

Effects of silicon on biochemical and physiological aspects in *Theobroma cacao* under inoculation with *Moniliophthora perniciosa*

Efeitos do silício sobre os aspectos bioquímicos e fisiológicos de *Theobroma cacao* inoculadas com *Moniliophthora perniciosa*

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ABSTRACT

This study aimed to verify the efficiency of silicon leaf application in the physiological and biochemical characteristics in a *Theobroma cacao* genotype inoculated with *Moniliophthora perniciosa* fungus, which causes the witches' broom disease, important cocoa disease in Brazil and worldwide. Seedlings of Catongo genotype were used. The experimental design used was a randomized block design with four replicates, including two inoculation factors (inoculated and non-inoculated plants), each one with two levels of silicon application (0.0 mg and 1.0 mg mL⁻¹ with AgriSil containing 98% of SiO₂). We observed that plants that were inoculated with *M. perniciosa* and subjected to leaf application with silicon presented enhanced photosynthesis, peroxidases, chitinase, polyphenoloxidases activities and stomatal density. Results have shown that silicon application is beneficial and increases the defenses against *Moniliophthora perniciosa* in cocoa seedlings.

Keywords: chitinase, cocoa tree, cocoa farming, gas exchange, witches' broom.

RESUMO

Objetivou-se avaliar a eficácia da aplicação foliar de silício nos aspectos fisiológicos e bioquímicos em um genótipo de *Theobroma cacao* inoculado com *Moniliophthora perniciosa*, fungo causador da vassoura-de-bruxa, importante doença da cacauicultura. Mudanças do genótipo Catongo foram submetidas a um experimento com dois fatores (inoculação e não inoculação das plantas com *M. perniciosa*) cada um com dois níveis de Si (0.0 mg e 1.0 mg mL⁻¹ de AgriSil contendo 98% de SiO₂ aplicado por pulverização). Verificou-se que plantas inoculadas e submetidas à aplicação foliar com Si apresentaram aumento na fotossíntese, das atividades de peroxidases, quitinases e polifenoloxidases e da densidade estomática. Os resultados indicam que a aplicação de silício é benéfica aumentando as defesas contra *Moniliophthora perniciosa* em mudas de cacauzeiros.

Palavras-chave: quitinase, cacauzeiro, cacauicultura, trocas gasosas, vassoura-de-bruxa.

INTRODUCTION

An alternative that has been used in disease management is induction of resistance (Bettiol, 1991) through the activation of the plant's latent resistance mechanisms. Such mechanisms might

be structural, such as lignification and papilla formation, or biochemical, such as chitinases accumulation and other proteins related to pathogenesis (Pascholati and Leite, 1994). The induction of resistance might be obtained through treatments that use silicon-based products, which besides

being effective in the protection against some diseases, also improve the plant's nutritional aspect, increasing the production and quality of agricultural products.

Existing studies on silicate leaf fertilization have pointed out the potential of silicon to increase resistance against pathogens (Carré-Missio *et al.*, 2012; Catalani *et al.*, 2016). Silicon is placed on the leaves in the form of amorphous silica on the epidermis, creating a mechanical resistance barrier against fungi getting into the plant interior after its polymerization on the leaf surface (Korndörfer *et al.*, 2004). In addition to that, silicon provides an induced active protection, beginning a sequence of defense biochemical reactions in the plant, which leads to peroxidase and polyphenoloxidase production (precursors of synthesis compounds of the plant defense) (Gomes *et al.*, 2008), besides accumulating chitinases, which create a structure able to protect and resist to microbial attack (Epstein, 1999).

Peroxidases are enzymes that polymerize monomers used in the formation of lignin, which along with other polysaccharides found in the cell wall, work as a physical barrier to fungi penetration (Vance *et al.*, 1980; Cai *et al.*, 2006). Chitinases, in turn, are lytic enzymes that hydrolyze the chitin, one of the main components of fungus cell wall. These enzymes release inductors through the pathogen's cell wall, allowing the plant to detect it and activate the defenses (Pascholati and Leite, 1994).

Plants infected by pathogens usually have their photosynthesis reduced, presenting chlorosis and necrosis, which interfere in the photosynthetic area and in pigment quantity (Agrios, 2005). Fertilization with Si improves leaf architecture so as to allow a higher sunlight penetration, CO₂ absorption and reduction of excessive transpiration, promoting increased photosynthetic activities (Gomes *et al.*, 2008).

Witches' broom, caused by *Moniliophthora perniciosa* fungus is one of cocoa's (*Theobroma cacao*) most destructive diseases, characterized by overgrowth of leaf releases, with proliferation of lateral buds and the swelling of infected tissues, this can cause losses of nearly 90% of the production (Evans, 1981).

Cocoa tree is a perennial species found in low lands in moist forests under the protection of tallest trees, as well as in forests of different altitudes, with less moisture, and with lower density trees (Almeida and Valle, 2007). Cocoa fruit seeds represent the main importance in cocoa growing, since they are used for the chocolate production, pharmaceutical products and cosmetics (Menezes and Carmo-Neto, 1993).

Based on the above, the aim of this study was to verify the efficiency of silicon leaf application in *Theobroma cacao*'s seedlings, Catongo genotype, inoculated with *Moniliophthora perniciosa* fungus, through morphophysiological and biochemical analysis.

MATERIAL AND METHODS

Seminal seedlings of *Theobroma cacao* L. Catongo genotype were planted in tubes containing Plantmax® substrate and grown in greenhouse and after 40 days, were underwent silicon treatment. The solution applications were performed in the adaxial and abaxial surfaces of the leaves. Around 6mL were applied per plant up to the point of running off, four applications in total, with intervals of 15 days and performed before inoculation.

The inoculation was performed in a humidified chamber, applying a suspension of 30 µL of 2x10⁵ viable basidiospores of *Moniliophthora perniciosa* (isolated CPK CEPEC/CEPLAC-Cocoa Research Center/ Cocoa Cultivation Executive Commission), on the 90-days-seedlings' apical meristem. Then the plants were maintained in humidity chamber during 36 h. For the basidiospores viability test, Petri dish with water agar culture medium was used. After being inoculated with the deposition of 4 drops of basidiospores suspension (120 µL), the dishes were kept in a humidified chamber and in incubators (25°C) for 24 hours and afterwards the counting of germinated spores was performed, dividing the dish in 4 quarters (Silva *et al.*, 2000). At 45 days after inoculation, the incidence of disease was determined by visual evaluation of symptoms in the aerial part of seedlings.

For the silicon leaf content, the leaves were washed with distilled water and dried in kiln at 70°C, for 72

hours and milled, in accordance with Korndörfer *et al.* (2004). The reading was performed in spectrophotometer (ThermoScientific®, Genesys 10S, US) at a wavelength of 410 nm. For the measurement, a standard curve was performed from a 1000ppm standard solution of Si, obtained with pure sodium silicate.

The gas exchange measurements were performed in fully expanded 135-days-seedlings' leaves, with the use of atmospheric CO₂ concentration and room temperature. The measures were obtained in closed system using a portable infrared gas analyzer *LI-6400 portable photosynthesis system* (LI-COR, Nebraska, US), in irradiance 1000 μmol photons m⁻² s⁻¹. The following variables were evaluated: CO₂ assimilation (*A*, μmol CO₂ m⁻² s⁻¹), transpiration (*E*, mol H₂O m⁻² s⁻¹), stomatal conductance (*g_s*, mol H₂O m⁻² s⁻¹) and intercellular CO₂ concentration (*C_i*, μmol CO₂ mol ar⁻¹).

For the extraction of total protein and determination of enzymatic activity, the extract was obtained from the same leaves used for the analysis of gas exchanges. 0.4g of foliar surface was homogenized in potassium phosphate buffer 100mM (pH 6.8), EDTA-Na₂ 0.1mM and polyvinylpyrrolidone (PVPP) 1% (p/v), using chilled mortar and pestle. The homogenized was centrifuged to 12000x g for 15min at 4°C (Parida *et al.*, 2004). The supernatant was used for measuring total protein and polyphenoloxidase, chitinase and peroxidase enzyme assay activities.

Total proteins quantification was performed in accordance with Bradford (1976). 100 μL of the extract supernatant was mixed with 2000 μL of Bradford Reagent. After 5 minutes, the absorption was determined at 595 nm in spectrophotometer (ThermoScientific®, Genesys 10S) and the results were expressed as mg of protein/g fresh tissue (mg ptn/g MF), containing bovine serum albumin as standard protein.

The reaction of peroxidase activity of guaiacol (POD) was performed in accordance with Cakmak *et al.* (1993), with modifications. We used 10 μL of extract and 200 μL of sodium phosphate buffer (100 mM, pH 6.8) with guaiacol 20 mM and 100 μL of hydrogen peroxide 20 mM. The absorption increase due to guaiacol oxidation was registered

at 470 nm after 2 minutes reaction using spectrophotometer ThermoScientific®, Genesys 10S. The results were expressed in absorption units/min/mg of protein, using extinction coefficient of H₂O₂ with value 26.6 mM⁻¹ cm⁻¹ (μmol H₂O₂/min/mg ptn).

Polyphenoloxidase (PPO) activity was obtained through the conversion of catechol into quinone in compliance with Cañal *et al.* (1988), with modifications, using 20 μL of extract, 1 mL of catechol 20 mM and 1 mL of sodium phosphate buffer 100 mM pH 6.8. After 2 minutes of reaction, a reading at 420 nm in spectrophotometer ThermoScientific®, Genesys 10S was carried out. The results were expressed in absorption units/min/mg of protein (UA/min/mg ptn).

Chitinase activity (CHI) was determined by the release of soluble fragment of carboxymethylated chitin marked with remazol brilliant blue ("Chitin azure" – SIGMA), according Stangarlin *et al.* (2000), with modifications. We used 150 μL of extract, 650 μL of sodium acetate buffer 50 mM pH and 200 μL of "Chitin azure". After incubation at 40°C for 20min, the reaction was stopped with 200 μL de HCl 1M and afterwards, centrifuged at 10000 g/10 min. at 4°C. The absorption was determined at 550nm through spectrophotometric analysis (spectrophotometer ThermoScientific®, Genesys 10S). The results were expressed in absorbance/min/mg unit protein (AU/min/mg ptn).

The determination of stomatal density (n°/mm²) was performed through the epidermal impression of abaxial leaf surface in microscope glass slide with instant adhesive (Super Bonder®). The observations and photographic documentation were made in photomicroscope Eclipse 50i, (Nikon, Japan). The quantitative analyses were performed with the use of TSview image analysis software.

The experimental design used was randomized block (RBD), with four replicates, in 2x2 factorial design, including two inoculation factors (inoculated and non-inoculated plants), each one with two levels of silicon application: 0.0 mg Si (water sprayed control) and 1.0 mg mL⁻¹ with AgriSil containing 98% of SiO₂, resulting in 16 treatments with 10 plants each. The data collected went through analysis of variance (ANOVA) and the means were compared via Turkey's test at 5. All

analysis was carried out using Assisat 7.6 beta (2012) program, UAEG-CTRN-UFCC Campina Grande – PB.

RESULTS AND DISCUSSION

Inoculated plants presented brooms, as a consequence of hypertrophy of the plants issues, loss of apical dominance and proliferation of axillary shoots, resulting in the formation of abnormal stems, characteristic of green brooms.

The beneficial relation of silicon against pathogens is reported in studies with different vegetal species (Zambolim *et al.*, 2009; Carré-Missio *et al.*, 2012; Guerra *et al.*, 2014; Polanco *et al.*, 2014; Santos *et al.*, 2014; Catalani *et al.*, 2016). There was statistical interaction between inoculation and silicon application factors to all variables analyzed, except for Si leaf content (Table 1) and the internal carbon dioxide concentration (Table 2).

Silicon application caused increase of photosynthesis in all plants. Without Si application, inoculated plants presented lower photosynthesis and stomatal conductance values compared to the non-inoculated plants (Table 2). The increase in the gas exchange values due to Si application points out the benefit of this element concerning photosynthesis (Hattori *et al.*, 2005; Carvalho *et al.*, 2008; Zanão Júnior, 2009). Silicon fosters improvement in plants' architecture, optimizing sunlight interception and resulting in a better efficiency in CO₂ assimilation (Gomes *et al.*, 2008; Souza *et al.*, 2010).

Silicon has influenced the increase in stomatal conductance only for non-inoculated plants and there was no statistical difference for inoculated plants (Table 2). The lower photosynthetic activity in the inoculated plants studied reinforces the influence that pathogens have on plants. The stomatal conductance reduction; the toxin production by pathogens; and the hormonal imbalance, among other things, compromise the photochemical and biochemical stages of photosynthesis (Daly, 1976; Machado *et al.*, 2006). This is possibly due to the lower radiation interception, because of photosynthetic leaf area loss, resulting from damage caused by pathogen in the leaf structure and biochemical reactions.

For transpiration, Si application has caused value decrease only for inoculated plants, no difference was observed for the non-inoculated ones (Table 2). The higher transpiration found in inoculated plants without Si application might be associated with the cuticle destruction, increase in permeability of the leaf cell membrane and inhibition of stomata closure (Duniway and Durbin, 1971).

With Si application, there was increase in POD and PPO activities in inoculated plants and PPO reduction in non-inoculated plants. For CHI, Si has tripled this enzyme's activity in inoculated plants and increased nearly tenfold the activity

Table 1 - Silicon leaf content in *T. cacao* plants under influence of inoculation with *M. perniciosa* and treated with silicon

Treatments	SiO ₂ (mg mL ⁻¹)	
	0.00	1.00
Inoculated	0.82	0.76 ns
Non-inoculated	0.95	0.96

Averages followed by the same letter in the column do not differ statistically by Tukey test at 5% probability. ns - non-significant at 5% probability.

Table 2 - Parameters of gas exchanges in *T. cacao* plants under influence of inoculation with *M. perniciosa* and treated with silicon

Treatments	SiO ₂ (mg mL ⁻¹)	
	0.00	1.00
	A (μmol CO ₂ m ² s ⁻¹)	
Inoculated	2.65 bB	4.72 bA
Non-inoculated	4.84 aB	5.90 aA
	E (mol H ₂ O m ² s ⁻¹)	
Inoculated	0.51 aA	0.34 bB
Non-inoculated	0.44 aA	0.46 aA
	g _s (mol H ₂ O m ² s ⁻¹)	
Inoculated	0.05 bA	0.07 bA
Non-inoculated	0.11 aB	0.22 aA
	Ci (μmol CO ₂ mol ar ⁻¹)	
Inoculated	221.00	294.00 ns
Non-inoculated	307.00	339.00

Averages followed by the same by the same small letter in the column and capital letter in the line do not differ statistically by Tukey test at 5% probability. ns - non-significant at 5% probability. A: photosynthesis; g_s: stomatal conductance; E: Transpiration; Ci: CO₂ internal concentration.

in non-inoculated plants. Without Si application, inoculated plants presented lower POD and PPO activities when compared to non-inoculated plants. Whereas for CHI, the highest activity was observed in inoculated plants (Table 3).

The increase of POD, PPO and CHI activities, observed in the treatments with Si confirm once more the action of this element as a promoter of increase resistance in infected plants, as reported in other studies (Silva *et al.*, 2008; Carré-Missio *et al.*, 2012; Soratto *et al.*, 2012). Silicon affects the signs between host and pathogen, activating defense organisms more quickly and extensively, acting as elicitor of the induced resistance process. Thus, Si increases transcription in some genes that codify POD and PPO, in addition to increasing lytic CHI activity, fungal cell wall (Datnoff *et al.*, 2007).

As far as POD activity is concerned, we expected to find higher values in inoculated plants, different from what was observed in this study. The higher POD activity in inoculated plants has possibly occurred in the beginning of the infection and reduced in the period in which its evaluation was conducted, at the end of the experiment. Boudjeko *et al.* (2005) observed higher POD activity two days after the inoculation, with subsequent activity reduction. Peroxidases act mainly as indicators of beginning of resistance response evolution than as elements dedicated to execute the defense (Asada, 1992). This POD increase over time might also be

resulting from leaf maturation. Due to the fact that these enzymes are involved in the formation of plant cell wall, specially leaves, having their activity reduced with complete leaf expansion (Macagnan *et al.*, 2008).

Although PPO activity is expected to improve plant resistance to stress, being increased in tissues infected by pathogens, its low activity, as observed in the present study, points out little level of cellular degradation, as observed by Farah and Donangelo (2006). PPO is found in latent forms in chloroplast and mitochondria membranes, having no contact with phenolic compounds, contained in the vacuoles. When there is chloroplast and vacuoles rupture, PPO does not oxidize the phenols (Yoruk and Marshall, 2003).

The highest chitinase enzyme activity in inoculated plants is a previously reported in cocoa trees (Macagnan *et al.*, 2008) and in other inoculated plants (Agrios, 2005; Campos *et al.*, 2009). Since they are enzymes related to pathogenesis, peroxidases and chitinases, they are activated by SAR (systemic acquired resistance), which is induced by pathogens or chemical inductors (Siegrist *et al.*, 2000). The accumulation and synthesis of chitinase in the plant tissues have also been frequently associated to defense mechanisms against diseases, which might be triggered by pathogens, metabolites derived from microorganisms or chemicals that act as resistance inductors (Roulin and Buchala, 1995).

Silicon has allowed density increase in inoculated and non-inoculated plants. In treatments without Si, inoculated plants presented higher stomatal density compared with those non-inoculated (Table 4). Higher stomatal density is reported as

Table 3 - Activity of enzymes POD, PPO and CHI in *T. cacao* leaves under influence of inoculation with *M. perniciosa* and treated with silicon

Treatments	SiO ₂ (mg mL ⁻¹)	
	0.00	1.00
	POD (mmol min ⁻¹ mg protein ⁻¹)	
Inoculated	0.15 bB	0.38 aA
Non-inoculated	0.26 aA	0.34 aA
	PPO (U min ⁻¹ mg protein ⁻¹)	
Inoculated	0.004 bB	0.049 aA
Non-inoculated	0.048 aA	0.026 bB
	CHI (U mg protein ⁻¹)	
Inoculated	1.07 aB	2.93 aA
Non-inoculated	0.17 bB	1.59 bA

Averages followed by the same by the same small letter in the column and capital letter in the line do not differ statistically by Tukey test at 5% probability.

Table 4 - Average values of stomatal density in *T. cacao* leaves under influence of inoculation with *M. perniciosa* and treated with silicon

Treatments	SiO ₂ (mg mL ⁻¹)	
	0.00	1.00
Inoculated	103.00 aB	114.00 aA
Non-inoculated	85.00 bB	90.00 bA

Averages followed by the same small letter in the column and capital letter in the line do not differ statistically by Tukey test at 5% probability.

a characteristic of resistance to diseases (Grewal *et al.*, 1999) and, along with silicon accumulation, it represents morphological changes that might occur and affect the host-pathogen interactions (Chakraborty *et al.*, 2000).

Silicon application did not affect Si leaf content in the studied specie. Similar results were also reported by other genotype of cocoa tree (Zanetti *et al.*, 2016) and other vegetal species infected by fungi (Zambolim *et al.*, 2009; Zano Junior, 2009; Carré-Missio *et al.*, 2012). In all treatments, the plants had Si content lower than 1%. Although Pinto *et al.* (2011) has observed content over 1% in Catongo leaves, according to which the plant was categorized as silicon accumulator (Ma *et al.*, 2001), the results obtained in the present study do not confirm these data.

CONCLUSION

Silicon leaf application contributed to assuage the effects caused by witches' broom disease in *T. cacao* allowing photosynthetic activity increase as well as increase in chitinase, polyphenoloxidase and peroxidase enzymes in inoculated plants.

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