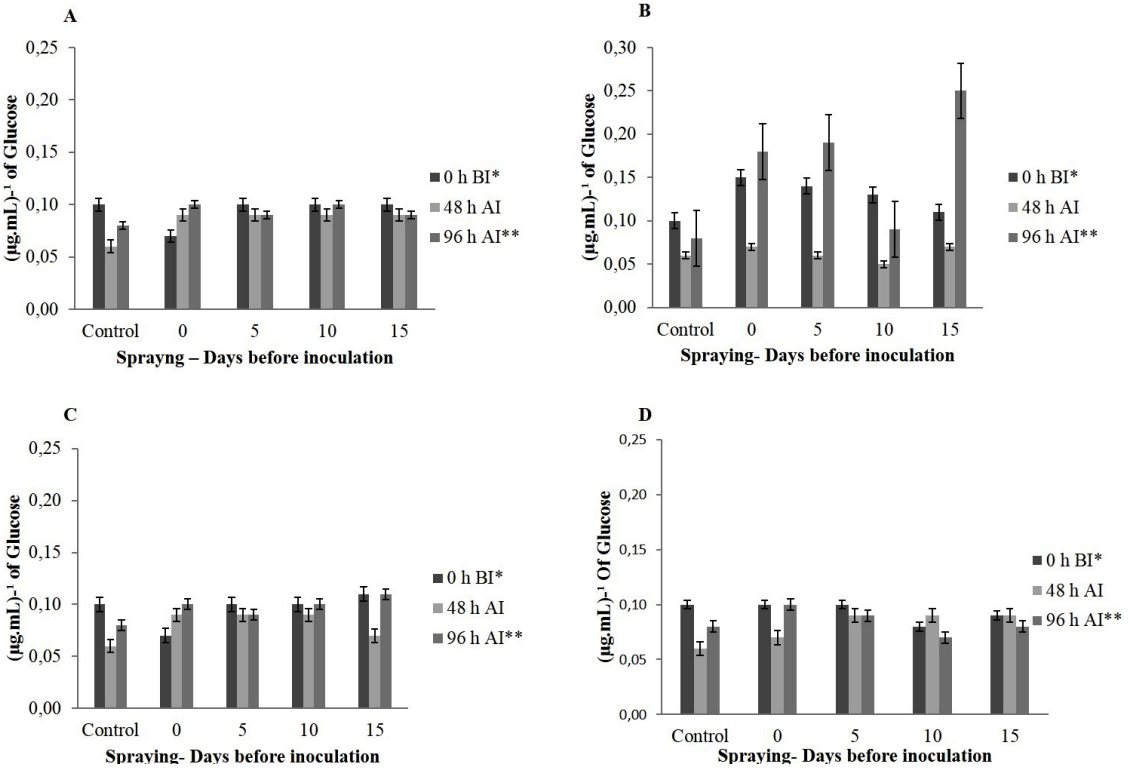

FIGURE 4 - β-1,3 glucanases in grapevine plants (cv. Redglobe) applied with A (*Saccharomyces cerevisiae* 2.50 mL 100 L^{-1}), B (Acibenzolar-S-Methyl 3 g 100 L^{-1}), C (Organic acids and polyphenols 4.50 mL 100 L^{-1}), and D (Silicate potassium 6.50 ml 100 L^{-1}) at 0, 5, 10 e 15 days before inoculation with *Xanthomonas campestris* pv. *viticola*. AI = after inoculation; BI = before inoculation.

TABLE 1 - Severity and area under the disease progress curve (AUDPC) of bacterial canker in grapevine plants (cv. Redglobe) treated with: *Saccharomyces cerevisiae* (SC), Acibenzolar-S-Methyl (ASM), organic acids and polyphenols (OAP) and potassium Silicate (SiK) at 0, 5, 10 and 15 days before inoculation (DBI).

Treatments	Aplication (DBI)							
	Severity (%)				AUDPC			
	0	5	10	15	0	5	10	15
SC	19.08aBC	40.83aA	14.25bc	16.75bBC	45.36aC	37.95abC	97.65aA	67.71aI
ASM	16.08bA	11.63bB	15.41bB	11.50cB	42.50aA	29.20bB	38.16bAB	18cC
OAP	22.50aB	15.17bC	45.83aA	31.83aB	46.37aA	53.47aA	30.13bB	54.47a
SiK	22.50aA	15.33bB	16.33bB	19.08bAB	51.52aA	34.90bB	40.60bAB	32.30bI
Oxychloride Copper				37.33b				73.95b
Relative Control				42.50a				116.75a
CV(%)				16.24				10.96

*Means followed by the same lowercase letters in the column and uppercase letters in the line do not statistically differ by the Dunnett test ($p \leq 0.05$).

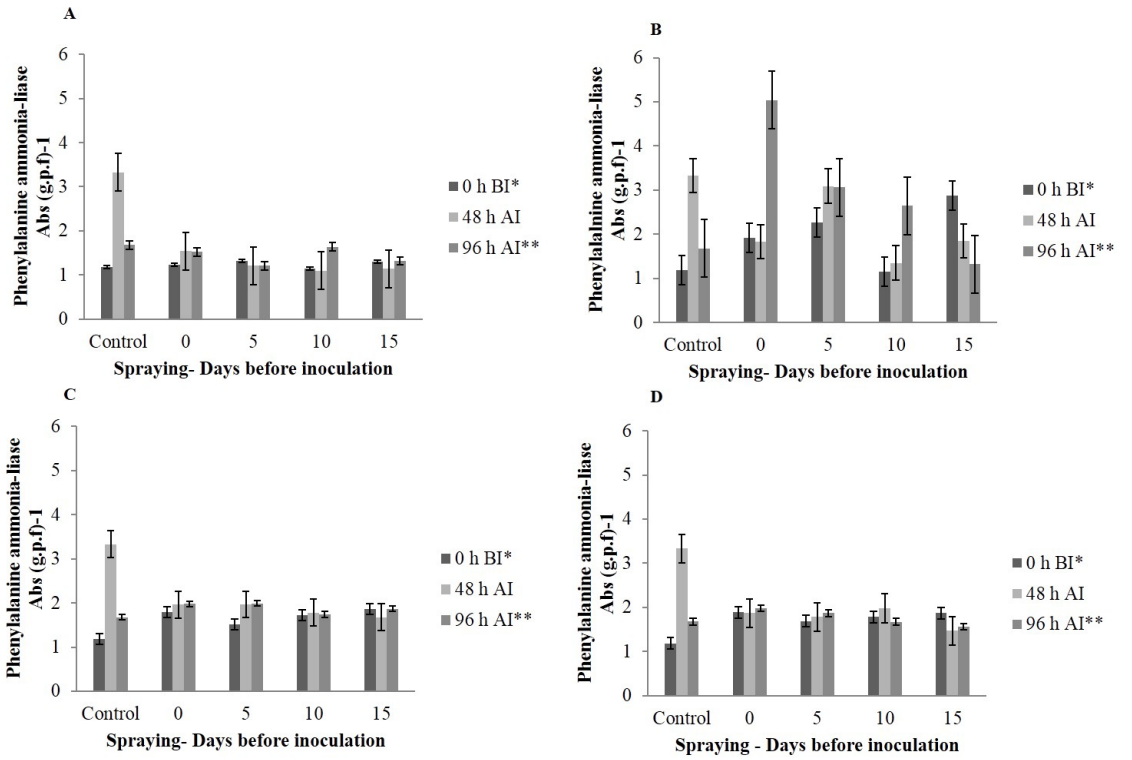

FIGURE 5- Phenylalanine ammonia-lyase activity in grapevine plants (cv. Redglobe) applied with A (*Saccharomyces cerevisiae* 2.50 ml 100 $L^{-1)}$, B (Acibenzolar-S-Methyl 3 g 100 L^{-1}), C (organic acids and polyphenols 4.50 mL 100 L^{-1}), and D (Silicate potassium 6.50 ml 100 L^{-1}) at 0, 5, 10 e 15 days before inoculation with *Xanthomonas campestris* pv. *viticola.*

CONCLUSION

Grapevine plants treated with Acibenzolar-S-Methyl presented significant reduction in the values of epidemiological variables of the disease from the minimum rate (3.0g 100 L^{-1}), applied 15 days before inoculation with the pathogen.

There was an increase in enzyme activity of phenylalanine ammonia-lyase and β-1,3 glucanases in plants treated with Acibenzolar-S-Methyl, denoting a potential resistance inducer in grapevine plants to the studied pathogen.

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