Silicon-Mediated Accumulation of Flavonoid Phytoalexins in Cucumber

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ABSTRACT

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The controversial role of silicon in plant disease resistance, described mostly as a passive mechanical protection, has been addressed. Conclusive evidence is presented that silicon is involved in the increased resistance of cucumber to powdery mildew by enhancing the antifungal activity of infected leaves. This antifungal activity was attributable to the presence of low-molecular-weight metabolites. One of these metabolites,

described here as a phytoalexin, was identified as a flavonol aglycone rhamnetin (3,5,3',4'-tetrahydroxy-7-O-methoxyflavone). This is the first report of a phytoalexin for this chemical group in the plant kingdom and of a flavonol phytoalexin in cucumber, a chemical defense long believed to be nonexistent in the family Cucurbitaceae. The antifungal activity of leaf extracts was better expressed after acid hydrolysis, extending to another plant species the concept that some phytoalexins are synthesized as glycosylated phytoalexins or their precursors.

Additional keywords: induced resistance, phenolics.

Silicon (Si) is the second most abundant atom in the earth's crust. Biologically, it is a common but generally minor element in the majority of living organisms, occurring as amorphous silica ($\text{SiO}_2 \cdot n\text{H}_2\text{O}$) and soluble silicic acid ($\text{Si}(\text{OH})_4$) (52). Its physiological essentiality is recognized in several protists and vertebrates, but in higher plants, its biological role is controversial (11,24,35,58, 64). Si is required as a nutrient for normal growth in wetland species of the families Gramineae, Equisetaceae, and some Cyperaceae, but in dicotyledons and other grasses, its role remains elusive (5,36,45,61). There are several hypotheses concerning the role of Si in dicots and nonaccumulator grasses including a positive effect on reproduction, alleviation of metal toxicity and nutrient imbalance, provision of structural rigidity, and increased resistance to fungal diseases such as powdery mildews and root rots (3,24).

The exact means by which Si influences these different physiological processes is unknown. The enhanced resistance of Si-treated host plants to pathogenic fungi has been suggested to be the result of a greater resistance to pathogen penetration of host tissue due to the specific accumulation and polymerization of $\text{Si}(\text{OH})_4$ in cell walls (4,32,42,62). Recent work, however, contended that Si may act by stimulating the natural defense mechanisms of the plant (3). Indeed, Chérif et al. (6) demonstrated that, regardless of Si accumulation (10), several enzymes including chitinases had increased activity in cucumber plants treated with Si and challenged by *Pythium ultimum*. While this finding supported a role of Si in activating induced resistance in cucumber, it failed to explain the precise mechanism by which the plant restricted fungal infection, since chitinases cannot yield a detrimental effect on *P. ultimum* or powdery mildew haustoria, which do not contain chitin (7,29). On the other hand, microscopic studies indicated that cells attacked either by *P. ultimum* or *Sphaerotheca fuliginea* (Schlechtend.:Fr.) Pollacci accumulated phenolic material toxic to fungal structures (9,47), in a manner very much similar to that described for phytoalexins (60).

The participation of phytoalexins in plant resistance is well established for the families Solanaceae and Leguminosae (2,17,20) but not for the family Cucurbitaceae, although it has been actively sought (30,40,53). However, a recent study provided evidence that cucumber plants can, in fact, produce antifungal compounds (16).

In this work, we investigated the possible role of Si as an inducer of disease resistance in the interaction between cucumber and *S. fuliginea*, causal agent of powdery mildew. Our objective was to find a link between Si and the expression of defense reactions in plants, using the cucumber-*S. fuliginea* interaction as a model since it is one of the most studied with regard to Si amendments (3). To achieve our goal, we wanted to target a defense response actively contributing to resistance of cucumber to *S. fuliginea*. Mechanisms of disease resistance of the family Cucurbitaceae against powdery mildews have been scarcely investigated. Deposition of autofluorescent material, possibly of phenolic nature, and lignification of cell walls have been recorded during *S. fuliginea* infection of *Cucumis melo* and *Cucumis sativus* and are believed to play a major role in limiting pathogen development in race-specific resistance, as well as induced resistance (13,15). Similar host responses were reported for the hop-*S. humuli* pathosystem (25), while in the rose-*S. pannosa* interaction, resistance was associated with elevated phenolic derivatives in the infected cells (14). Likewise, the accumulation of autofluorescent compounds derived from the phenylpropanoid pathway has been proven to largely participate to the background and the race-specific resistance of temperate cereals against *Erysiphe* spp. (1,43,65). This potential role of phenolic compounds in plant resistance against powdery mildews is further emphasized by microscopic observations in which epidermal cells of Si-treated cucumber plants accumulated phenolic-like material detrimental to fungal cells (48).

Thus, the implication of Si inducing defense mechanisms was verified by determining antifungal activity in cucumber leaves (*Cucumis sativus* L.), with special attention to phenolic derivatives. We provide conclusive evidence that Si plays an active role in disease resistance by inducing the production and accumulation of antifungal low-molecular-weight metabolites during pathogenesis in cucumber. These Si-induced metabolites represent the first evidence of flavonoid phytoalexins in cucumber and, to our knowl-

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edge, of flavonol-derived phytoalexins in plants. These results, together with previous microscopic observations (48), suggest that synthesis of phytoalexins represent a critical defensive element of cucumber plants against powdery mildew.

MATERIALS AND METHODS

Plant material and pathogen inoculation. Cucumber seeds (*Cucumis sativus*) cv. Mustang were sown in rockwool cubes and watered with distilled water until emergence. Nutrient solutions containing 0 or 100 ppm of Si were prepared as previously described (8). Emerging seedlings were transferred individually to 500-ml glass jars filled with the Si- or Si+ nutrient solution and placed in a growth chamber (16-h photophase of approximately $145 \mu\text{E m}^{-2} \text{s}^{-1}$, 23°C day/ 19°C night) for the duration of the experiment. Plants were grown until the third leaf had fully expanded and were subsequently inoculated with the pathogen. The experiment included four treatments: noninfected Si- and Si+ plants (Si-PM-, Si+PM-), and infected Si- and Si+ plants (Si-PM+, Si+PM+). Each treatment was replicated twice.

Powdery mildew conidia were collected from naturally infected leaves of cucumber plants. Source leaves were shaken 24 h before conidial harvest to ensure a high level of viability of the inoculum. Conidia were collected in 500 ml of distilled water to which two drops of Tween 80 was added. Cucumber leaves were sprayed with the conidial suspension (approximately 1,000 conidia per ml) until run-off. At this stage, noninfected control plants were transferred to a similar growth chamber to avoid cross-infection.

For each treatment, the first four inoculated leaves of three plants were harvested at intervals of 0, 24, 48, 72, and 96 h and 6, 8, and 10 days after inoculation. Leaves were immediately frozen in liquid N_2 and stored at -80°C until analysis.

Analysis of phenolic compounds and fungitoxic activity of leaf samples. A modified extraction method of Derks and Buchenauer (21) to determine free and glycosidic-bound phenolics was used to compare the patterns of phenolic compounds in leaf extracts. Harvested foliar material was ground to a fine powder in liquid N_2 and extracted in 80% MeOH (10-g fresh weight per 100 ml). Homogenate was filtered, and the residue was concentrated under reduced pressure at 40°C . The aqueous residue was adjusted to pH 2.0 and partitioned against light petroleum ether to remove lipophilic compounds. The aqueous phase containing the phenolic constituents was further partitioned against ethyl acetate (vol/vol) and then subjected to acid hydrolysis (4 N HCl [vol/vol]) for 1 h at 100°C . The hydrolysate was cooled and partitioned against ethyl acetate. The two ethyl acetate fractions obtained were dried and the residues, designated as 'free phenolic fraction' and 'conjugated phenolic fraction,' respectively, were resuspended in absolute methanol (2.5-g fresh weight per ml).

Samples (400 μl for free phenolic fractions and 800 μl for conjugated phenolic fractions) were first analyzed on aluminum-baked silica gel thin-layer plates (Silica Gel 60F₂₅₄, VWR Scientific, Mississauga, Ontario, Canada) developed with dichloromethane/EtOAc (6:4, vol/vol). Patterns of compounds from both fractions were first recorded under UV light (254 and 366 nm) and their retention values (R_f) calculated.

Because of its biotrophic status, *S. fuliginea* could not be used directly on thin-layer plates to analyze antifungal activity of cucumber leaf extracts. Instead, *Cladosporium cucumerinum* Ellis & Arth., responsible for cucumber scab and routinely used for screening antifungal molecules because of its dark pigmentation, was selected as a biotest pathogen. Antifungal activity was directly evaluated on developed thin-layer plates. After cautious drying, plates were overlaid with a conidial suspension of *C. cucumerinum* in half potato dextrose agar and subsequently incubated for 48 to 72 h in a humid chamber at room temperature. Zones of fungal growth inhibition appeared as white spots against a grey background formed by mycelium and spores of *C. cucumerinum*.

Conjugated phenolic fractions (10 μl) were further analyzed using high-performance liquid chromatography (HPLC) on a C-18 reverse-phase column as described previously (16).

Isolation and identification of phytoalexins. Leaves from infected cucumber plants fed with 100 ppm of Si, collected after 48, 72, and 96 h of infection, were pooled (approximately 100-g fresh weight) and submitted to the phenolic extraction method described previously. The conjugated phenolics fraction obtained was subjected to several steps of purification including preparative thin-layer chromatography (TLC) (0.5-mm silica gel plates) and open silica gel column chromatography, using sequentially solvents of increasing polarity (hexane-dichloromethane-ethylacetate-methanol). Sets of fractions obtained after each purification step were analyzed for antifungal activity using the *C. cucumerinum* bioassay to eliminate inactive fractions.

Active fractions were subjected to standard spectral analysis (47), and one prominent fraction was purified sufficiently for identification. UV spectra were recorded on a UV-Vis Beckman spectrophotometer (Beckman Instruments, Inc., Montréal, Quebec, Canada). Nuclear magnetic resonance (^1H NMR) spectrum was recorded on a Bruker AMX-300 spectrometer (Bruker Instruments Inc., Billerica, MA); the sample was dissolved in deuteriated methanol. The structure of the purified fraction was further confirmed by cochromatography with an authentic standard, using HPLC and TLC (dichloromethane/EtOAc [6:4, vol/vol]).

RESULTS

Effect of Si on powdery mildew development. The restrictive effect of Si on powdery mildew was observed 5 days after inoculation, as the first signs of powdery mildew colonies appeared on Si--inoculated plants only. At that time, Si-PM+ plants showed mycelium growth on both cotyledons and leaves. The first visible signs of *S. fuliginea* development appeared on Si+PM+ plants 3 days later. By 10 days, colonies of *S. fuliginea* were clearly present on inoculated Si- plants, covering 15% of the surface area, whereas inoculated Si+ plants averaged only 4% of the infected surface area. In addition, secondary infections had developed on newly formed leaves of Si- plants, but not on leaves of Si+ plants. No colonies were observed on either Si- or Si+ leaves of non-inoculated plants.

Analysis of phenolic compounds and fungitoxic activity of leaf samples. Separation of the compounds contained in the free phenolic fractions on silica gel thin-layer plates revealed no distinct difference among treatments when observed under UV light or after antifungal activity was assessed (data not shown).

Analysis of aglycones (obtained from the acid-hydrolyzed phenolic fractions) highlighted several compounds presenting different characteristics under UV light (Fig. 1). Comparison of control plants (PM-) showed a remarkably similar pattern of phenolic compounds both in Si- and Si+ treatments throughout the experimental period (Fig. 1A). This pattern was essentially identical over time in Si-PM+ plants, but a striking difference appeared in the UV fluorescence profile of infected plants as a result of Si fertilization (Fig. 1B). This difference was characterized by the accumulation of distinctive bands between $R_f = 0.4$ and 0.6 specific to Si+PM+ plants that appeared in leaf samples within 24 h of inoculation and remained visible until 96 h after infection (Fig. 1B, arrowheads). The UV behavior of these bands suggested the presence of flavonoids. By comparison, controls and Si-PM+ plants only displayed a greenish fluorescent zone in a similar region of the chromatogram (Fig. 1A and B, arrow). Interestingly, the presence of these distinctive bands subsided in samples observed 6 days after inoculation, and at that time, the pattern of phenols resumed an appearance similar to that observed earlier in control and Si-PM+ plants (Fig. 1C).

When tested for their antifungal activity, leaf extracts separated on thin-layer plates of controls (Si-PM- and Si+PM-) never

showed major differences, nor did those of Si-PM+ plants (Fig. 2A and B) regardless of the sampling time, an observation that corroborated their similar UV profiles. By contrast, the accumulation of distinctive bands between 24 and 96 h after inoculation in Si+PM+ plants gave rise to an extensive zone of fungitoxicity (Fig. 2B, open arrow). Using lower concentrations of extracts, the strongest fungitoxic activity was associated with the two upper bands (data not shown). In addition, another unique zone of fungitoxicity appeared at $R_f = 0.0$ between 24 and 96 h in Si+PM+ plants (Fig. 2B, arrow). As expected from their similar UV profiles, no difference in antifungal activity was observed between leaf samples of Si-PM+ and Si+PM+ plants collected 6 days after inoculation (Fig. 2C).

Isolation and identification of phytoalexins. The nature of the metabolites induced by Si was examined using Si+PM+ leaf extracts. After extensive purification, we were able to isolate one compound in sufficient quantity for characterization. This compound was identified as rhamnetin (3,5,3',4'-tetrahydroxy-7-O-methoxyflavone) (Fig. 3) based on NMR spectra similar to those described by Mabry et al. (46) for rhamnetin and on UV spectra consistent with the work of Markham (47). Confirmation of the structure was further achieved by HPLC and TLC cochromatography with a standard. When plated individually, this compound had an R_f corresponding to the upper band with strong antifungal activity particularly well defined in total leaf extracts of the Si+PM+ treatment (Fig. 1B), it presented the same UV characteristics, and it also exhibited strong fungitoxicity. When biotested separately, rhamnetin showed antifungal activity at a quantity as low as 20 μ g, thus ranging within the physiological level reported for other phytoalexins.

Presence of rhamnetin within total extracts was further verified by HPLC comparison of leaf extracts from control Si-PM- and

Si+PM+ plants (Fig. 4). The chromatograms corroborated the lack of signal for the presence of rhamnetin in control plants (Fig. 4A) and its distinctive appearance in Si+ plants subjected to an infection by powdery mildew (Fig. 4B). In addition, they revealed the presence or increase in concentrations of several peaks in Si+PM+ plants (Fig. 4B, arrowheads).

DISCUSSION

For a number of years, speculations have surrounded the mode of action of Si as a factor contributing to disease resistance (3,24). Our results provide conclusive evidence that Si increases the accumulation of specific fungitoxic metabolites in a pattern typical of phytoalexins (2,17,20). Thus, we suggest that this emergence of fungitoxic compounds stimulated by Si contributes to the enhanced resistance of cucumber to powdery mildew, which would explain, in part, its reported prophylactic properties (3).

This first finding of a flavonol phytoalexin in the family Cucurbitaceae is of particular interest, since no phytoalexin was recorded to be part of the mode of resistance of cucumber despite numerous studies conducted on the mechanism of systemic acquired resistance (SAR) with this plant (30,31,50,57). However, it is consequent with the recent report by Daayf et al. (16) who observed the accumulation of several unidentified antifungal compounds in cucumber leaves following a prophylactic treatment with a plant extract. Since we also found an accumulation of several postinfectious antifungal compounds during the Si-induced resistance, it appears that cucumber does synthesize phytoalexins in response

Fig. 1. Thin-layer chromatograms taken under long-wave UV light (366 nm) of acid hydrolyzed phenols extracted from cucumber leaves (conjugated phenolic fractions). Plates were developed with $\text{CH}_2\text{Cl}_2/\text{EtOAc}$ (6:4, vol/vol). **A**, Comparison of the phenolic pattern between extracts from control plants (PM-) fertilized (Si+) or not (Si-) with soluble silicon. Patterns appear similar namely at $R_f = 0.6$. **B**, Comparison of the phenolic pattern between extracts from plants inoculated with *Sphaerotheca fuliginea* (PM+) and fertilized (Si+) or not (Si-) with soluble silicon. Samples were taken 96 h after inoculation. Distinctive bands (arrowheads) specific to inoculated leaves of silicon-fed cucumber plants (Si+PM+) can be observed. A similar pattern was found in samples taken 24, 48, and 72 h after inoculation. **C**, Comparison of the phenolic pattern between extracts from plants inoculated with *S. fuliginea* (PM+) and fertilized (Si+) or not (Si-) with soluble silicon. Samples were taken 6 days after inoculation. Bands present in **B** have now disappeared and been replaced by the same band found in all other extracts (arrow).

Fig. 2. Thin-layer chromatograms of Figure 1 after development of the bioassay for assessing the fungitoxicity of extracted phenolics. Plates were sprayed with a conidial suspension of *Cladosporium cucumerinum* in 20 g/liter of potato dextrose agar. Plates were then incubated for 48 to 72 h in a humid chamber at room temperature. Zones of inhibition appear as whitish spots against a grey background formed by mycelium and spores of *C. cucumerinum*. **A**, Comparison of fungitoxic activity between extracts from control plants (PM-) fertilized (Si+) or not (Si-) with soluble silicon. Patterns appear similar with a light background activity. **B**, Comparison of fungitoxic activity between extracts from plants inoculated with *Sphaerotheca fuliginea* (PM+) fertilized (Si+) or not (Si-) with soluble silicon. Samples were taken 96 h after inoculation. The zone corresponding to the induced distinctive bands in Figure 1B blend into an extensive antifungal region (open arrow). Another zone of intense fungitoxicity specific to Si+PM+ plants was at $R_f = 0.0$ (arrow). **C**, Comparison of fungitoxic activity between extracts from plants inoculated with *S. fuliginea* (PM+) and fertilized (Si+) or not (Si-) with soluble silicon. Samples were taken 6 days after inoculation. Antifungal activity is now minimal and comparable to controls in **A**. All results are representative of an analysis performed on three different plants for each treatment.

to an eliciting treatment, and that this response is chemically complex, with the emergence of a variable set of antifungal compounds. This presence of phytoalexins in cucumber was unveiled in both cases with a particular method of analysis of antifungal activity that includes acid hydrolysis of the extracts. This hydrolysis is responsible for the liberation of aglycones from their glycosidic substitutions. Conjugation of chemicals by plants is generally considered as a means of deactivation of otherwise biochemically active compounds (44,51,56). In light of recent findings that preformed and neosynthesized conjugates, both play an important role in the biosynthesis of phytoalexins (26,27,33); such a hydrolysis appears to be of primary importance in the antifungal activity analysis, as it reveals all the elements of plant chemical defense. In the current study, we demonstrated that cucumber possesses such a pool of conjugates that was raised or modified by Si treatment, and from which new antifungal metabolites could be released. The Si-induced compounds were not detected in Si-PM- plants, suggesting that they derive essentially from neosynthesized conjugates. This discovery of phytoalexins in cucumber is significant, as it opens an area of research ignored in the analysis of SAR in cucurbits. Incidentally an induced defense reaction, unrelated to lignification, has been recently reported to occur during cucumber SAR, which reduced the development of the pathogen inside the

tissues (19,39). This could well be explained by a direct antimicrobial activity in the infected cells, expressed by the accumulation of the same kind of antifungal compounds found in the current study.

The phytoalexins identified in this work belong to the group of flavonols that represents, to our knowledge, the first report of a phytoalexin within this chemical group. Several flavones and flavonols have been tested in antimicrobial screening studies (49,63), and they have been reported to occur as preformed antifungal compounds, but never as postinfectious metabolites (28,59). This finding adds a new class of flavonoids to the chemically diverse family of phytoalexins that already encompasses the well-known isoflavonoids among its representatives (22). More importantly, with the recent developments of genetic manipulation of plants for flavonoid synthesis (23), this provides further evidence to the importance of these secondary metabolites in crop improvement.

The detection and analysis of antifungal activity in infected cucumber leaves completes microscopical study of the cucumber-powdery mildew interaction, in which Si prompted the accumulation of a phenolic-like material deleterious to the haustorium (48). The isolated phytoalexin rhamnetin, together with other antifungal compounds induced by Si, are probably responsible for the buildup of this antifungal material, which also reached its highest point at 96 h postinfection. A similar case of resistance, in which accumulation of an autofluorescent material, possibly of phenolic nature, could be associated with deteriorated haustoria, has already been reported for resistant genotypes of *Cucumis melo* to *S. fuliginea* (12,13). Taken together, these results suggest that synthesis of phytoalexins could be a determinant in the resistance of the family Cucurbitaceae to powdery mildews. It is interesting to note that resistance to powdery mildews has also been closely linked with phenolic compounds in other plants. Thus, in rose, resistant and susceptible cultivars differed in their capacity to synthesize large amounts of soluble phenolic compounds, while deposition of callose and lignin were similar (14). Moreover, a phytoalexin activity was suggested to be related to primary and secondary resistance in barley leaves infected with *E. graminis* f. sp. *hordei* (1). There-

Fig. 3. Chemical structure of the phytoalexin rhamnetin isolated from the methanolic extract of cucumber leaves infected with *Sphaerotheca fuliginea* and fertilized with soluble silicon.

Fig. 4. High-performance liquid chromatography analysis of acid-hydrolyzed phenolics (conjugated phenolic fraction) from **A**, control (Si-PM-, 0 h) and **B**, infected Si-treated (Si+PM+) cucumber leaves 96 h after inoculation. Several peaks appear or increase in Si+PM+ leaves (arrowheads). The purified and identified phytoalexin flavonol rhamnetin is absent from the control (the small peak at approximately 97 min is not rhamnetin), while its accumulation is evident in the Si+PM+ leaf extract. Another peak corresponds to *p*-coumarate methyl ester (CME), one of the compounds previously reported to increase in infected cucumber plants treated with plant extracts (16). Retention times: CME, 91.9 min; rhamnetin, 96.8 min.

fore, phytoalexin synthesis could represent a defense reaction essential in resistance of plants to powdery mildew, especially in the case of postpenetration resistance, in which haustoria would be the ideal target for phytoalexin fungitoxic activity.

By providing evidence that Si amplifies the chemical defense of cucumber, this work raises the challenge to understand the physiology of Si in plant resistance and also to reexamine its role in monocots, in which its known prophylactic properties are still attributed to a passive effect. As already suggested, resistance induced by Si presents similarities with SAR. In both cases, the defense potentials of the plant are increased and maximally expressed following infection. However, Si-induced resistance is quickly lost when the Si source is removed (54), while SAR is characterized by a long-lasting effect (18,41). This difference could be the result of the properties of Si itself in plants, in which it is required in a soluble state to have its beneficial effect on disease resistance, but is constantly being transformed to a polymerized form (54). Recently, it was suggested that the efficacy of a chemical to condition SAR could be related to a high resistance to degradation, leading to its continuous presence under an active form in the plant (37). Also, salicylic acid loses its inducing properties by its natural conjugation in the plant (51). By analogy, polymerization of Si leads to its inactivation as an inducer of resistance. Thus Si-induced resistance and SAR could be more similar than previously stated (34,38), as illustrated by the work of Schneider and Ullrich (55).

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