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Infection process of *Bipolaris sorokiniana* on wheat leaves is affected by silicon

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ABSTRACT

The supply of silicon (Si) to wheat plants may potentiate their resistance against infection by *Bipolaris sorokiniana*. Considering that there is no information regarding how Si affects the infectious process of this fungus on wheat leaves, this study intended to investigate, using light and scanning electron microscopy, the effects of Si supply on the infection process of *B. sorokiniana* on wheat leaves at tissue level. For that, leaf segments of -Si and +Si plants were prepared. The number of necrotic brown cells and the degree of browning of necrotic cells were determined for each infection site. As results, it was found that the number of brown epidermal cells and the frequency of infection sites showing browning were significantly lower on leaves of +Si plants. On the leaves of -Si plants, an intense dissolution of the wax layer also occurred and hyphae were found inside the disorganized epidermal cells. On leaves of +Si plants, degradation of the wax layer was observed only in areas close to conidia. Within 96 hours after inoculation, a dense network of hyphae had colonized the leaf surface of -Si plants, in striking contrast to +Si plants, for which only sparse hyphae were found.
Key words: light microscopy, SEM, spot blotch.

Spot blotch, a disease caused by *Bipolaris sorokiniana* (Sacc.) Shoemaker [teleomorph: *Cochliobolus sativus* (Ito & Kuribayashi) Drechs. Ex Dastur], affects wheat plants worldwide (Misra, 1979). The process by which *B. sorokiniana* infects the plant starts with conidium germination on the leaf surface. A rudimentary appressorium forms from the apex of the germ tube and directly penetrates the cuticle and epidermal cell wall via infection hyphae that colonize the inter- and intracellular leaf tissue (Kumar et al., 2002). *Bipolaris sorokiniana* produces non-selective toxins and several hydrolytic enzymes, which quickly destroy the leaf tissue to generate nutrients for continued fungal growth (Gayad, 1961). Helminthosporol, the major non-specific toxin produced by *B. sorokiniana*, affects the permeability of the leaf cell plasma membrane so that the fungus can nourish of the electrolytes leaked and then to grow and colonize the host tissue (Briquet et al., 1998; Wisniewska et al., 1998).

Although wheat breeding programs have made important advances in recent years in creating cultivars resistant to spot blotch, epidemics of the disease continue to cause reductions in yield (Duveiller & Sharma, 2008). Silicon (Si) application has been shown to be a viable method of enhancing the resistance of several plant species to root and foliar pathogens (Datnoff et al., 2007). According to Yoshida et al. (1962), a thick layer of silica formed beneath the cuticle of rice epidermal cells when the monosilicic acid in the plant was polymerized. Kim et al. (2002) demonstrated that the double layer of cuticle Si can decrease the capacity of *Pyricularia grisea* appressoria to penetrate the rice leaf epidermis and, consequently, decrease the number of lesions formed on the leaf blades. It is known that rice plants supplied with Si respond more efficiently against *P. grisea* infection through an increase in the production of phenolic-like compounds and phytoalexins belonging to the diterpenoids class, in addition to the strong activation of some PR-genes (Rodrigues et al., 2004, 2005). As wheat plants can contain up to 1.5 dag kg^{-1} of Si (dry weight basis) in their shoots, Si amendment may contribute to enhanced host resistance against pathogens attack (Bélanger et al., 2003; Xavier Filha et al., 2011; Guevel et al., 2007). Until recently, few studies have demonstrated the potential of Si to reduce the intensity of wheat diseases (Datnoff et al., 2007; Domiciano et al., 2010; Xavier Filha et al., 2011). According to Domiciano et al. (2010), the area under the spot blotch progress curve decreased and the activities of chitinases and peroxidades was higher for leaves of wheat plants that had been supplied with Si. Studies showing the effect of Si on the infection process of *B. sorokiniana* in wheat leaves are, to the best of our knowledge, missing. Therefore, the current study investigated the effect of Si supply on the infection process of *B. sorokiniana* on the leaves of wheat plants using light and scanning electron microscopy.

For that, an experiment was arranged in a completely randomized design consisting of two treatments (-Si and +Si plants) and seven replications. Each replication consisted of one plastic pot containing 1 kg of soil and two plants. The soil type used in the experiment was a Si-deficient typical Acrustox red yellow latosol collected at "Triangulo Mineiro" savanna area with 530 g kg^{-1} of clay; pH in KCl = 4.8; P (Mehlich-1) = 0.5 g

dm^{-3}; K (Mehlich-1) = 13 mg dm^{-3}; Al^{3+}, Ca^{2+}, Mg^{2+} and $H+Al^{3+}$ = 0.1, 0.0, 0.0 and 3.8 $cmol_c$ dm^{-3}, respectively; base saturation = 2% and organic matter = 2.3 dag kg^{-1}. The concentration of available Si (extraction in $CaCl_2$) was 11.8 mg dm^{-3}. Plastic pots were filled with 1 kg of air-dried, sieved (5 mm) soil. The mineral wollastonite used as the Si source (calcium silicate; Vansil, EW-20; Ipiranga Chemical Co.) is composed of 24.2% Si and 31% Ca. Wollastonite was incorporated into each pot at the rates of 0 and 1.25 g kg^{-1} of soil, which corresponded, respectively, to 0 and 0.3 g of elemental Si per pot. Calcium carbonate (40% Ca; Sigma Aldrich) was added, at the rate of 0.97 g kg^{-1} of soil, to equilibrate the amount of Ca in the treatment that did not receive 1.25 g of wollastonite. In this way, the amount of Ca in both treatments was fixed at 0.39 g pot^{-1}. Before sowing, the soil in each pot was incubated for 60 days with humidity fixed at approximately 65%.

Wheat seeds from the cultivar BR-18, which is susceptible to *B. sorokiniana*, were surface sterilized in 10% (v/v) NaOCl for 1.5 min, rinsed in sterilized water for 3 min and sowed at the rate of four seeds per pot. Five days after the seedlings emerged, each pot was thinned to two plants. The soil in each pot was fertilized before sowing with 1.63 g of calcium phosphate per kg of soil and with 30 mL of a nutrient solution containing in g l^{-1}: 6.4 KCl; 3.48 K_2SO_4; 5.01 $MgSO_4.7H_2O$; 2.03 $(NH_2)_2CO$; 0.009 $NH_4MO_7O_{24}.4H_2O$; 0.054 H_3BO_3; 0.222 $ZnSO_4.7H_2O$; 0.058 $CuSO_4.5H_2O$; and 0.137 $MnCl_2.4H_2O$ (Domiciano et al., 2010). The nutrient solution was applied every week after seedling emergence. A volume of 15 mL of a solution containing 0.27 g $FeSO_4.7H_2O$ and 0.37 g of EDTA bisodic l^{-1} was also applied after the seedlings emerged, and re-applied when the plants were 35 days old (growth stage 35) (Zadoks et al., 1974). The plants were watered as needed.

A pathogenic isolate of *B. sorokiniana* (UFV-DFP-01) obtained from symptomatic wheat leaves was used to inoculate the plants. This isolate was preserved in glass vials containing autoclaved wheat seeds. Infested seeds were transferred to Petri dishes containing potato-dextrose-agar (PDA) medium. After three days, PDA plugs containing fungal mycelia were transferred to new Petri dishes containing PDA media. These Petri dishes were kept in a growth chamber at 25°C with a 12-hour photoperiod for seven days. After this period, the Petri dishes were washed with a sterile water solution containing gelatin (1%, w/v) and scraped with a rubber policeman to remove mycelia and conidia. Plants were inoculated with a conidial suspension of *B. sorokiniana* (2×10^4 conidia mL^{-1}) 45 days after emergence (growth stage 45; seven leaves) (Zadoks et al., 1974). A volume of 20 mL of suspension was applied as a fine mist to the adaxial leaf blades of each plant using a VL Airbrush atomizer (Paasche Air-brush Co.). Gelatin (1%, w/v) was added to the sterile water to aid conidial adhesion to the leaf blades. Immediately after inoculation, plants were transferred to a mist chamber at 25 ± 2ºC, relative humidity of 90 ± 2%, and an initial 12-hour dark period. After this 12-hour period, plants received a 12-hour photoperiod of 225 $\mu E/m^2/s$ provided by cool-white fluorescent lamps. The plants were kept inside the mist chamber until the end of the experiment.

Spot blotch severity was assessed in all leaves of the main tiller of each plant per replication and treatment at 12, 24, 36, 48, 72 and 96 hours after inoculation (hai) by using a scale based on the percentage of the leaf showing disease symptoms (IRRI, 1996). The area under the spot blotch progress curve (AUSBPC) for each leaf in each plant was computed using the trapezoidal integration of the spot blotch progress curve over time according to the formula proposed by Shaner & Finney (1977).

For the light microscopy analyses, at each time point of 12, 24, 36 and 48 hai, the 5th and 6th leaves of one plant of each treatment was collected. Therefore, all light microscopy analyses relied on four plants per treatment. Each collected leaf was cut into 50 pieces of 0.25 cm^2 (only at the last sampling time the leaves were cut into 60 instead of 50 pieces due to need of experimental material for the scanning electron microscopy). Thirty pieces (for each treatment and sampling time) were randomly selected and cleared in boiling 95% ethanol (v/v) for approximately 15 min followed by two weeks in saturated chloral hydrate solution (50 g mL^{-1}) (Sigma Aldrich). The cleared leaf pieces were mounted adaxial side up on glass slides containing 2-3 drops of modified Hoyer's mounting medium (Cunningham, 1972) (one leaf per glass slide). The glass slides were then examined individually until 50 infection sites randomly chosen were found. Those infections sites were examined to determine the number of brown epidermal cells (NBEC) and the degree of browning under a Carl Zeiss Axio Imager A1 microscope (400 × magnification) equipped with differential interference contrast optics. The degree of browning on the examined fifty infection sites was grouped into four categories according to the following classifications: category 1 - the cell wall of epidermal cells shows no browning; category 2 - the cell wall of epidermal cells appears pale yellow; category 3 - cells evince a slightly brown cell wall; and 4 - whole epidermal cells are deeply browned, with adjacent cells showing a slightly brown cell wall. The number of infection sites for each category was converted to percentages based on the total number of infection sites examined. Treatment means (n = 50) were then compared using t-test ($P < 0.05$) with the aid of the statistical software SAS (SAS Institute). For scanning electron microscopy analyses, a total of 24 leaf pieces (0.5 cm^2 in size) were collected from the fifth and sixth leaves of the main tiller of one plant per treatment at 48, 72 and 96 hai, following the procedure earlier described for light microscopy. The leaf pieces were carefully transferred to glass vials containing 15 mL of fixative (2.5% v/v glutaraldehyde in 0.1 mM sodium cacodylate buffer, pH 7.2). The glass vials containing the samples were stored at 4°C for 10 days and then carefully washed with a sodium cacodylate buffer (0.1 M), dehydrated in a graded ethanol series and critical point dried in CO_2 (Bal-tec, model CPD 030; Electron Mycroscopy Sciences). Four specimens from each sample (each leaf samples was divided into four pieces) were mounted on aluminum stubs, sputter coated with gold (Balzers Union, model FDU 010; Electron Mycroscopy Sciences), examined qualitatively and photographed with a LEO scanning electron

microscope (SEM) (model 1430 VP) operating at 10 kV and with a working distance ranging from 10 to 30 mm. For each treatment, four stubs with four specimens each were examined by SEM.

After the experiment, all the leaves of each plant, replication and treatment were collected, washed in deionized water, dried for 72 hours at 65°C and ground to pass through a 40 mesh screen with a Thomas-Wiley mill. The foliar concentrations of Ca and Si were determined according to Domiciano et al. (2010).

Diseases that have an economic impact on barley, corn, cucumbers, grapes, oats, rice, rye, sorghum, strawberries and wheat can be efficiently controlled by supplying Si to plants (Datnoff et al., 2007). In the present study, the foliar Si concentration significantly increased ($P < 0.001$) by 105% for the +Si plants (1.2 dag kg^{-1}) compared to the -Si plants (0.60 dag kg^{-1}). There was no significant difference ($P = 0.05$) between -Si and +Si plants for Ca concentrations on leaf tissue (0.91 and 0.97 dag kg^{-1}, respectively). The AUSBPC for +Si plants (500.5) was significantly reduced in comparison to -Si plants (1005). Because the Ca concentration in wheat leaf tissues did not change, it can be concluded that reductions in spot blotch was due to Si itself. Corroborating this result, Domiciano et al. (2010) showed that higher Si concentration in the leaves of wheat plants from cultivars BR-18 and BRS-208 decreased spot blotch progression and the number of lesions per cm^2 of leaf area, while the incubation period increased.

The present study presents the first cytological features associated with the increase in wheat resistance to spot blotch mediated by Si. The NBEC was significantly lower for the +Si plants compared to the -Si plants at 24, 36 and 48 hai (Figure 1).

It seems, therefore, that in areas of heavy Si deposition, such as below the cuticle and in the plant cell wall, fungal ingress was delayed and tissue colonization by *B. sorokiniana* was reduced. Rice resistance against blast has been associated with the density of silicified cells in the leaf epidermis or the cuticle-silica double layer formed upon deposition and polymerization of monosilicic acid beneath the cuticle (Kim et al., 2002).

At the infection sites examined, most of the cell walls became pale yellow and some slightly brown (categories 2 and 3) at 24 hai for -Si plants (Figure 2a), while for +Si plants, the majority of cell walls did not exhibited any browning signs and some became pale yellow (categories 1 and 2) (Figure 2b). For the +Si plants, cell walls became slightly brown only at 36 hai (category 3), while for –Si plants at 36 hai, even though reduced, the majority of the cells continued to be those characterized was pale yellow (the number of slight brown cells did increase). For both -Si and +Si plants,

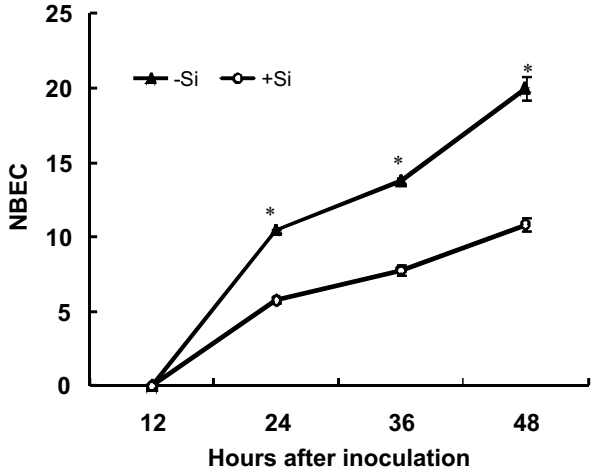

FIGURE 1 - Number of brown epidermal cells (NBEC) by infection site present in fragments (0.5 cm^2) of leaves obtained from wheat plants grown in soil non-amended (-Si) or amended (+Si) with silicon (+Si). Error bars represent the standard deviation of means. Means from -Si and +Si treatments followed by an asterisk (*) are significantly different ($P \leq 0.05$) by *t*-test. $n = 7$.

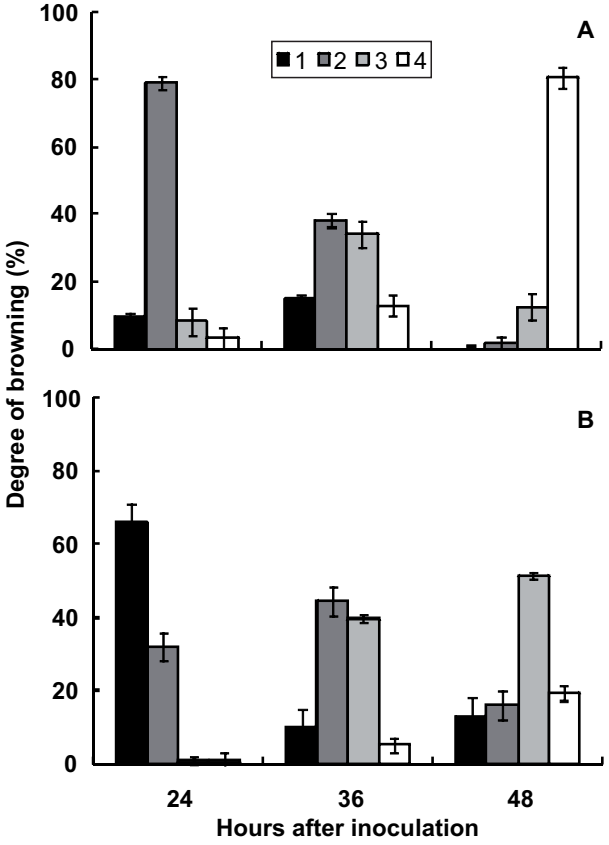

FIGURE 2 - Degree of browning of epidermal cells from leaves of wheat plants grown in soil non-amended (-Si) (A) or amended (+Si) (B) with silicon (Si) at different time-points after inoculation with *Bipolaris sorokiniana*. The degree of browning was grouped into four categories according to the following classifications: category 1 - the cell wall of epidermal cells shows no browning; category 2 - the cell wall of epidermal cells appears pale yellow; category 3 - cells evince a slightly brown cell wall and 4 - whole epidermal cells are deeply browned, with adjacent cells showing a slightly brown cell wall. Error bars represent standard deviation of the means. $n = 50$.

the increase in the frequency of infection sites showing browning (category 3) by 36 hai was followed by a decrease in the number of cells characterized like this, and also by a sharp increase in the frequency of infection sites with whole epidermal cells deeply brown and adjacent cells with slightly brown cell walls (category 4) at 48 hai (Figure 2ab). The frequency of infection sites showing browning (category 4) was lower for +Si plants (Figure 2b) than for -Si plants (Figure 2a) because of the high frequency of apressorial sites exhibiting either no browning of the epidermal cell walls (category 1) or pale yellow or slightly brown cell walls (categories 2 and 3). It is noteworthy that the overall distribution of the cells according to the browning degree at 36 hai is very similar for both -Si and +Si treatments. On the opposite direction, the distribution of the browning degrees at previous and later stages (24 and 48 hai) was very distinct between -Si and +Si treatments. *Bipolaris sorokiniana* causes browning of epidermal cells, most likely due the action of the non-specific toxins belonging to the helminthosporol family, which are citotoxic (Kumar et al., 2002).

In the present study, the higher number of brown epidermal cells on -Si plant leaves resulted from an unlimited fungal growth, compared to what was obtained for +Si plants where fungal growth was greatly restricted. Rodrigues et al. (2005) noted that a decrease in the number of blast lesions possibly owes to the fact that some apressoria from *P. grisea* cannot overcome the physical impediment created by the cuticle-silica double layer; however, the presence of silica cells and silica bodies that are not uniformly distributed in the adaxial epidermis of rice leaves (Figure1) may allow fungus penetration and successful infection.

By 48 hai, conidia of *B. sorokiniana* germinated and formed germ tubes with intense dissolution of the wax layer on the leaves of -Si plants (Figure 3a). By contrast, on the leaf surface of +Si plants at 48 hai, conidia germinated

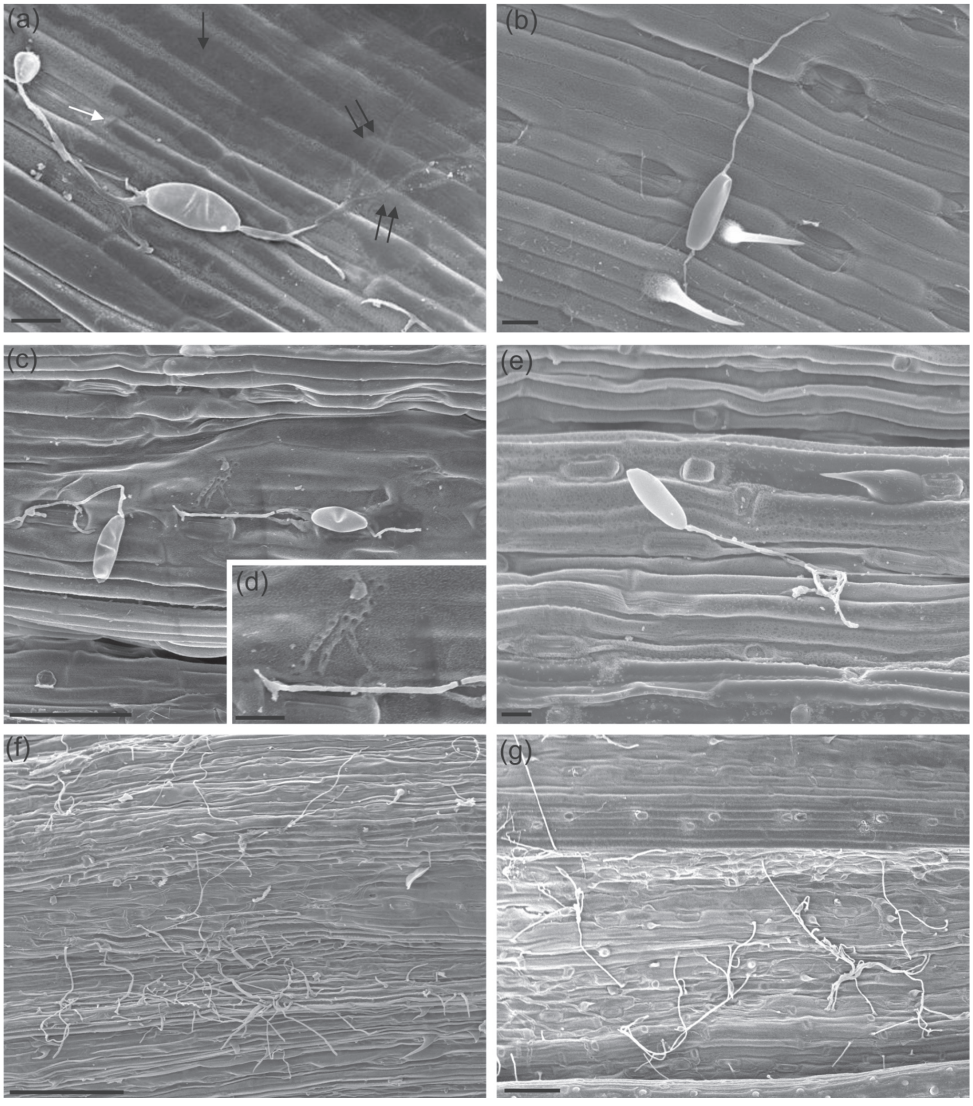

FIGURE 3 - Scanning electron microscopy of wheat leaves obtained from plants grown in soil non-amended (-Si) or amended (+Si) with silicon (Si) and inoculated with *Bipolaris sorokiniana*. (a) -Si plants at 48 hours after inoculation (hai): conidium with germ tube in an area with intense wax degradation (arrows); (b) +Si plants at 48 hai: conidium with germ tube without apparent wax degradation; (c) -Si plants at 72 hai: intense epidermal cells disorganization; (d) -Si plants at 72 hai: detail of fungal hyphae inside the epidermal cells; (e) +Si plants at 72 hai: epidermal cells without any degree of disorganization; (f) -Si plants at 96 hai: intense hyphal growth on the leaf surface; (g) +Si plants at 96 hai: restricted hyphal growth on the leaf surface. Bars: a = 30 µm; b, d and e = 20 µm; c and g = 100 µm; f = 200 µm.

and produced short germ tubes in an area where the wax layer seemed to be quite intact (Figure 3b). In +Si plants, substantial degradation of the wax layer was only observed in regions close to conidia, most likely because the double layer of cuticle-Si on leaves had reduced the diffusion of degrading enzymes to the epidermal cells. Hyphae tropic growth was observed mainly in the grooves between the epidermal cells of leaves from -Si and +Si plants. On the leaf surface of -Si plants at 72 hai, an intense dissolution and disorganization of the epidermis (Figure 3c) with fungal growth inside the leaf tissue was observed (Figure 3d), while for +Si plants such disorganization was less pronounced (Figure 3e). Hyphae of *B. sorokiniana* often crossed and were attached to the epidermal ridges on the leaves of -Si plants (Figure 3f). Conidiophore-like hyphae were often observed from these hyphae with the absence of conidia on leaf surfaces from both -Si and +Si plants. On the leaf surface of -Si plants, *B. sorokiniana* formed a dense network of hyphae at 96 hai (Figure 3f) in striking contrast to what was seen on the leaf surfaces from +Si plants (Figure 3g).

Necrotrophic foliar pathogens such as *B. sorokiniana* do not only kill their hosts' tissue by secreting non-specific toxins and inducing the production of reactive oxygen species but also show remarkable cutinase activity and the action of non-specific esterases (Deising et al., 1992; Geimba et al., 1999). Cell wall degradation most likely maximizes fungal colonization, in addition to being a source of carbohydrates (Delgado-Cerezo et al., 2012). Dallagnol et al. (2011), studying a rice mutant deficient in Si uptake (*lsi*1), observed that the area under the brown spot progress curve for plants from cv. Oochikara (wild-type) and the *lsi1* mutant was reduced in the presence of Si. The reduced number of brown epidermal cells on leaves from cv. Oochikara and the *lsi1* mutant plants supplied with Si contributed to decreased lipid peroxidation and electrolyte leakage during the infection process of *Bipolaris oryzae*.

This study provides novel microscopic evidence of the negative effect of Si on the infection process of *B. sorokiniana* by reducing the number of necrotic cells and also the intensity of the browning of the cells and, consequently, in reduction of spot blotch symptoms.

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