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ORIGINAL ARTICLE

Potassium silicate-induced resistance against blackfly in seedlings of *Citrus reticulata*

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Abstract – Introduction. Citrus trees in Brazil are often attacked by the blackfly, *Aleurocanthus woglumi* Ashby. The induction of resistance to control this pest is required to maintain the sanitary and nutritional quality of the crop. The aim of this study was to evaluate the potential of silicon in the form of potassium silicate (K_2SiO_3) to modify the activity of enzymes involved in the defence of *Citrus reticulata* and to find any correlation between the activity of these enzymes and the development of *A. woglumi*. **Materials and methods.** The study was conducted in a greenhouse using seedlings of *C. reticulate* cv. 'Dancy' in the following one-application treatments: T_1: Infestation with *A. woglumi* and no K_2SiO_3 (control, "C"); T_2: No *A. woglumi* and no K_2SiO_3 (absolute control "AC"); T_3: 17 g L^{-1} K_2SiO + *A. woglumi*, T_4: 35 g L^{-1} K_2SiO + *A. woglumi*; T_5: 52 g L^{-1} K_2SiO + *A. woglumi*; and T_6: 70 g L^{-1} K_2SiO + *A. woglumi*. To perform the enzymatic analyses, one leaf was removed separately from each mandarin seedling after 10, 30, 50, 70, and 90 days of continuous feeding of *A. woglumi*. The activity of peroxidase, polyphenol oxidase and phenylalanine ammonia-lyase was assessed. **Results and discussion.** The correlation between peroxidase activity and *A. woglumi* development was positive. The peroxidase and polyphenol activities indicated strong induction of plant defences against *A. woglumi*. The increase in peroxidase and polyphenoloxidase activity revealed the induction of synthesis of compounds for plant defence against *A. woglumi,* but this effect depended on the time of *A. woglumi* feeding and on the concentration of silicon. **Conclusion**. Silicon was shown to be an elicitor that potentiates the defence mechanisms of *C. reticulata* to *A. woglumi*.

Keywords: Brazil / mandarin *(Citrus reticulate)* / blackfly (*Aleurocanthus woglumi)* / resistance inductor / peroxidase / polyphenol oxidase / phenylalanine ammonia-lyase

Résumé – Résistance aux aleurodes noires des agrumes induite par le silicate de potassium dans les semis de mandarinier (*Citrus reticulata*). Introduction. Les agrumes au Brésil sont fréquemment attaqués par des aleurodes *(Aleurocanthus woglumi* Ashby). L'induction de résistance pour lutter contre ce ravageur est nécessaire pour maintenir la qualité sanitaire et nutritionnelle de la récolte. Le but de cette étude était d'évaluer le potentiel du silicium sous la forme de silicate de potassium (K_2SiO_3) dans la modification de l'activité des enzymes impliquées dans la réaction de défense du mandarinier *(Citrus reticulata*), et aussi de trouver une corrélation entre l'activité de ces enzymes et le développement des aleurodes. **Matériel et méthodes.** L'étude a été menée en serre en utilisant des plants de mandarinier cv. Dancy. Les traitements suivants ont été appliqués : T_1 : infestation par *A. woglumi* et pas de K_2SiO_3 (contrôle, "C" ; T_2 : pas *d'A. woglumi* et pas de K_2SiO_3 (contrôle absolu "AC") ; T_3 : 17 g L^{-1} K_2SiO + *A. woglumi*, T4 : 35 g L^{-1} K_2SiO + *A. woglumi* ; T5 : 52 g L^{-1} K_2SiO + *A. woglumi* ; et T_6 : 70 g L^{-1} K_2SiO + *A. woglumi*. Pour effectuer les analyses enzymatiques, une feuille de mandarinier a été prélevée séparément de chaque plante après 10, 30, 50, 70 et 90 jours d'alimentation continue en aleurodes des agrumes. L'activité de la peroxydase, de la polyphénol oxydase et de la phénylalanine ammonia-lyase (PAL) a été évaluée. **Résultats et discussion.** La corrélation entre l'activité de la peroxydase et le développement d'*A. woglumi* s'est montrée positive. L'activité de la peroxydase et celle de la polyphénol oxydase ont indiqué une forte induction des défenses de la plante contre *A. woglumi*. L'augmentation de l'activité polyphénol oxydase et peroxydase a révélé l'induction de la synthèse de composés actifs dans la défense

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des plantes contre *A. woglumi*, mais cet effet dépendait de la durée 'alimentation des aleurodes et de la concentration en silicium. **Conclusion.** L'étude a montré que le silicium pouvait jouer le rôle d'éliciteur en stimulant des mécanismes de défense de *C. reticulata* contre *A. woglumi*.

Mots clés : Brésil / mandarine *(Citrus reticulate)* / aleurode des agrumes (*Aleurocanthus woglumi)* / mécanismes de défense / peroxidase / polyphénol oxydase / phénylalanine ammonia-lyase.

1 Introduction

The citrus blackfly, *Aleurocanthus woglumi* Ashby, 1915 (Hemiptera: Sternorrhyncha: Aleyrodidade), is a pest that affects approximately 300 species of host plants. *A. woglumi* is native to Asia and is considered a pest of great importance in several countries due to crop damage or loss, which reduces the quality and/or quantity of production in a large number of crops [1].

The search for ways to control insect pests that reduce the use of agrochemicals, increased public awareness about environmental issues, and the promotion of food security and sustainability, especially integrated pest management have gained prominence in recent years. The use of products that induce resistance has also been gaining importance in the control of plant pests. Resistance induction corresponds to the activation of latent defence systems in plants when they come into contact with agents called elicitors. Among the elicitors, silicon has attracted the attention and interest of researchers [2]. Some of these products in the form of potassium silicate, calcium silicate and sodium silicate have gained importance because they are alternatives to chemical control. Conferring resistance also provides nutritional benefits and increases the production and quality of agricultural products [3]. As one of the most abundant elements on earth, silicon is wellknown to protect plants against a suite of pests with different lifestyles and modes of infection. The resistance induced by silicon is expressed in different ways, such as the lignification of cell walls, the formation of buds and the induction of several defence proteins [4]. Silica is an effective defence against folivorous insects, acting as a feeding deterrent probably by increasing abrasiveness reducing the digestibility of plant leaves [5]. However silicon is never found in a plant available form and is always combined with other elements. Silicon is absorbed by plants in the form of uncharged silicic acid, $Si(OH)_4$, and is ultimately irreversibly precipitated throughout the plant as amorphous silica [6].

Induced defence in plants is due to the formation of mechanical barriers and/or by changing the biochemical plant responses to herbivore attack via an increase in the synthesis of toxins that can act as inhibitors or repellents [7]. Some studies report that the application of silicon can reduce the rate of population growth, causing increased mortality in insects. Additionally, silicon may stimulate growth and crop production through various indirect actions, which may be related to the presence of chemical defences that may have adverse effects on the biology and behaviour of insects [8–10]. These resistance mechanisms may include the accumulation of phenolic compounds, phytoalexins and pathogenesis-related proteins (such as β-1,3-glucanase, chitinase, peroxidase, phenylalanine ammonia-lyase and polyphenol) [11]. The induction of resistance is a promising alternative to control insect pests and in this context, the aim of the present study was to evaluate the effects of silicon dose on the activity of the enzymes peroxidase, polyphenol oxidase and phenylalanine ammonia-lyase (PAL), known to be involved in the defence system of *Citrus reticulata* to *A. woglumi*.

2 Materials and method

2.1 Experimental design

The experiment was conducted in a greenhouse. We used seedlings of mandarine (*Citrus reticulata* cv. 'Dancy') with the following treatments:

T_1: infestation with *A. woglumi* and no K_2SiO_3 (control, "C")
T_2: without *A. woglumi* and without K_2SiO_3 (absolute control "AC")
T_3: 17 g L^{-1} K_2SiO + *A. woglumi*
T_4: 35 g L^{-1} K_2SiO + *A. woglumi*
T_5: 52 g L^{-1} K_2SiO + *A. woglumi*
T_6: 70 g L^{-1} K_2SiO + *A. woglumi*.

In this study was used the randomised block design with four blocks and six treatments, and the experimental unit was made up with four citrus seedlings. Plants were used at six months of age. These plants were budded on *Citrus limonia* cv. 'Cravo'. Each plant received 20 mL of treatment solution, applied once manually with a sprinkler up to the point of dripping. The isolated effect of potassium was eliminated by balancing soil fertilisation with potassium sulphate (K_2SO_4).

Cages of galvanised iron were used to maintain mating insects. Each cage was with a screen (80 cm in length and 24 cm in diameter), with a total of 96 cages. The infestation of *A. woglumi* on citrus seedlings was performed five days after foliar application of potassium silicate with the release of 100 adult insects per plant. After 72 h of infestation, adult insects were removed. Leaves with eggs of *A. woglumi* were evaluated daily under a stereoscopic microscope, counting the developmental time (in days) of the nymphal phase of *A. woglumi* and comparing it to the enzymatic activity of peroxidase, polyphenol oxidase and phenylalanine ammonia-lyase (PAL).

2.2 Biochemical analyses

Biochemical analyses were performed at the laboratory. For the analyses, one leaf was removed from each mandarin seedling after 10, 30, 50, 70, and 90 days with continuous feeding by citrus blackfly adults. These samples were stored individually in aluminium foil, frozen in liquid nitrogen (N_2) and then stored at −20 °C before experimental analysis. Enzyme assays were performed in triplicate for each leaf extract.

2.2.1 Obtaining protein extracts

For the assessment of protein extracts 0.25 g of citrus leaves were weighed and mechanically homogenised in 4 mL of 100 mM sodium acetate buffer (pH 5.0), with the aid of a mortar. The homogenate was centrifuged at 12,000 rpm for 20 min at 4 °C, and the supernatant was used to determine the activity of peroxidase, polyphenoloxidase and PAL.

2.2.2 Peroxidase activity

Peroxidase activity was determined using a direct spectrophotometric method by measuring the conversion of guaiacol to tetraguaiacol at 470 nm [12]. The reaction mixture contained 0.01 mL of the enzyme extract with 0.25 mL guaiacol and 0.25 mL hydrogen peroxide in 0.75 mL of 0.1 M phosphate buffer (pH 6.0). The peroxidase activity is expressed as specific activity (absorbance units (a.u.) min^{-1} mg^{-1} protein).

2.2.3 Polyphenoloxidase activity

The activity of polyphenol was determined using the methodology proposed by Duangmal and Apenten [13]. The test consisted of measuring the oxidation of catechol converted into quinone, a reaction mediated by the enzyme in this study. The substrate was composed of catechol at a concentration of 20 mM dissolved in 100 mM sodium phosphate buffer (pH 6.8). The reaction was developed by mixing 0.25 mL of substrate with 0.5 mL of enzyme extract and 0.75 mL of reaction buffer. The reaction temperature was 40 °C for 15 min, and the reaction stopped after this time by adding 0.05 mL of 5 N HCl. Absorbance was read on a spectrophotometer at 420 nm. The results are expressed as a.u. min^{-1} mg^{-1} protein.

2.2.4 Phenylalanine ammonia-lyase (PAL) activity

The activity of PAL was determined by colorimetric quantification of trans-cinnamic acid released from the substrate phenylalanine [14]. The reaction mixture was incubated at 40 °C for 2 h with 0.5 mL enzyme extract, 1.5 mL of 25 mM Tris HCl (pH 8.8) and 0.5 mL substrate. The absorbance of the samples was determined at 290 nm *versus* extraction buffer, i.e. each value of the sample had the value of the control subtracted from it (this control corresponded to a mixture of 0.5 mL of the enzyme extract and 1.5 mL Tris HCl buffer). The reaction was stopped by adding 0.05 mL of 5 N HCl. Enzymatic activity is expressed in absorbance units: a.u. min^{-1} mg^{-1} protein.

2.2.5 Determination of soluble proteins

A volume of 50 µL standard solutions of the extracts was separated from the samples, then 50 µL distilled water and 1 mL of Bradford reagent were pipetted into a tube, mixed and incubated for 10 min. Readings were performed on a spectrophotometer at a wavelength of 595 nm.

Table I. Correlation coefficient of Spearman (r) involving the development time (in days) of the nymphal phase of *A. woglumi* with enzyme activity of peroxidase, polyphenol oxidase and phenylalanine ammonia lyase (PAL).

Parameter	Enzyme activity		
	Peroxidase	Polyphenol oxidase	PAL
Development (days)	0.9000	0.4000	0.5270
$P > r$[1]	0.0374*	0.5046[ns]	0.3615[ns]

[1]Probability values > correlation coefficient of Spearman; [ns] Spearman coefficient not significant; * Spearman coefficient significant.

2.3 Data analysis

Data for the development of the nymphal phase with the activity of enzymes (peroxidase, polyphenol oxidase and PAL) using doses of potassium silicate were analysed with the Spearman correlation (r) using PROC CORR [15]. A model was used to test the interaction involving the data on enzyme activity in response to time after infestation and in response to the dose of potassium silicate. Data were subsequently subjected to polynomial regression using PROC GLM [15]. For the comparison of enzymatic activity between the absolute control and the other treatments, Dunnett's test was used ($P = 0.05$).

3 Results and discussion

3.1 Correlation between enzyme activity and insect development

The correlation between peroxidase activity and the development of *A. woglum*i was positive (*table I*). Changes in peroxidase activity following treatment with elicitors may indicate the induction of resistance in plants [16], because one of the most rapid responses in plant cells following attack by pests is an oxidative burst of reactive oxygen species. In soybean, oxidative responses are increased with herbivory [17]. Peroxidases participate in various physiological processes, catalysing the oxidation and polymerisation of hydroxycinnamic alcohol in the presence of hydrogen peroxide, producing lignin, an important physical means of plant defence [18, 19], which contributes by strengthening the cell walls of the host.

The rigidity and pectin content of the middle lamella seem to play a role as a physical barrier to insect stylet penetration [20]. Sucking insects like *A. woglumi* are mainly phloem feeders and locating the sieve elements requires for them to avoid physical and chemical barriers. Silicon deposits on cell walls act as a mechanical barrier to stylet penetration. Non-preference and low reproduction of *Schizaphis graminum* (Rond.) (Hemiptera: Aphididae) were observed after the application of silicon on sorghum [21]. Silicon treatment did not induce a physical barrier in wheat plants because the stylet penetration of *S. graminus* was not affected by this treatment, but honeydew excretion was highly reduced indicating lower sap ingestion rate or greater sap retention inside the body [22].

There was no correlation between the activity of polyphenol oxidase and PAL in the development of *A. woglumi*. Therefore, the pattern of enzymatic activity with the development

Table II. Covariance[1] analyses of the effects of dose (*D*), time (*T*) and their interaction (*D***T*) in the activity of the following enzymes: peroxidase, polyphenol oxidase and phenylalanine ammonia lyase (PAL).

Model	Enzymes		
	Peroxidase	Polyphenol oxidase	PAL
Doses (*D*)	$F = 2.33$; $P = 0.0485$	$F = 6.00$; $P < 0.0001$	$F = 2.39$; $P = 0.0437$
Time (*T*)	$F = 29.53$; $P < 0.0001$	$F = 26.26$; $P < 0.0001$	$F = 16.28$; $P < 0.0001$
*D***T*	$F = 2.72$; $P = 0.0007$	$F = 2.49$; $P = 0.0018$	$F = 4.28$; $P < 0.0001$

[1] Analysis of covariance conducted using the ProcMixed [15].

ofcitrus blackfly depends on the enzyme. With other species of arthropods, induced defence depends on an enzyme complex. Similarly to the current study, damage caused by spider mites increased lipid peroxidation, lipoxygenase and peroxidase levels, but did not affect the activity of the antioxidant enzymes catalase and superoxide dismutase [23]

3.2 Effects of silicon dose on enzyme activity at different time points

3.2.1 Peroxidase activity

The silicon dose and the time point after infestation had an effect on the activity of the studied enzymes, and the interactions involving dose *versus* time for peroxidase, polyphenol oxidase and PAL were found to be significant (*table II*). There were different patterns of peroxidase, polyphenol oxidase and PAL activity in response to time after infestation of citrus blackfly for each dose of silicon used

In the case of peroxidase activity, the curves confirmed that there were differences in the response time after the application of potassium silicate (*figure 1*). The highest peaks of the activity of this enzyme were concentrated between 30 and 55 days after insect infestation and the highest activities were observed when the dose was 70 g L^{-1} (662.80 min^{-1} mg^{-1} protein) (*figure 1F*). The results show that silicon is not only involved in mechanical restraints against insect damage but also with biochemical changes, because there was a significant difference in peroxidase activity at 30 days after infestation when the different doses were compared with the absolute control over time. The dose of 17 g L^{-1} K_2SiO provided the highest enzyme activity compared to the absolute control (*figure 2B*) at 90 days. Doses of 17, 35 and 52 g L^{-1} K_2SiO caused the greatest enzymatic activity of the peroxidase enzyme in relation to the absolute control treatment (*figure 2E*). The highest activity of peroxidase was probably associated with a progressive incorporation of phenolic compounds within the cell wall, thus affecting the feeding of *A. woglumi*, besides this indicates that reduction on peroxidase activity remains capable of activating various mechanisms during the process of resistance induction so an additional application of potassium silicate may be required. This can also be associated with the factors of defence responses, such as the speed of expression, type and concentration of the plant elicitor used and the duration of the protective effect [24].

3.2.2 Polyphenol activity

The lowest activity of polyphenol oxidase for the absolute control treatment was estimated on the 85th day and the maximum was observed on the 33rd day (*figure 1A*). Similarly, for the treatments 0, 50 and 70 g L^{-1} the minimum activity was concentred between days 80-90, while for treatments at 17 and 35 g L^{-1}, the minimum polyphenol oxidase activity was estimated on the 10th day (*figure 1B–1E*). In general the maximum activity was estimated between days 30–40. Therefore, the curves of enzyme activity indicate that there were differences in the response time after the application of potassium silicate for polyphenol oxidase (*figure 1*). The highest enzymatic activity of polyphenol oxidase occurred between day 31 and 59, suggesting that increased activity of this enzyme is an event associated with the induction of resistance in *C. reticulata* to *A. woglumi* as the plant catalyses the oxidation of phenolic compounds, thus decreasing the quality of leaf tissue and reducing protein digestibility, thereby harming the insect. Besides being involved in the lignification process, polyphenol is also responsible for catalysing the oxidation of phenols to quinones, which become complexed with proteins, thus decreasing the nutritional quality of leaf tissue making it difficult to digest proteins [25, 26]. However, the activity of polyphenol oxidase differed among all the doses of silicon used, including the control (*C*) (0 g L^{-1}) on the 30th day after infestation, revealing lower enzyme activity compared to the absolute control treatment (*figure 2B*). The same was observed on the 50th day for the absolute control, 17, 35 and 70 g L^{-1} (*figure 2C*) and 52 g L^{-1} on the 70th day (*figure 2D*).

3.2.3 PAL activity

The estimation of the maximum activities of PAL were concentrated on time points within 10 days with infestation. With application of the treatment at 52 g L^{-1} K_2SiO, PAL activity declined to levels close to zero (day 73). Therefore, these results indicate that PAL did not induce any defence response in the plant (*figure 1*). Similar results to those found in the present study were found pre-infestation with aphids and/or with the application of silicon-induced defence responses in wheat; however, increased activities of peroxidase and polyphenol were also found that triggered the processes of plant defence, thus acting as an inductor. In regard to phenylalanine, no effect of silicon as an activator of this enzyme was observed [8]. However, this enzyme is of the great importance in some physiological pathways in other crops. As observed in this study, a pattern of phenylalanine activity that was dependent on time was observed in strawberry. Furthermore, strawberry fruit show development-dependent expression of phenylalanine activity and accumulation of phenolic substances derived from the phenylpropanoid pathway [27].

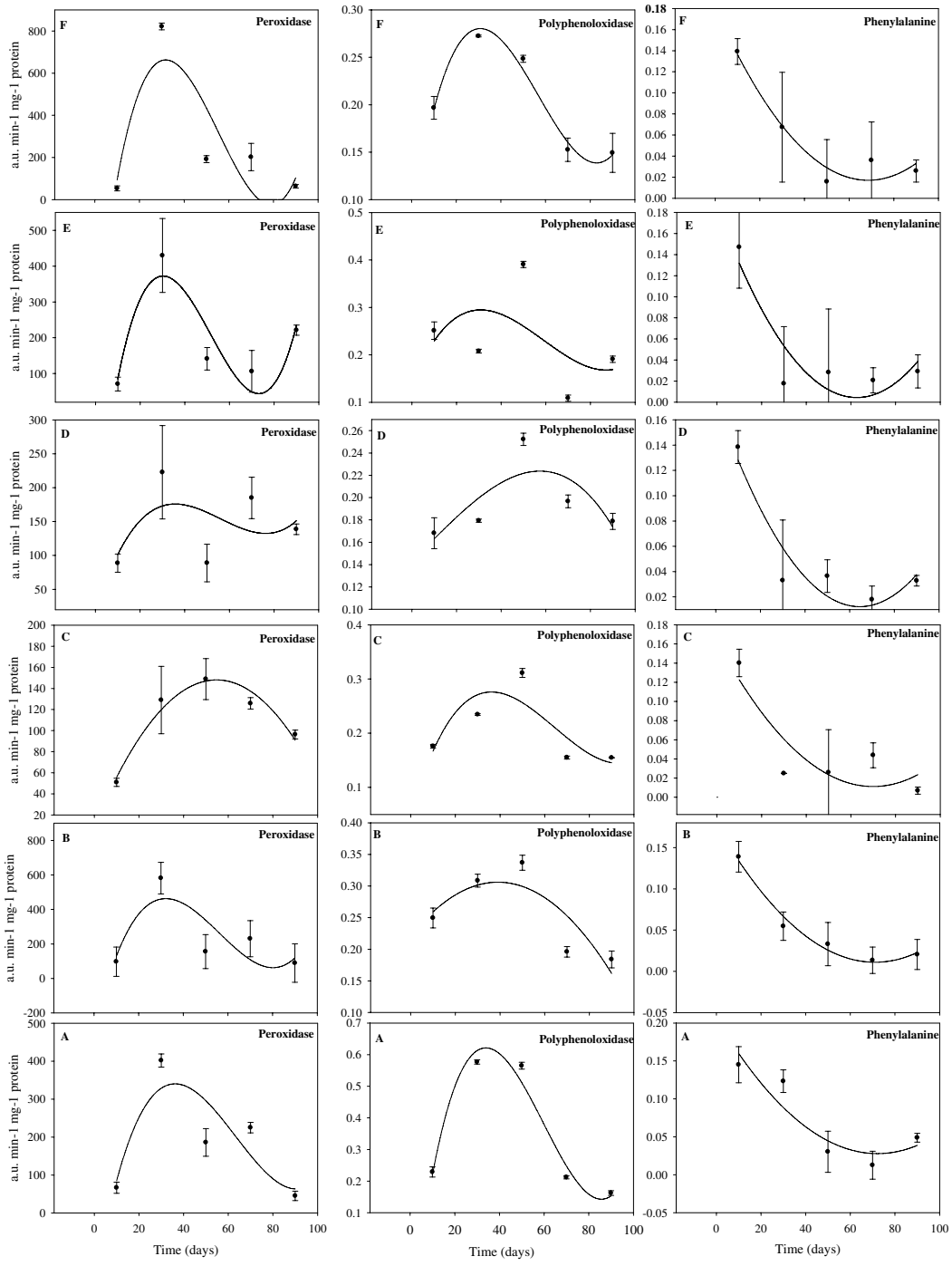

Figure 1. Activity of peroxidase, polyphenoloxidase and phenylalanine ammonia lyase (PAL) in response to the time after application of doses of potassium silicates: A = Absolute control; B = 0 g L^{-1}; C = 17 g L^{-1}; D = 35 g L^{-1}; E = 52 g L^{-1} and F = 70 g L^{-1}.

The activity of phenylalanine in treatments with doses 0 and 35 g L^{-1} K_2SiO differed from the absolute control, with lower enzymatic activities recorded at 10 days after infestation of *A. woglumi* (*figure 2A*). At 30 days, the activity at doses of 17 and 52 g L^{-1} K_2SiO differed significantly from the control treatment (*figure 2B*). The same effect was observed at 90 days when silicon was applied at a dose of 17 g L^{-1} K_2SiO (*figure 2E*) compared to the absolute control.

There is a wide range of silicon effects on plant species; this variation can be mainly attributed to differences in the specific characteristics of Si uptake and transport. Active Si uptake has been demonstrated in gramineous species [28]; however, some of these species, such as oats take up silicon passively [29]. Passive Si uptake has been demonstrated in a few dicots [28], and the molecular mechanisms underlying Si uptake in *Citrus* plants are poorly known. The results of

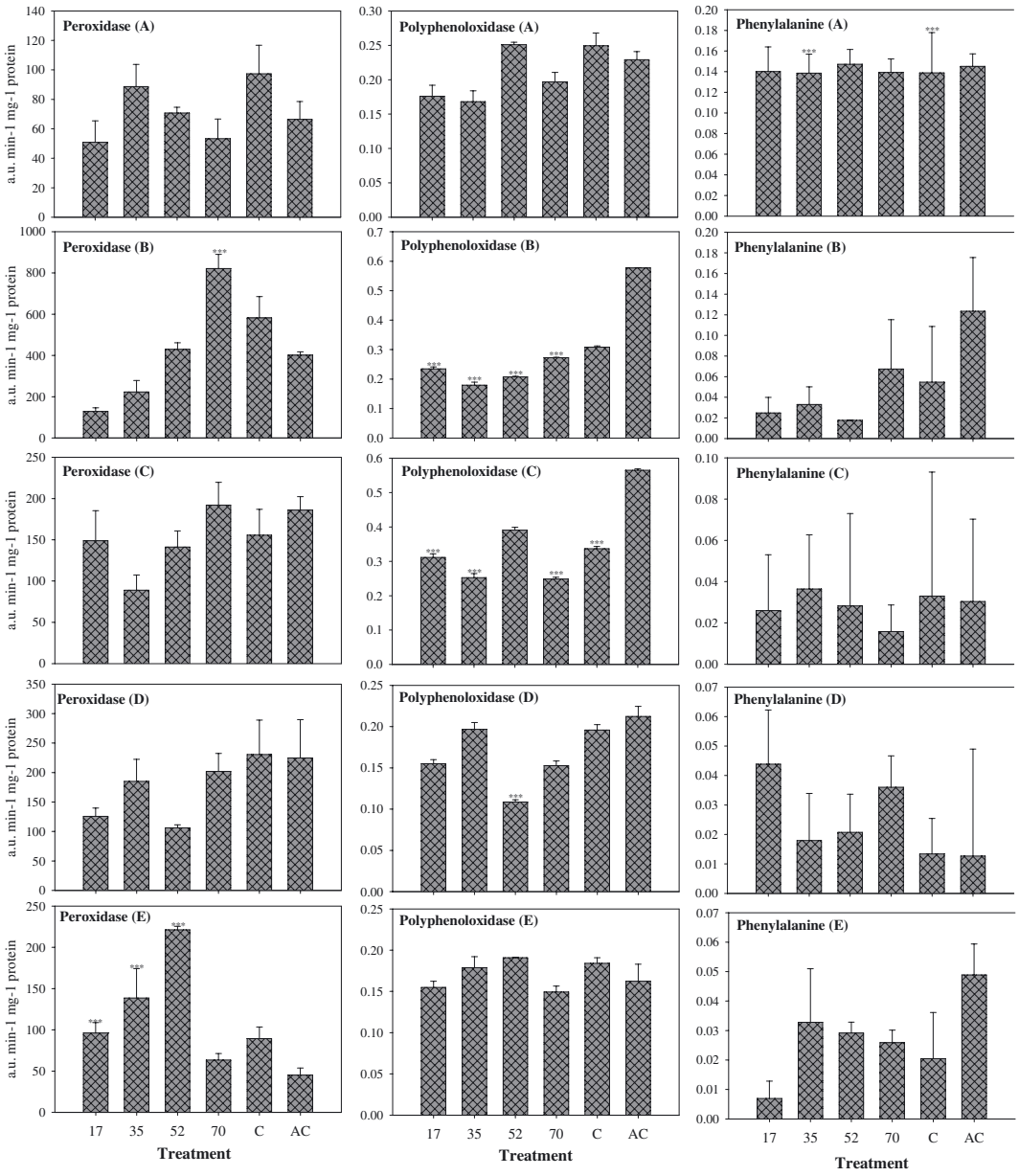

Figure 2. Peroxidase, polyphenoloxidase and phenylalanine ammonia lyase (PAL) activity as response to dose of potassium silicate (17 g L^{-1}; 35 g L^{-1}; 52 g L^{-1}, 70 g L^{-1}, AC = absolute control, C = 0 g L^{-1} with *A. woglum*i) after infestation respectively in the following time periods: A = 10 day; B = 30 days; C = 50 days; D = 70 days and E = 90 days. Bars represent the mean ś standard error. Means followed by asterisks (***) indicate significant difference compared with the absolute control by Dunnett's test (P = 0.05).

the current study reveal that peroxidase and polyphenol are probably involved in the activation of defence mechanisms of *C. reticulate* against *A. woglumi* after the use of silicon because there was a significant increase in the enzymatic activity of these enzymes. The role of exogenous silicon is of great importance because it can act as a modulator influencing the timing and extent of plant defence responses in a manner reminiscent of the role of secondary messengers in induced systemic resistance. It can also bind to hydroxyl groups of proteins strategically involved in signal transduction, and it can interfere with cationic co-factors of enzymes influencing pathogenesis-related events [4]. In addition there is evidence showing that silicon enhances plant resistance to various abiotic stressors such as salinity, drought, metal toxicity and ultraviolet radiation [3].

4 Conclusion

Potassium silicate is an elicitor that potentiates the defence mechanisms of *C. reticulate* against *A. woglumi*. The increase in peroxidase and polyphenoloxidase activities revealed the induction of compound synthesis for plant defence against *A. woglumi,* but this effect was dependent on the time of *A. woglumi* feeding and on the concentration of silicon.

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