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). Helminthosporal, 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 chitinas and peroxidases 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
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 1% (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 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 carbonate per kg of soil and with 30 mL of a nutrient solution containing g l⁻¹: 6.4 KCl; 3.48 K₂SO₄; 5.01 MgSO₄.7H₂O; 2.03 (NH₄)₂CO₃; 0.009 NH₄NO₃; 0.054 H₂BO₃; 0.222 ZnSO₄.7H₂O; 0.058 CuSO₄.5H₂O; and 0.137 MnCl₂.4H₂O (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 FeSO₄.7H₂O and 0.37 g of EDTA bisodic 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.

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Spots 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 cm² (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 leaves were placed 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 evice 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 cm² 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 CO₂ (Bal-tec, model CPD 030; Electron Microscopy 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 Microscopy Sciences), examined qualitatively and photographed with a LEO scanning electron microscope.
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 \((1.2\ \text{dag kg}^{-1})\) compared to the -Si plants \((0.60\ \text{dag kg}^{-1})\). There was no significant difference \((P = 0.05)\) between -Si and +Si plants for Ca concentrations on leaf tissue \((0.91\ and\ 0.97\ \text{dag kg}^{-1},\ respectively)\). 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 cm² 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,
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

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**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*), 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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**REFERENCES**

Bélanger RR, Benhamou N, Menzies JG (2003) Cytological evidence of an active role of silicon in wheat resistance to powdery mildew (*Blumeria graminis f.sp. tritici*). Phytopathology 93:402-412.

Briquet M, Vilret D, Goblet P, Mesa M, Eloy MC (1998) Plant cell membranes as biochemical targets of the phytotoxin helminthosporol. Journal of Bioenergy and Biomembranes 30:285-295.

Cunningham JL (1972) A miracle mounting fluid for permanent whole mounts of microfungi. Mycologia 64:906-11.

Dallagnol LJ, Rodrigues FA, Mielli VBM, Pereira SC (2011) Deficiency in silicon uptake affects cytological, physiological, and biochemical events in the rice-*Bipolaris oryzae* interaction. Phytopathology 101:92-104.

Datnoff LE, Rodrigues FA. Seebold KW (2007) Silicon and Plant Disease. In: Datnoff LE, Elmer WH, Huber DM (Eds.) Mineral nutrition and plant disease. St Paul MN, USA. APS Press. pp. 233-246.

Deising HB, Horbach R, Ludwig N, Münch S, Schweizer P (2009) Mechanisms of fungal infection. In: Feldmann F, Alford DV, Furk C (Eds.). Crop plant resistance to biotic and abiotic factors: Current potential and future demands. Braunschweig Germany. DPG Selbstverlag, pp. 290-301.

Delgado-Cerezo M, Rodriguez CS, Escudeiro V, Miedes E, Fernandez PV, Jordá L, Hernandez-Blanco C, Sanchez-Vallet A, Bednarek P, Schulze-Lefert P, Somerville S, Estevez JS, Persson S, Molina A (2012) Arabidopsis heterotrimeric G-protein regulates cell wall defense and resistance to necrotrophic fungi. Molecular Plant Pathology 5:98-114.

Domiciano GP, Rodrigues FA, Vale FXR, Xavier Filha MS, Moreira WR, Andrade CCL, Pereira SC (2010) Wheat resistance spot blotch potentiated by silicon. Journal of Phytopathology 158:334-343.

Duveiller EM, Sharma RC (2009) Genetic improvement and crop management strategies to minimize yield losses in warm non-traditional wheat growing areas due to spot blotch pathogen *Cochliobolus sativus*. Journal of Phytopathology 157:521-534.

Gayad SK (1961) Production of symptoms of barley leaf spot disease by cultural filtrates of *Cochliobolus sativus*. Nature 191:725-726.

Geimba MP, Riffel A, Agostini V, Brandelli A (1999) Characterization of cellulose-hydrolysing enzymes from the fungus *Bipolaris sorokiniana*. Journal of the Science of Food and Agriculture 79:1849-1854.

Guevel MH, Menzies JG, Bélanger RR (2007) Effect of root and foliar applications of soluble silicon on powdery mildew control and growth of wheat plants. European Journal of Plant Pathology 119:429-436.

IRRI (1996). Standard Evaluation System for Rice. 4th ed. Manila The Philippines. International Rice Research Institute. Kim SG, Kim KW, Park EW, Choi D (2002) Silicon-induced cell wall fortification of rice leaves, a possible cellular mechanism of enhanced host resistance to blast. Phytopathology 92:1095-1103.

Kumar J, Schafer P, Huckelhoven R, Langen G, Baltruschat H,
Infection process of *Bipolaris sorokiniana* on wheat leaves is affected by silicon

Stein E, Nagarajan S, Kogel KH (2002) *Bipolaris sorokiniana*, a cereal pathogen of global concern: cytological and molecular approaches towards better control. Molecular Plant Pathology 3:185-195.

Misra AP (1979) Variability, physiologic specialization and genetics of pathogenicity in graminicolous *Helminthosporium* affecting cereal crops. Indian Phytopathology 32:1-22.

Rodrigues FA, McNally DJ, Datnoff LE, Jones JB, Labbe C, Benhamou N, Menzies JG, Bélanger RR (2004) Silicon enhances the accumulation of diterpenoids phytoalexins in rice: a potential mechanism for blast resistance. Phytopathology 94:177-183.

Rodrigues FA, Jurick WM, Datnoff LE, Jones JB, Rollins JA (2005) Silicon influences cytological and molecular events in compatible and incompatible rice-*Magnaporthe grisea* interactions. Physiological and Molecular Plant Pathology 66:144-159.

Shaner G, Finney RE (1977) The effect of nitrogen fertilization on the expression of slow mildewing resistance in Knox wheat. Phytopathology 67:1051-1056.

Wisniewska H, Wakulinski W, Chelkowski J (1998) Susceptibility of barleys to *Bipolaris sorokiniana* seedling blight determined by disease scoring and electrolyte leakage. Journal of Phytopathology 146:563-566.

Yoshida SS, Ohnishi Y, Kitagishi K (1962) Chemical forms, mobility, and deposition of silicon in rice plant. Soil Science and Plant Nutrition 8:15-21.

Xavier Filha MS, Rodrigues FA, Domiciano GP, Oliveira HV, Silveira PR, Moreira WR (2011) Wheat resistance to leaf blast mediated by silicon. Australasian Plant Pathology 40:28-38.

Zadoks JC, Chang TT, Konzac CF (1974) A decimal code for the growth stages of cereals. Weed Research 14:415-421.