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SHORT COMMUNICATION / *COMUNICAÇÃO*

Silicon reduces bacterial speck development on tomato leaves

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ABSTRACT

This study aimed to evaluate the effect of silicon (Si) on the resistance of tomato plants (cv. Santa Clara) to bacterial speck, caused by *Pseudomonas syringae* pv. *tomato* (*Pst*). Tomatoes were grown in soil without calcium silicate (control), in soil without calcium silicate and sprayed with Supa Sílica® (2 mL/L) (SS) and in soil with calcium silicate (0.16 g/kg of soil) (CS). The effect of SS on the growth of *Pst* was evaluated in *vitro*. There was no significant difference among the treatments for foliar Si concentration and incubation period. No significant differences were observed between the control and CS for the number of lesions per plant (NLP) and bacterial speck severity estimated by the software QUANT. The NLP was significantly reduced with SS spray. There was a negative linear response of *in vitro Pst* growth to the SS rates. Peroxidase, polyphenoloxidase and glucanase activities were significantly higher for plants sprayed with SS and grown in soil with CS compared to the control treatment. Phenylalanine ammonia-lyase and lypoxygenase activities were significantly higher for plants grown in soil with CS compared to the control. The concentration of malondialdehyde was significantly higher for plants sprayed with SS compared to the control at 0 and 7 dai. The results of this study indicate that the symptoms of bacterial speck are reduced when plants are sprayed with SS, which can be linked to the direct effect of this product against *Pst* rather than the potentation of host defense responses.

Key words: *Pseudomonas syringae* pv. *tomato*, *Solanum lycopersicum*, host resistance, plant nutrition

Bacterial speck caused by *Pseudomonas syringae* pv. *tomato* (*Pst*) is an economically important disease on tomato crops (Silva & Lopes, 1995). Yield losses of up to 30% are reported in environments with high relative humidity and temperature ranging from 20 to 25ºC (Silva & Lopes, 1995). The control of bacterial diseases is limited to the availability of chemicals. Products containing copper and the inducer of resistance acibenzolar-S-methyl are the most used to control bacterial speck (Lopes & Santos, 1994). Also, the use of cultivars containing the resistant *Pto* gene, such as the cultivar BRS Tospodoro, is highly recommended to the growers (Lopes & Santos, 1994). Other control methods for bacterial speck remain to be investigated. Silicon (Si) has been gaining attention in the control of certain fungal diseases, such as powdery mildew in muskmelon and tomato (Dallagnol et al., 2012; Yanar et al., 2011). Dannon & Wydra (2004) obtained reduction in the symptoms of bacterial wilt (*Ralstonia solanacearum*) in tomato plants from susceptible and moderately resistant genotypes supplied with Si. According to Diogo & Wydra (2007), the reduction in the symptoms of bacterial wilt occurred due to changes in cell wall structure of the xylem vessels in plants supplied with Si. In the same pathosystem, Ghareeb et al. (2011) found that the expression of genes related to basal defense of tomato was induced in plants supplied with Si and inoculated with *R. solanacearum*, thereby, demonstrating the importance of Si in enhancing host defense responses.

This study aimed to evaluate the potential of foliar application and supply of Si to the roots of tomato plants on the reduction of bacterial speck symptoms.

The soil type used in the experiments was a Si-deficient typical Acrustox red-yellow latosol collected at the 'Triângulo Mineiro' savanna area with the following characteristics: 530 g kg^{-1} of clay; pH 4.8 in KCl 1 M; 0.5 mg dm^{-3} P (Mehlich-1); 13 mg dm^{-3} K (Mehlich-1); Al^{3+}, Ca^{2+}, Mg^{2+}, $H^{+}+Al^{3+}$ of 0.1, 0.0, 0.0 and 3.8 $cmol_c$ dm^{-3}, respectively; 2% base saturation and 2.3 dag kg^{-1} organic matter. The concentration of available Si (extraction in $CaCl_2$) was 11.8 mg dm^{-3}. Each plastic pot (Ecovaso) was filled with 5 kg of air-dried, 5 mm-sieved soil. The calcium silicate (CS) used as the Si source (AgroSilício®, Harsco Minerais Ltda.) was composed of 10.5% (w/w) Si, 25% (w/w) Ca and 6% (w/w) Mg. The CS was mixed with the soil before filling of each pot at the rates of 0 and 1.5 g kg^{-1} of soil, which corresponded, respectively, to 0 and 0.16 g of elemental Si per kg of soil. Calcium carbonate [40% (w/w) Ca, Sigma-Aldrich] and magnesium carbonate ($MgCO_3$, Sigma-Aldrich) were added at the rates of 0.96 and 0.78 g kg^{-1} of soil, respectively, to the pots that did not receive

Si in order to equilibrate the amounts of Ca and Mg in this treatment with the amounts present in the pots that received 1.5 g of calcium silicate. The amounts of Ca and Mg among the treatments were fixed at 0.39 and 0.09 g, respectively, per pot. The soil in each pot was incubated for 30 days with an approximate humidity of 65%.

Six seeds of tomato (cv. Santa Clara), susceptible to *Pst*, were sowed per pot containing 5 kg of soil. After emergence, seedlings were thinned to two per pot and received 50 mL of nutrient solution containing, in mg L^{-1}: 192 KCl, 104.42 K_2SO_4, 150.35 $MgSO_4.7H_2O$, 61 urea, 100 NH_4NO_3, 0.27 $NH_4MO_7O_{24}.4H_2O$, 1.61 H_3BO_3, 6.67 $ZnSO_4$, 1.74 $CuSO_4.5H_2O$, 4.10 $MnCl_2.4H_2O$, 4.08 $FeSO_4.7H_2O$ and 5.58 disodium EDTA. The solution was also applied at 22 and 29 days after sowing. The plants were kept in a greenhouse (temperature ranging from 18 to 27°C and relative humidity of 75±5%) and irrigated daily. Tomato plants with the third leaf, from base to the apex, fully expanded (34 days after sowing) and at 24 hours before inoculation with *Pst*, the plants were sprayed with Supa Silica® (SS) (Agrichem; 23.7% K_2O + 10% Si, pH 9.42) at the concentration of 2 mL L^{-1}. All leaves of plants from the replications of each treatment were sprayed with 25 mL of SS solution. Subsequently, the plants were kept in mist chamber with temperature of 25°C, relative humidity of 85±5% and photoperiod of 12 hours, where they remained for 24 hours before being inoculated with *Pst*.

The strain of *Pst* (UFV-01), obtained from tomato plants (cv. Santa Clara) with symptoms of bacterial speck, was streaked and grown on Kado 523 medium for 24 hours at 28°C (Kado & Heskett, 1970). After this period, 0.85% saline (Romeiro, 2001) was added to each Petri dish containing the bacteria in active growth and the concentration of the suspension was adjusted with spectrophotometer at 540 nm absorbance according to predetermined equation where: A_{540}=0.2 is equivalent to 1×10^8 CFU mL^{-1}. Tomato plants were inoculated 24 hours after SS spraying using a suspension of *Pst* at a concentration of 1×10^8 CFU mL^{-1} using a VL Airbrush atomizer (Paasche Airbrush Co.). After inoculation, plants were placed in a chamber with temperature of 25±2°C, relative humidity of 80±5% and photoperiod of 12 h light for 24 h. At 24 h after inoculation (hai) the plants were transferred to the greenhouse (temperature ranging from 18 to 27°C and relative humidity of 75±5%).

In order to test the effect of SS on *Pst* growth *in vitro*, the rates of 0, 0.125, 0.25, 0.5 and 1 µL of SS were added separately to 250 µL of *Pst* suspension present in the holes of Elisa plates. Plates containing only Kado 523 medium served as the control treatment. The plates were incubated for 24 hours at 28°C and the absorbance was determined at 540 nm. The concentration of 0.5 µL of SS per 250 µL of *Pst* suspension corresponded to the concentration of 2 mL of SS/L used to spray the tomato leaves.

The incubation period (IP), the number of lesions per plant (NLP) and the severity of bacterial speck were evaluated on five leaflets of leaves 1 and 2 fully expanded, from the base to the apex, of the plants of each replication per treatment. The IP (days) was obtained by evaluating the onset of symptoms of bacterial speck (necrotic lesions with chlorotic halo) on leaves every 24 hai. The NLP was assessed 12 days after inoculation (dai) and soon after, the leaves of each plant were collected and scanned at 300 dpi resolution. The images were processed with the software QUANT (Liberato, 2003) to quantify the diseased leaf area (chlorosis and necrosis), which was denominated severity estimated by the software QUANT (SEQ).

After the experiment (12 dai), all leaves from each plant, replication and treatment were collected, washed in deionized water, dried for 72 h at 65°C and ground to pass through a 40 mesh screen with a Thomas-Wiley mill. The foliar Si concentration was determined according to Dallagnol et al. (2011).

A separate experiment was carried out to obtain leaf samples for the biochemical analysis. Fully expanded leaves 1 and 2, from the base to the apex, of the replications of each treatment were collected at 2, 5, 7 and 10 dai. Samples from leaves not inoculated with *Pst* served as the control treatment (0 dai). Leaf samples were quickly frozen in liquid nitrogen (N_2) and stored at -80°C for further analysis. To obtain the extracts used to determine the activities of peroxidases (POX, EC1.11.1.7), polyphenoloxidases (PPO, EC 1.10.3.1), β-1,3-glucanases (GLU, EC 3.2.1.39) and phenylalanine ammonia-lyases (PAL, EC 4.3.1.5), 0.3 g of leaf tissue was macerated with liquid N_2 in a mortar with the addition of polyvinylpyrrolidone (PVP) 1% (w/vol) to obtain a fine powder. The powder was homogenized in 2 mL of 50 mM sodium phosphate (pH 6.5) containing 1 mM phenylmethylsulfonicfluoride (PMSF). The homogenized material was centrifuged at 20,000 × *g* for 25 min at 4°C and the supernatant was used to determine the enzyme activity.

POX and PPO activities were determined by the oxidation of pyrogallol according to the method of Kar & Mishra (1976). For POX activity, a mixture of 300 µL of distilled water, 280 µL of 100 mM potassium phosphate buffer (pH 6.8), 200 µL of 100 mM pyrogallol and 200 µL of 100 mM hydrogen peroxide was added to 20 µL of the extract. For PPO activity, the mixture was composed of 300 µL of distilled water, 280 µL of 100 mM potassium phosphate buffer (pH 6.8) and 200 µL of 100 mM pyrogallol, which was added to 20 µL of the extract. The absorbance was measured by a spectrophotometer (Evolution 60, Thermo Scientific) at 420 nm every 10 seconds for 1 min after addition of the extract to the mixture in a total of five readings. The molar extinction coefficient of 2.47 mM^{-1} cm^{-1} was used to calculate POX and PPO activities (Chance & Maehley, 1955), which were expressed in mM purpurogallin produced min^{-1} mg^{-1} of protein. Throughout the process, the microcentrifuge tubes were covered with aluminum foil to protect the mixture from oxidation by light. GLU activity was determined as described by Lever

(1972). The reaction was initiated by the addition of 20 µL aliquots of the supernatant to a mixture of 230 µL of buffer 100 mM sodium acetate (pH 5.0) and 250 µL of the substrate laminarin (Sigma-Aldrich) in a concentration of 4 mg mL^{-1}. The reaction mixture was incubated in a water bath for 30 min at 45°C. After the incubation period, the amount of reducing sugars was determined by adding 250 µL of dinitrosalicylic acid to the mixture and then incubating the resulting mixture in a water bath for 15 min at 100°C. The reaction was interrupted by cooling the samples in an ice bath. In the control samples, the reaction mixture was the same, except that the extract was added after heating the mixture at 100°C. The absorbance of the product released by GLU was measured at 540 nm and the activity of GLU was expressed in absorbance units min^{-1} mg^{-1} of protein. PAL activity was determined by adding 100 µL of the extract to a mixture containing 400 µL of 25 mM Tris-HCl (pH 8.8) and 500 µL of 100 mM *L*-phenylalanine. The reaction mixture was incubated in a water bath at 30°C for 4 h. In the control samples, the *L*-phenylalanine was replaced with 1 mL of Tris-HCl buffer. The reaction was finalized by adding 60 µL of 6 N HCl. The absorbance of the *trans*-cinnamic acid derivatives was measured in a spectrophotometer at 290 nm and the molar extinction coefficient of 10^4 mM^{-1} cm^{-1} (Zucker, 1965) was used to calculate PAL activity, which was expressed in µM min^{-1} mg^{-1} of protein.

To obtain the extract for enzymatic determination of lipoxygenases (LOX, EC 1.13.11.12), 0.2 g of leaf tissue was macerated with liquid N_2 in a mortar to obtain a fine powder. The powder was homogenized in 2 mL buffer of 20 mM sodium phosphate (pH 6.8) containing Triton X-100 1% (v/v) and PVP 1% (w/v). The homogenized material was centrifuged at 15,000 × *g* for 10 min at 4°C. The supernatant was used as the extract for the determination of LOX activity. The reaction was started by adding 7 µL of the extract to a mixture containing 790 µL of buffer, 50 mM sodium phosphate (pH 6.5), and 5 µL of a 10 mM sodium linoleate substrate. The LOX activity was determined according to the method described by Axelrod et al. (1981). The absorbance of the product released by LOX was measured in a spectrophotometer at 234 nm. The molar extinction coefficient of 25,000 M^{-1} cm^{-1} was used to determine LOX activity, which was expressed as µmol min^{-1} mg^{-1} of protein. The protein concentration in each sample was determined according to the method of Bradford (1976).

Oxidative damage to lipids was estimated as the content of the total 2-thiobarbituric acid (TBA) reactive substances and expressed as equivalents of malondialdehyde (MDA), according to Cakmak & Horst (1991), with few modifications. Briefly, a total of 0.2 g of leaf tissue was homogenized in 2 mL 0.1% (w/v) trichloroacetic acid (TCA) solution at 4°C. After centrifugation at 10,000 × *g* for 15 min, 250 µL of the supernatant was reacted with 750 µL TBA (0.5% in 20% TCA) for 20 min in a boiling water bath. After this period, the reaction was stopped by immersion in an ice bath. The samples were centrifuged at 13,000 × *g* for 4 min and the absorbance of the supernatant was recorded at 532 nm. The concentration of MDA formed in each sample was calculated by using the extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as µmol of MDA per kg^{-1} of fresh weight (FW).

One experiment was arranged in a completely randomized design with three treatments and six replications to evaluate the IP, NLP, SEQ and foliar Si concentration. This experiment was repeated once. Data from each component of resistance from the two experiments were pooled for statistical analysis because homogeneity of variances was confirmed by Cochran's test. Moreover, the experiment-treatment interactions were not significant ($P \geq 0.05$) when compared with the main effects of treatments. The activities of POX, PPO, GLU, PAL and LOX were studied in one separate experiment. Data were submitted to analysis of variance (ANOVA) and treatments means comparisons by Tukey's test ($P \leq 0.05$) using SAS (SAS Institute Inc.).

It is known that several diseases affecting important economic crops had their intensities decreased by supplying Si to the plants which ideally fits in with environmentally friendly strategies for sustainable crop production including tomato. Even though the foliar Si concentration from plants grown in soil without calcium silicate (control), in soil without calcium silicate and sprayed with SS and in soil with calcium silicate ranged from 0.30 to 0.35 dag kg^{-1} of dry matter (Table 1), the symptoms of bacterial speak were reduce by the direct effect of the foliar Si application.

TABLE 1 - Incubation period (IP), number of lesions per plant (NLP), severity estimated by the software QUANT (SEQ) and foliar silicon (Si) concentration on tomato plants grown in soil without calcium silicate (control), in soil without calcium silicate and sprayed with Supa Sílica® (SS) and in soil with calcium silicate (CS) and inoculated with *Pseudomonas syringae* pv. *tomato*

Treatments	IP (days)	NLP	SEQ (%)	Si (dag/kg)
control	4.60	222.35 b	1.92 b	0.34
SS	4.47	118.24 a	0.74 a	0.35
CS	4.15	215.42 b	1.69 b	0.30
F values	0.93^{ns}	22.83^{**}	65.35^{**}	0.78^{ns}
CV (%)	19.30	22.77	18.10	28.10

Means followed by the same letter in each column are not significantly different ($P \leq 0.05$) as determined by Tukey's test. ns=nonsignificant. CV=coefficient of variation. n=12.

Quantification of components of resistance allows inferences about possible mechanisms involved in host resistance against pathogens attack (Parlevliet, 1979). In the present study, Si did not contribute to increase the IP (Table 1). Similar results were found by Silva et al. (2010) where both the IP and the latent period of bacterial leaf streak of wheat were not affected by Si application. Nevertheless, Dannon & Wydra (2004) and Diogo & Wydra (2007) found that in the presence of Si, there was a delay in the onset symptoms of wilt caused by *Ralstonia solanacearum* in tomato plants from cultivars susceptible and moderately resistant to this bacterium. The NLP and the SEQ significantly decreased with SS spray in comparison to the treatments control and plants grown in soil amended with calcium silicate (Table 1). There was no significant difference between the treatments control and plants grown in soil amended with calcium silicate for both NLP and SEQ (Table 1). In other pathosystems such as tomato-*Leveillula taurica*, bean-*Pseudocercospora griseola* and soybean-*Phakopsora pachyrhizi*, the foliar application of potassium silicate was effective in reducing the severity of the diseases caused by these pathogens (Pereira et al., 2009; Rodrigues et al., 2010; Yanar et al., 2011).

The relationship between *Pst* growth *in vitro* and the SS rates was negatively linear ($Y=0.318-0.201x$; $R^2=0.94$, $P=0.002$). It is believed that the high pH of the SS solution affected *Pst* growth *in vitro*. At the rate of 0.5 µL of SS the pH was 9.2 and *Pst* growth was inhibited by 31.4%. According to Oginsky & Umbreit (1959), pH ranging from 6.5 to 7.5 is the best for *Pst* growth. Amaral et al. (2008) showed that potassium silicate had fungitoxic activity against *Cercospora coffeicola* growth *in vitro*.

Although calcium silicate has contributed to increase the activity of defense enzymes, it was not effective in reducing the NLP and the SEQ. Probably only the increased activity of these enzymes was not sufficient for the plant to contain *Pst* infection so other defense mechanisms must be activated. In the present study, the foliar concentration of Si did not contribute to increase the resistance of tomato plants to infection by *Pst*. The activities of POX and PPO for plants sprayed with SS and supplied with calcium silicate increased during the infectious process of *Pst* (Figures 1A and B). Liang et al. (2005) obtained association of the high POX and PPO activities with an increase in cucumber resistance to *Podosphaera xanthii*.

The GLU activity for plants sprayed with SS or supplied with calcium silicate was significantly higher at 5 and 10 dai in comparison to the control treatment (Figure 1C). Increased activity of this enzyme has also been reported in tomato sprayed with ASM and inoculated with *X. campestris* pv. *vesicatoria* (Cavalcanti et al., 2006). The GLU has been also associated with induced resistance of tomato plants pre-treated with *Methylobacterium oryzae* to *Pst* (Indiragandhi et al., 2008).

The activity of PAL was only significantly higher in plants supplied with calcium silicate at 5 dai compared to the control treatment (Figure 1D) and apparently had no effect on tomato resistance against *Pst*. By contrast, Liang et al. (2005) linked the increase in PAL activity with the higher resistance of cucumber plants supplied with Si to powdery mildew. Kavitha & Umesha (2008) found that a higher activity of PAL resulted in increased resistance of tomato to *Xanthomonas campestris* pv. *vesicatoria* and *Clavibacter michiganensis* subsp. *michiganensis*.

Little is known in the literature about the effect of Si on LOX activity. Nevertheless, in the present study, there was increased activity of LOX in plants supplied with calcium silicate and inoculated with *Pst* with significant differences only at 2, 7 and 10 dai (Figure 1E). Similar results were found by Koch et al. (1992), whom observed the highest activity of LOX in tomato plants infected with *Pst* and *P. syringae* pv. *syringae*. Slusarenko et al. (1991) found that this enzyme is involved in cell membrane damage in incompatible interactions. During the infection process, physical damage occurs to cell membranes and due to this, a sequential degradation of lipids can be initiated by LOX (Jalloul et al., 2002). Thus, plants were analyzed for the concentration of MDA, which is an indicator of damage to cell membranes in response to various types of stress (Jalloul et al., 2002). The MDA concentration was significantly higher for the foliar spray of SS at 0 and 7 dai in comparison to the CS and control treatments (Figure 1F) indicating a possible effect of this product in damaging the cell membrane even though with the reduction of bacterial speak symptoms.

A preliminary experiment to determine enzyme activities was carried out and showed a similar trend for the treatments discussed above (data not shown).

From the results of the present study, three hypotheses can be proposed to explain the effect of foliar spray of SS in reducing the bacterial speck symptoms: i) the possible deposition of SS on the leaf surface and at the base of the trichomes could have hindered the penetration of bacteria into the plant tissue since *Pst* epiphytically is capable of surviving in the depressions between epidermal cells at the base of trichomes, along the ribs and around stomas on tomato leaves (Mariano & McCarter, 1991), ii) the effect of osmotic potential generated by the presence of SS on the leaves surface, which may affect the multiplication of bacteria and iii) the possible effect of the pH of the SS. Dallagnol et al. (2012) found by X-ray microanalysis, deposition of Si on the surface of melon leaves forming a physical (after water evaporation) and chemical barrier (due to increases in both osmotic potential and pH), which reduced the powdery mildew symptoms.

In conclusion, the results of the present study demonstrate that the spray of a source of Si to tomato leaves can decrease bacterial speck symptoms probably due to its direct effect on *Pst* without any clearly contribution of host defense enzymes. This information may be valuable to be used in an integrated bacterial speck management program and, therefore, reduce yield losses caused by this disease.

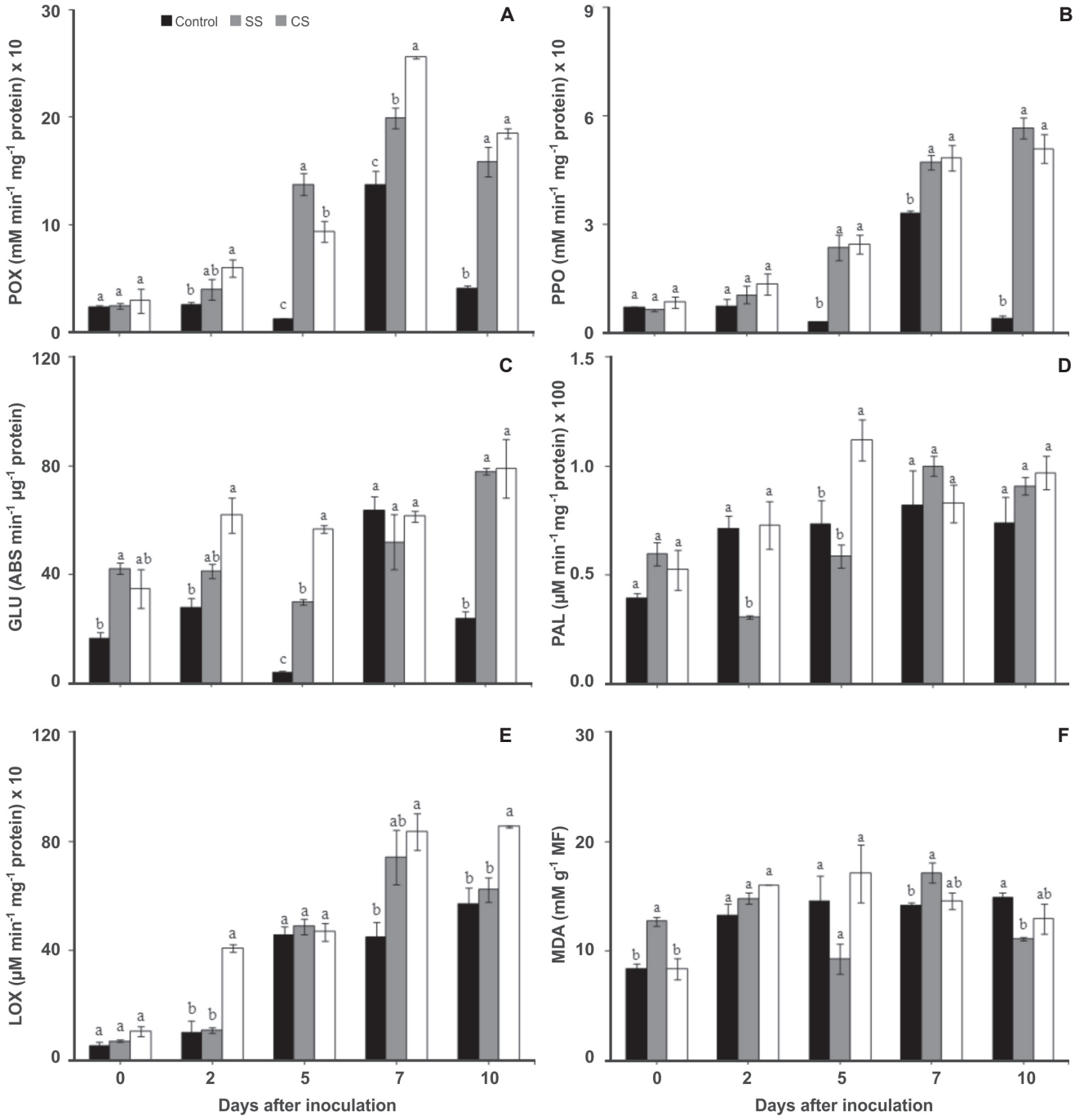

FIGURE 1 - Activities of peroxidases (POX) (**A**), polyphenoloxidases (PPO) (**B**), *β*-1,3-glucanases (GLU) (**C**), phenylalanine ammonia-lyases (PAL) (**D**), lypoxigenases (LOX) (**E**) and concentration of malondialdehyde (MDA) (**F**) on leaves of tomato plants grown in soil without calcium silicate (control), in soil without calcium silicate and sprayed with Supa Sílica® (SS) and in soil with calcium silicate (CS) and inoculated with *Pseudomonas syringae* pv. *tomato*. Means followed by the same letter for each evaluation time are not significantly different ($P \leq 0.05$) as determined by Tukey's test.

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