Silicon Enhances the Accumulation of Diterpenoid Phytoalexins in Rice: A Potential Mechanism for Blast Resistance

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ABSTRACT

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Although several reports underscore the importance of silicon (Si) in controlling *Magnaporthe grisea* on rice, no study has associated this beneficial effect with specific mechanisms of host defense responses against this fungal attack. In this study, however, we provide evidence that higher levels of momilactone phytoalexins were found in leaf extracts from plants inoculated with *M. grisea* and amended with silicon (Si⁺) than in leaf extracts from inoculated plants not amended with silicon (Si⁻)

or noninoculated Si⁺ and Si⁻ plants. On this basis, the more efficient stimulation of the terpenoid pathway in Si⁺ plants and, consequently, the increase in the levels of momilactones appears to be a factor contributing to enhanced rice resistance to blast. This may explain the lower level of blast severity observed on leaves of Si⁺ plants at 96 h after inoculation with *M. grisea*. The results of this study strongly suggest that Si plays an active role in the resistance of rice to blast rather than the formation of a physical barrier to penetration by *M. grisea*.

Additional keywords: antifungal compounds, calcium silicate slag, induced resistance, *Oryza sativa*, secondary metabolites.

Rice blast, caused by *Magnaporthe grisea* (T.T. Hebert) Yaegashi & Udagawa) Barr (anamorph *Pyricularia grisea* (Cooke) Sacc.), is the most important fungal disease of rice (*Oryza sativa* L.) and causes significant yield losses worldwide (25). Planting rice cultivars with partial resistance, early sowing and transplanting of seedlings in the field, adequate water and nitrogen management, and seed treatment or foliar application of fungicides (3,23) are currently the most effective strategies used to control blast under conducive environmental conditions.

The application of silicon (Si) to rice fields to control blast is an alternative approach that is gaining increased interest, especially when rice is grown in soils deemed to be low or limiting in plant-available Si (9,29). Silicon application increases rice resistance to blast on both partially resistant and susceptible cultivars (30), but the mechanisms underlying this phenomenon remain poorly understood. Silicification of epidermal cells (15,16,33) or the formation of a cuticle-silica double layer in the apoplast (35), preventing fungal penetration and colonization, are hypotheses linked to the Si-mediated blast resistance. Kim et al. (18) recently studied Si accumulation in rice leaves and its possible contribution to blast resistance using electron microscopy and X-ray microanalysis. According to their results, Si was deposited in the outer regions of the cell walls of epidermal cells and was associated with a possible cellular mechanism of enhanced resistance of rice to *M. grisea*. In an attempt to gain further insight into the role of Si in rice blast resistance, Rodrigues et al. (26) investigated the ultrastructural outcome of the rice-*M. grisea* interaction upon Si treatment. The authors observed an abundant occurrence of empty

fungal hyphae surrounded or trapped in phenolic-like compounds in the epidermis, mesophyll, and vascular bundles of Si-treated plants. From that study, Rodrigues et al. (26) concluded that Si may modulate rice defense responses by influencing the metabolism or the synthesis of antifungal compounds upon penetration of *M. grisea* within epidermal cells.

The observations made by Rodrigues et al. (26) are consistent with several reports demonstrating that rice produces several phenolic compounds as well as phytoalexins in response to *M. grisea* infection (6,11,22). Rice leaf phytoalexins are chemically diverse and comprise both flavonoid and diterpenoid constituents. The diterpene isoprenoid phytoalexins, synthesized via the terpenoid pathway, are momilactones A and B (7), oryzalexins A to F (1,17,31,32), oryzalexin S (19), and phytocassanes A to D (21). Sakuranetin, a flavonoid phytoalexin, is synthesized through the phenylpropanoid or phenylpropanoid-acetate pathway (20).

According to Dillon et al. (11), the accumulation of the phytoalexins sakuranetin, momilactone A, and oryzalexin S was associated with an increase in resistance of rice cultivars susceptible and partially resistant to blast. Dillon et al. (11) also noted that genetic differences among rice cultivars may affect phytoalexin production in both quantity and timing of expression and, consequently, the level of resistance to blast. However, it remains unclear which phytoalexin plays the most important role in rice blast resistance. It is likely that the elicitation and the effectiveness of phytoalexins depend on a number of factors including cultivars, elicitors, and environmental conditions. In addition, phytoalexins may act in a synergistic manner where the quantity and rate of production of each phytoalexin influences the infection process by *M. grisea* (11).

Fawe et al. (12) reported that the increased resistance to powdery mildew (*Podosphaera xanthii*) in cucumber plants amended with Si was associated with the production of a flavonol phyto-

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alexin. However, no such link has ever been reported in monocots, and direct evidence for the possible involvement of Si in a chemical defense response elaborated by rice plants against *M. grisea* infection is, so far, lacking in the literature. Accordingly, the main objective of this study was to test the hypothesis that an alteration in the development of *M. grisea* in leaf tissues of rice plants amended with Si could be associated with an enhanced production of phytoalexin.

MATERIALS AND METHODS

Plant and fungal material. Rice cv. M201 (CI 9980) was chosen for this study because it has no known major or minor genes for resistance to race IB-49 of *M. grisea* (30). Plastic pots (12 cm in diameter) were filled with 2 kg of peat Premier Pro-Mix (Premier Horticulture, Québec, Canada) and amended with calcium silicate slag (22% available soluble Si) (Calcium Silicate Corp., Lake Harbor, FL) at the rates of 0 and 10 g pot⁻¹ (10 t ha⁻¹) 5 days before sowing. Seeds were sown at the rate of eight seeds per pot, and at 3 days after emergence, each pot was thinned to two seedlings. Seedlings were then fertilized by adding 100 ml of a nutrient solution to each pot containing (in milligrams per kilogram of peat Premier Pro-Mix): 100 N, 300 P, 150 K, 85 Ca, 70 Mg, 40 S, 0.81 B, 1.33 Cu, 3.66 Mn, 0.15 Mo, and 4.00 Zn (27). A second application of nutrient solution was made 25 days later. Iron deficiency was avoided by adding 10 ml of a solution containing 300 g of FeSO₄ liter⁻¹ per pot after thinning. Plants were kept under flooded conditions until the end of the experiments.

Inoculation of rice plants with *M. grisea*. Inoculations were made at the time of emergence of the seventh leaf from the main tiller (24). A conidial suspension (5 × 10⁴ conidia ml⁻¹) of race IB-49 of *M. grisea* (isolate 793) was applied as a fine mist to the two plants per pot until runoff with an aerosol sprayer (Crown Spra-Tool; Fisher Scientific Co., Pittsburgh, PA). Gelatin (1%, wt/vol) was added to the sterile water used to prepare the conidial suspension to aid conidial adhesion to the rice leaf blades. The noninoculated rice plants were sprayed with a gelatin-sterile water (1%, wt/vol) solution until runoff and served as a control. Inoculated and noninoculated plants were covered with plastic bags and transferred to a growth chamber (Conviron Model PGR 15; Controlled Environments Ltd., Winnipeg, Canada) configured to the following conditions: photoperiod of 8 h dark at 23°C and 16 h light at 25°C with approximately 162 µE m⁻² s⁻¹ provided by cool-white fluorescent lamps. Pots were placed in water-filled plastic trays that maintained the relative humidity inside the growth chamber at approximately 85% for the remainder of the experiment. Inoculated and noninoculated plants were submitted to an initial 24 h dark period, and after 48 h, the plastic bags were removed from the plants.

Experimental design, sampling of treatments, and evaluation of blast lesion development. Two 2 × 2 factorial experiments, consisting of rice plants amended with silicon (Si⁺) or not (Si⁻) and inoculated with *M. grisea* (*M. grisea*⁺) or not (*M. grisea*⁻), were arranged in a completely randomized design with three replicates each. Because rice susceptibility to blast is affected by leaf age (25), only the third, fourth, fifth, and sixth leaves were collected from each plant to insure the use of leaves with similar susceptibility among the treatments Si⁻ *M. grisea*⁻, Si⁺ *M. grisea*⁻, Si⁻ *M. grisea*⁺, and Si⁺ *M. grisea*⁺. Leaves were collected at 96 h after inoculation with *M. grisea* from both inoculated and noninoculated rice plants. Representative leaves from each treatment were photographed with a digital camera to record rice blast severity.

Extraction of leaf material. Harvested leaf material was placed into plastic bags and immediately stored at -80°C. Leaf samples were freeze-dried (Labconco Corporation, Kansas City, MO) for approximately 72 h, blended to a fine powder, and trans-

ferred to plastic tubes that were stored at -80°C until further analysis.

The methodology developed by Derks and Buchenauer (10) as modified by Daayf et al. (8) was used to analyze free and glycosylated phenolic compounds, diterpenoids, as well as aglycones released after acid hydrolysis. Briefly, a representative sample of 10 g (dry weight) of powdered leaf material, obtained from combined leaves from the three replications of each treatment, was transferred to an Erlenmeyer flask containing 800 ml of 80% MeOH. The homogenate solution was protected from oxidation by replacing oxygen with nitrogen and light was eliminated by covering the flask with aluminum foil, and the solution was extracted for 24 h on a rotary shaker (125 rpm) at 25°C. The dark green extract was filtered in a Büchner apparatus with Whatman No. 1 filter paper under vacuum. Methanol was removed by roto-evaporation at 38 ± 2°C under vacuum giving a final volume of approximately 160 ml. The aqueous residue was first partitioned against hexane (2 × 500 ml) to remove chlorophylls, carotenoids, lipids, waxes, and other nonpolar compounds, and was named fraction I (FI). In the following step, the aqueous phase was further partitioned against anhydrous ethyl ether (2 × 625 ml) to obtain fraction II (FII), which contained free phenolics as well as other classes of nonglycosylated compounds such as diterpenoids.

For analysis of aglycones, the remaining aqueous residue containing glycosylated compounds was transferred to an Erlenmeyer flask, acidified with an equal volume of 4 N HCl, and subjected to hydrolysis (2 N HCl, vol/vol; 90 min; 100°C). The hydrolysate was cooled and partitioned against ethyl acetate (5 × 50 ml) and ethyl ether (5 × 50 ml) to recover aglycones. Both organic fractions containing aglycone compounds were combined and named fraction III (FIII). Trace amounts of water were removed from FIII by adding anhydrous magnesium sulfate.

Fractions I, II and III were then evaporated to dryness by roto-evaporation at 38 ± 2°C under vacuum and the brown solid residue obtained for each fraction was resuspended in HPLC grade MeOH (1 ml of MeOH g⁻¹ [dry weight] of leaves).

Bioassay of FI, FII, and FIII against *M. grisea*. Fractions I, II, and III obtained from each treatment were bioassayed to test for fungitoxicity against *M. grisea*. Petri dishes were filled with 20 ml of potato dextrose agar (PDA) and a hole (10 mm in diameter) was made in the center of each dish. A volume of 150 µl of each fraction was transferred to each hole and allowed to dry for 30 min. In separate petri dishes, 150 µl of methanol was added to each hole and served as the control. Two agar plugs (15 mm in diameter), obtained from the margin of an actively growing colony of *M. grisea*, were placed 1.5 cm from the center of each hole. Plates were transferred to an incubator (25°C; 12 h light/12 h dark), and growth of *M. grisea* was monitored daily until the two fungal colonies reached confluence in the control treatment. This confluence occurred at approximately 120 h after the agar plugs containing the fungus were transferred to the petri dishes. The bioassay was repeated twice with two petri dishes per each leaf extract.

Thin-layer chromatogram and high-performance liquid chromatography analysis of FI, FII, and FIII. Authentic standards for rice phytoalexins momilactone A, momilactone B, oryzalexin A, and oryzalexin C were supplied by K. Yoheyama (Utsunomiya University, Japan) and sakuranetin was obtained from a commercial source (Sigma-Aldrich, Mississauga, Ontario, Canada). Fractions I, II, and III from all treatments in addition to the five phytoalexin standards were analyzed with aluminum silica gel thin-layer chromatogram (TLC) plates (0.2-mm pre-coated silica gel 60 F₂₅₄; Merck & Co. Inc., Whitehouse Station, NJ) developed with ethanol/chloroform (1:1, vol/vol). The TLC bands were observed under short- and long-wavelength UV illumination (short wavelength UV illumination 254 nm, Model UVS-11 Lamp; and long-wavelength illumination 366 nm, Model UVL-21; UV Products Inc., San Gabriel, CA), and the retention

value of each band was calculated. In addition, chromatographic bands were visualized by spraying TLC plates with vanillin solution (6 g of vanillin, 3 ml of H₂SO₄, and 197 ml of EtOH) prior to heating at 110°C for 5 min and with anisaldehyde-sulphuric acid solution to confirm the presence of diterpenoids (4). The resulting color changes of the chromatophoric bands for extracts and standards were observed under UV light (long and short wavelengths) and visible light.

Compounds present within FI, FII, and FIII prepared from all treatments were analyzed using high-performance liquid chromatography (HPLC) with an autosampler (Waters Model 717; Waters Corp., Milford, MA), photodiode array detector (Waters Model 996), and fitted with a C₁₈ reverse-phase column (Waters Model RCM; 8 × 100 mm reverse phase C-18). Fifty microliters of FI, FII, and FIII from all treatments was injected, and the column was eluted with a gradient of acetonitrile/water acidified with H₃PO₄ (Table 1). All HPLC analyses were subjected to three separate injections to ensure the reproducibility of the results which were analyzed using the program Millennium 2.1 software (Waters Corp., Milford, MA).

Semipurification of FII from Si⁻ *M. grisea*⁻, Si⁺ *M. grisea*⁺, Si⁻ *M. grisea*⁺, and Si⁺ *M. grisea*⁺ treatments. In order to facilitate further analysis of the most apolar metabolites present within FII of Si⁻ *M. grisea*⁺, Si⁺ *M. grisea*⁺, and control treatments (Si⁻ *M. grisea*⁻ and Si⁺ *M. grisea*⁻), leaf extracts were semipurified using Sep-Pak C-18 cartridges (Waters Corp.). The Sep-Pak C-18 cartridges were first conditioned with 20 ml of acidified MeOH followed by 20 ml of acidified water. Five milliliters of crude, concentrated FII leaf extract from each treatment was loaded in a cartridge and rinsed sequentially with 10 ml of acidified water, 10 ml of acidified water-acidified MeOH (75:25), 10 ml of acidified water-acidified MeOH (50:50), 10 ml of acidified water-acidified MeOH (25:75), and 10 ml of acidified MeOH. The solvents were acidified with 2.5% glacial acetic acid to a final pH of 2.5 to suppress ionization of extracted compounds (4). The five subfractions (SF) obtained from each crude extract were collected individually in 10-ml aliquots, dried under a nitrogen stream, and resuspended in HPLC grade MeOH (1 ml of MeOH g⁻¹ [dry weight] of leaves).

HPLC co-chromatography experiment. The presence of phytoalexins within SF5 (the most apolar SF) prepared from FII of Si⁺ *M. grisea*⁺ treatment was unambiguously determined by HPLC co-chromatography with the five standards. A few milligrams of each standard was solubilized in 5 ml of HPLC grade MeOH. Twenty-five microliters of SF5 was injected simultaneously with 25 µl of each standard and eluted with a gradient of acetonitrile/water acidified with H₃PO₄ (Table 1).

Bioassay of the five FII SFs from Si⁻ *M. grisea*⁺ and Si⁺ *M. grisea*⁺ treatments. The antifungal activity of the five FII Sep-Pak SFs prepared from Si⁻ *M. grisea*⁺ and Si⁺ *M. grisea*⁺ treatments was tested against *Cladosporium cucumerinum* Ellis & Arth and *M. grisea*.

TABLE 1. Solvent gradient of acetonitrile/water acidified with H₃PO₄ used in high-performance liquid chromatography elutions of leaf extracts^a

Time (min)	Flow (ml min ⁻¹)	% A	% B
0	1.0	0	100
40	0.9	15	85
50	0.9	25	75
60	0.6	25	75
70	0.5	40	60
80	0.5	50	50
85	0.5	70	30
90	0.9	80	20
95	1.0	80	20
105	1.0	100	0
110	1.0	0	100

^a A = acetonitrile acidified with 0.1% H₃PO₄. B = water acidified with 0.1% H₃PO₄.

For bioassays with *C. cucumerinum*, 150 µl of each FII Sep-Pak SF from Si⁻ *M. grisea*⁺ and Si⁺ *M. grisea*⁺ treatments was spotted on a silica-gel TLC plate using a glass capillary tube (0.4 mm i.d. × 75 mm, Drummond Scientific Co., Broomall, PA). The TLC plate was then covered with a conidial suspension (9 × 10⁸ conidia ml⁻¹) of *C. cucumerinum* (1:1, vol/vol) in a 20 g liter⁻¹ solution of PDA (2). The TLC plate was placed in a dark and humid chamber at 25°C. The appearance of zones of fungal growth inhibition, as determined by the white spots against a gray background formed by mycelia and conidia of *C. cucumerinum*, was monitored from 6 to 72 h.

A volume of 150 µl of each FII Sep-Pak SF from Si⁻ *M. grisea*⁺ and Si⁺ *M. grisea*⁺ treatments was tested against *M. grisea* using the same procedure described for testing FI, FII, and FIII. The experiment was repeated twice using two petri dishes per each FII SF.

HPLC analysis of SF5 from FII of Si⁻ *M. grisea*⁻, Si⁺ *M. grisea*⁻, Si⁻ *M. grisea*⁺, and Si⁺ *M. grisea*⁺ treatments. Twenty-five microliters of SF5 from FII leaf extract of each treatment was analyzed by HPLC with a gradient of acetonitrile/water acidified with H₃PO₄ (Table 2). The presence of phytoalexins in leaf extracts was verified based on their retention time, ultraviolet absorbance spectra at 212 and 272 nm, as well as co-chromatography with the authentic standards. All HPLC analyses were subjected to three separate injections to ensure the reproducibility of the results which were analyzed using the program Millennium 2.1 software.

RESULTS

Effect of Si on development of leaf blast lesions. The development of leaf blast lesions on rice cv. M201 receiving silicon (Si⁺) or not (Si⁻) is illustrated in Figure 1. Differences were observed between Si⁻ and Si⁺-treated plants at 96 h after inoculation with *M. grisea*. Many large and coalescing lesions, often surrounded by a chlorotic halo, were noticed on leaves from Si⁻ plants. In contrast, discrete and small lesions, seemingly confined in their expansion, characterized leaves of Si⁺ plants. Additionally, infected leaves of Si⁻ plants displayed intense chlorosis compared with the leaves of Si⁺ plants.

TLC analyses and bioassay of FI, FII, and FIII. There were no qualitative differences among treatments for compounds contained in FI (chlorophylls, carotenoids, lipids, waxes, and other nonpolar compounds) and FIII (aglycones) as observed on TLC plates under UV light at both short and long wavelengths. In addition, FI and FIII did not show any antifungal activity against *M. grisea*.

In contrast, comparative analysis of FII (nonglycosylated compounds, naturally phenolic aglycones, and terpenoids) between the treatments Si⁻ *M. grisea*⁺ and Si⁺ *M. grisea*⁺ highlighted the presence of several bands with different characteristics under short and long UV light (data not shown). A distinct difference in the pattern of bands between Si⁻ *M. grisea*⁺ and Si⁺ *M. grisea*⁺ control treatments was not observed. Visual assessment of the fungitoxicity activity of FII against *M. grisea* in the petri dish bioassay indicated that the Si⁻ *M. grisea*⁺ and Si⁺ *M. grisea*⁺

TABLE 2. Solvent gradient of acetonitrile/water acidified with H₃PO₄ used in high-performance liquid chromatography elutions of subfractions 5 from fraction II^a

Time (min)	Flow (ml min ⁻¹)	% A	% B
0	1.0	30	70
15	0.5	70	30
45	1.0	100	0
50	1.0	30	70

^a A = acetonitrile acidified with 0.1% H₃PO₄. B = water acidified with 0.1% H₃PO₄.

Fig. 1. Development of leaf blast symptoms at 96 h after inoculation with *Magnaporthe grisea* in rice plants nonamended (Si^-) and amended (Si^+) with silicon.

Fig. 2. Bioassay of the fungitoxic activity of ethyl ether fractions against *Magnaporthe grisea* of leaf extracts from plants inoculated with *M. grisea* ($M. grisea^+$) and noninoculated plants ($M. grisea^-$) amended with silicon (Si^+) or not (Si^-). Growth of *M. grisea* was monitored daily in the four treatments until two fungal colonies reached confluence in the control (150 μl of methanol). The confluence occurred at approximately 120 h after the agar plugs containing the fungus were transferred to the plates. Note a difference in the radial growth of *M. grisea* especially in the area immediately surrounding the well.

treatments contained compounds that altered the pattern of the radial growth of *M. grisea*, especially in the area immediately surrounding the well, whereas leaf extracts from $\text{Si}^- M. grisea^-$ and $\text{Si}^+ M. grisea^-$ control treatments did not appear to have any effect on fungus growth (Fig. 2).

HPLC analyses of FII. The presence of metabolites in crude extracts of FII from all treatments was determined by HPLC over a 110 min elution time. Comparative analysis of all four chromatograms revealed similar secondary metabolite profiles for the first 100 min elution time (Fig. 3A to D, arrows) with the exception of a few singular metabolites present in the $\text{Si}^- M. grisea^+$

Fig. 3. High-performance liquid chromatography analysis of the ethyl ether fractions obtained from **A**, noninoculated plants not amended with silicon ($\text{Si}^- M. grisea^-$), **B**, noninoculated plants amended with silicon ($\text{Si}^+ M. grisea^-$), **C**, plants not amended with silicon and inoculated with *M. grisea* ($\text{Si}^- M. grisea^+$), and **D**, plants amended with silicon and inoculated with *M. grisea* ($\text{Si}^+ M. grisea^+$). Note that the four chromatogram profiles are quite similar in the pattern of secondary metabolites; however, in the section after 100 min, peaks show much higher quantities in leaf extracts from $\text{Si}^- M. grisea^+$ and $\text{Si}^+ M. grisea^+$ treatments than in the control treatments.

treatment (Fig. 3C, arrow). These unrelated compounds can be attributed, at least in part, to the release of wall bound or other cellular metabolites, including fungus compounds, in the soluble fraction of leaf tissues degraded by *M. grisea*. Based on the result of the bioassay against *M. grisea* (Fig. 2), the main zone of interest appeared to be concentrated in the section after 100 min. For that section, some compounds increased in concentration in both Si⁻ *M. grisea*⁺ and Si⁺ *M. grisea*⁺ treatments (Fig. 3C and D, arrows) compared with Si⁻ *M. grisea*⁻ and Si⁺ *M. grisea*⁻ control treatments (Fig. 3A and B, arrows).

Bioassays of FII SFs from Si⁻ *M. grisea*⁺ and Si⁺ *M. grisea*⁺ treatments, HPLC, and co-chromatography analysis. Analysis of FII from leaf extracts of Si⁻ *M. grisea*⁺ and Si⁺ *M. grisea*⁺ treatments revealed that of the five SFs collected, only SF5, which corresponded to compounds eluting after 90 min in the HPLC chromatograms, displayed antifungal activity against *C. cucumerinum* (Fig. 4A) and *M. grisea* (Fig. 4B). The SF5 from Si⁻ *M. grisea*⁻ and Si⁺ *M. grisea*⁻ treatments was not fungitoxic against *M. grisea* (Fig. 4B), whereas the SF5 from Si⁺ *M. grisea*⁺ treatment showed higher fungitoxicity against *C. cucumerinum* and *M. grisea* than SF5 from Si⁻ *M. grisea*⁺ treatment. SFs 1, 2, 3, and 4 had no apparent antifungal activity against *C. cucumerinum* (Fig. 4A) and *M. grisea* (data not shown) regardless of treatment. The presence of a clear white zone in the spot of SF1 from Si⁻ *M. grisea*⁺ treatment (Fig. 4A) is a common artifact often observed when the most polar fraction is first eluted with acidified water. Despite great care taken to evaporate the acid residues, they remained on the TLC plate and seemed to be toxic to *M. grisea*.

Fig. 4. Bioassay of the fungitoxic activity of five subfractions (SF) of the ethyl ether fraction. **A**, Comparison of the fungitoxic activity against *Cladosporium cucumerinum* on thin-layer chromatogram (TLC) plate among the five SF from leaf extracts obtained from plants amended with silicon (Si⁺) or not (Si⁻) and inoculated with *Magnaporthe grisea* (*M. grisea*⁺). Note the appearance of *C. cucumerinum* growth inhibition around the spots of SF5 on the TLC plate. **B**, Comparison of the fungitoxic activity of SF5 against *M. grisea* between leaf extracts of plants amended with silicon (Si⁺) and not (Si⁻) and inoculated with *M. grisea* (*M. grisea*⁺) or not (*M. grisea*⁻). Growth of *M. grisea* was monitored daily in the four treatments until two fungal colonies reached confluence in the control (150 µl of methanol). The confluence occurred at approximately 120 h after the agar plugs containing the fungus were transferred to the plates. Note a difference in the radial growth of *M. grisea* especially in the area immediately surrounding the well.

This particular finding is contrasted by inhibition zones of fungus growth around the spots of SF5 from Si⁻ *M. grisea*⁺ and Si⁺ *M. grisea*⁺ treatments as opposed to the brownish background represented by mycelia and conidia of *C. cucumerinum* all over the TLC plate (Fig. 4A). When standards of sakuranetin, oryzalexin A and C, and momilactones A and B were injected and analyzed by HPLC using a gradient of acetonitrile/water acidified with H₃PO₄ (Table 1), only the latter two phytoalexins migrated in the section between 100 and 110 min (data not shown). Consequently, SF5 of FII from the Si⁺ *M. grisea*⁺ treatment was spiked with momilactone A and B standards and results showed a similar UV spectra and co-elution of these two momilactones (Fig. 5, arrow) with the peaks eluting after 100 min in the Si⁺ *M. grisea*⁺ treatment (Fig. 3D, arrow).

Based on these observations, SF5 from FII was further analyzed by HPLC using a gradient of acetonitrile/water acidified with H₃PO₄ (Table 2). This allowed separation of the two momilactones based on their UV spectra and retention time (*R_t*) (momilactone A, *R_t* 46 min; and momilactone B, *R_t* 47 min). These compounds were present in minute or small quantities in non-inoculated treatments (Fig. 6A and B). By contrast, both products showed a two- to threefold increase in leaf extract from Si⁺ *M. grisea*⁺ treatment (Fig. 6D) compared with the lower levels observed in leaf extract from Si⁻ *M. grisea*⁺ treatment (Fig. 6C). Of particular interest, when using a more discriminant gradient for separation of the apolar SF (Table 2), it became apparent that these momilactone phytoalexins were in fact more prevalent in the leaf extract from the Si⁺ *M. grisea*⁺ treatment than analysis of total crude extract appeared to reveal (Fig. 3D).

DISCUSSION

Results from this study have shown that rice plants amended with Si exhibited an enhanced resistance against infection by *M. grisea*, and this resistance appears to be strongly associated with the production of momilactones A and B. This is, to our knowledge, the first report linking the beneficial role of Si against *M. grisea* with a specific induced defense mechanism in rice.

Several reports have described the importance of Si to control rice blast, but no study has associated this beneficial effect with an increase in the concentration of momilactones and their influence on some components of resistance such as lesion expansion.

Fig. 5. High-performance liquid co-chromatography analysis of the most apolar compounds present in the ethyl ether fraction of rice leaves collected from plants amended with silicon at 96 h after inoculation with *Magnaporthe grisea* co-chromatographed with momilactone A and B standards. Note that peaks previously observed after 100 min in the crude extract profile (Fig. 3C) co-eluted with the peaks corresponding to the two momilactones with identical UV spectra.

Fig. 6. High-performance liquid chromatography analysis of subfraction 5 (the most apolar subfraction) obtained from the ethyl ether fraction. **A**, Noninoculated plants not amended with silicon ($\text{Si}^- M. grisea^-$). **B**, Noninoculated plants amended with silicon ($\text{Si}^+ M. grisea^-$). **C**, Plants not amended with silicon and inoculated with *M. grisea* ($\text{Si}^- M. grisea^+$). **D**, Plants amended with silicon and inoculated with *M. grisea* ($\text{Si}^+ M. grisea^+$). Note the presence of two peaks in leaf extracts from $\text{Si}^+ M. grisea^-$ and $\text{Si}^- M. grisea^+$ treatments, which reached much higher quantities in the $\text{Si}^+ M. grisea^+$ treatment. These two peaks correspond to momilactones A and B based on their UV spectra (λ_{max} 212 nm) and retention times (momilactone A, R_t 46 min and momilactone B, R_t 47 min).

Based on early findings that silicic acid polymerizes in the rice epidermal cell walls (35), it was proposed that Si played a passive role as a mechanical barrier restricting penetration by *M. grisea*. Although a decrease in the number of blast lesions can be attributed to the fact that some appressoria cannot overcome the physical impedance offered by the cuticle-silica double layer, it is well known that the presence of silica cells and silica bodies is not uniformly distributed in the adaxial epidermis of rice leaf. This has led many authors to speculate that the protection conferred by Si must be more than a physical barrier (20,26,28). Results presented herein support this new hypothesis and complement the list of other host-pathogen interactions that Si is purported to play an active role in plant protection.

Many phytoalexins have been identified in rice and are reported to play a key role against infection by *M. grisea* (1,7,31). In this study, we have identified the increased presence of momilactones A and B, two common rice phytoalexins, as potential defense compounds altering fungal growth in leaf cells of rice plants amended with Si. The production of these diterpenoid phytoalexins represents a chemical defense strategy elaborated by the rice plant to counter further colonization of *M. grisea* after its penetration peg has entered the cytoplasm of either the epidermal or bulliform cells (5). In line with our observations, it has been reported that momilactones A and B reach their maximum concentration in rice tissues at approximately 72 to 96 h after infection by *M. grisea* (1) and efficiently arrest hyphal growth at very low concentrations (34). In cases where rice phytoalexins have been elicited with *M. grisea*, the outcome of the interaction is determined by the speed and concentration at which the phytoalexins accumulate ahead of fungal invasion (11). According to Hammerschmidt (14), phytoalexins have, among other roles, a measurable benefit for the plants producing them. This phenomenon was illustrated in our study in which rice plants not amended with Si and inoculated with *M. grisea*, in spite of releasing antifungal compounds including momilactones, were obviously not protected efficiently against fungal colonization. In contrast, rice plants amended with Si and inoculated with *M. grisea*, releasing higher amounts of momilactones probably earlier in the infection process, benefited from a lower level of rice blast severity. Considering that momilactones are often concentrated in areas of *M. grisea* colonization causing early cessation of its growth (5), the differences in their production between $\text{Si}^+ M. grisea^+$ and $\text{Si}^- M. grisea^+$ plants are most likely proportionally much higher than the results of this study. This suggests that the increase in the concentration of these two phytoalexins in leaf extracts obtained from rice plants amended with Si contributes to enhance resistance against *M. grisea* as shown by the visual differences in lesion size on the leaves of rice plants amended with this element.

While little is known about the mechanisms of resistance of rice plants amended with Si in response to *M. grisea* infection, two mutually agreeable hypotheses must be considered. On the one hand, it is possible that in certain areas of heavy Si deposition, delayed fungal ingress and colonization provides the rice plant enough time for momilactones, synthesized in response to infection by *M. grisea*, to accumulate to considerable levels and express their fungitoxicity within the zone of the infection site. On the other hand, as proposed by Fawe et al. (13), the soluble Si present in the plant cells may mediate some defense responses that are functionally similar to systemic acquired resistance. For instance, there is evidence that Si-increased resistance to powdery mildew (*Podosphaera xanthii*) in cucumber was associated with the production of a novel flavonol phytoalexin (12). Indeed, it has been shown that polymerization of Si results in its inactivation as a possible modulator of plant resistance which might explain the necessity of an uninterrupted, continuous availability of soluble Si to the plant to positively amplify its expression of defense responses against fungal attack (28).

Taken together, the results of this investigation are of particular significance because they support the concept of an active role of Si in the resistance of rice against *M. grisea*. On this basis, the capacity of Si-amended rice plants to respond more effectively to *M. grisea* attack through the production of momilactones must be regarded as a potentially important factor in the reduction of rice blast severity. This indicates that Si is involved in a more complex defense mechanism in rice than simply the formation of a physical barrier to penetration by *M. grisea*.

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