Effect of Silicon Absorption on Soybean Resistance to *Phakopsora pachyrhizi* in Different Cultivars

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Abstract

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Silicon (Si) is recognized for its prophylactic role in alleviating diseases when absorbed by plants and has been proposed as a possible solution against soybean rust, caused by *Phakopsora pachyrhizi*. However, little is known about its potential effects on soybean (*Glycine max*) because the plant's ability to absorb Si is poorly defined. In this work, our objectives were to evaluate and quantify the absorption of Si in leaves of different soybean cultivars and to determine if such absorption was able to enhance resistance to soybean rust. In a first set of experiments with cv. Williams 82, hydroponic plants were supplied or not with Si and inoculated with urediniospores of *P. pachyrhizi*. Chemical analyses revealed no significant differences in the plants' Si

content regardless of the treatment, which translated into no effect on rust incidence. However, in a second set of experiments with different cultivars, plants of Korean cultivar Hikmok sorip absorbed nearly four times more Si than those of Williams 82. At the same time, plants from this cultivar exhibited a near absence of disease symptoms when supplied with Si. This resistance appeared to be the result of hypersensitive (HR) reactions that were triggered when plants were fed with Si. These results support the concept that a plant's innate ability to absorb Si will dictate the benefits conferred by a treatment with Si and provide evidence that Si can protect soybean plants against soybean rust through mediated resistance.

Asian soybean rust, caused by *Phakopsora pachyrhizi* Syd. & P. Syd., was initially discovered and identified in Asia in 1902 (16). In 2001, the first occurrence of the disease was reported in Paraguay (12), and since then, severe yield losses have been recorded in South America, where soybean (*Glycine max*) is the largest crop by planting area (22,37). Soybean rust was first reported in the United States in 2004 and since then has been reported in 20 U.S. states (31), and its spread has progressed northward such that urediniospores were reported in Ontario, Canada in 2007 (34). Currently, repeated fungicide treatments are applied to control the disease, generating important economic losses and potential environmental damage. As commercial cultivars resistant to *P. pachyrhizi* are not yet available (14), research efforts are needed to develop alternative methods of combating this pathogen.

The application of silicon (Si) has been associated with many benefits to agricultural crops, namely as a preventive measure against a number of fungal diseases (8). The presence of amorphous silica ($\text{SiO}_2 \cdot n\text{H}_2\text{O}$) or soluble Si in plant shoots has been shown to enhance resistance to *Sphaerotheca fuliginea* and *Pythium* sp. in cucumber (3,9,23), *Blumeria graminis* f. sp. *tritici* in wheat (1,27), and *Magnaporthe grisea* in rice (29) in numerous studies. For reasons still debated, it appears that Si is particularly effective against biotrophic fungi (10). Given this evidence and the limited options to control soybean rust, it stands to reason that investigation of the potential of Si as a prophylactic treatment against other biotrophic pathogens such as fungi-causing rust diseases is warranted. In a previous attempt, foliar applications of Si were reported to reduce the intensity of soybean rust (28), but this work could not correlate the efficacy with Si absorption in soybean, a key component in reproducible and durable control. In a

more recent study, Lemes et al. (21) also reported encouraging results with Si and soybean rust. As previous work would suggest, they had better success with soil amendments than with foliar applications, showing that Si concentration only increased in plants fed Si through the roots. The authors could not explain how Si protected the soybean plants or why the two cultivars tested absorbed Si differently and recommended further research toward resolving these issues.

It is well known that plants will absorb Si to varying degrees (7,17,19,25) and there is a direct correlation between their ability to absorb this element and the benefits they draw from it. Little is known about these benefits for soybean because this plant's ability to absorb Si is poorly understood. Over the past decades, some studies have tried to establish the Si uptake capacity of soybean, showing that it will accumulate Si in its shoots in the presence of soluble Si in the soil solution (6,17,35). However, techniques available today to measure the silicon content of a plant, such as dosage by inductively coupled-plasma optical emission spectrometry (ICP-OES), are extremely precise and reliable (11). Considering the importance of the soybean-*P. pachyrhizi* interaction, we were interested in establishing the extent to which soybean was able to absorb Si and if Si could have a preventive effect against soybean rust.

The objectives of the present study were: (i) to evaluate and quantify by ICP-OES and X-ray microanalysis the absorption of Si in leaves of different soybean cultivars, and (ii) to evaluate if the absorption of Si by different cultivars varied in their resistance to soybean rust.

Materials and Methods

Plant material and growth conditions in hydroponics. The soybean cultivar Williams 82 was used as a highly susceptible control (30). Also used were the cultivars Hikmok sorip (Korea), Majesta (Canada), and Cristalina (Brazil). Seeds were germinated in plastic petri dishes (150 × 15 mm; Fisher Scientific) containing filter paper wetted with sterile distilled water. After 3 days, seedlings were transferred to rockwool in 9-cm pots (Hydroculture Dionne) at a rate of two seeds per pot. The plants were grown in a greenhouse maintained at 25/20°C with a relative humidity of 65% and a photoperiod of 14 h.

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Hydroponic systems were used to control Si dosage and mitigate external Si contamination. Roots were immersed in Hoagland solution for 15 min every 45 min. The final concentration of each trace mineral in the basal Si-free solution was (mg/liter): KNO₃ (101), KCl (221), CaCl₂ (235), K₂HPO₄ (17), MgSO₄·7H₂O (250), MgCl₂·6H₂O (145), H₃BO₃ (0.28), MnCl₂·4H₂O (0.4), CuSO₄·5H₂O (0.2), ZnSO₄·7H₂O (0.2), Na₂MoO₄·2H₂O (0.15), CoSO₄·7H₂O (0.05), Fe as Fe-EDTA (40), all pH 6.2. Buffering capacity was provided by adding 1.0 mM MES (pH 6.2).

Soluble Si was fed to the plants by amending the Hoagland solution with (i) no silicon (control), (ii) 0.4 mM, or (iii) 1.7 mM Si in the form of potassium silicate (Kasil #6, 23.6% SiO₂, National Silicates). Potassium levels were adjusted in the standard solution to compensate for the input of K from the Kasil #6. Treatment of the seedlings with the different solutions began 2 weeks after seedling emergence (day 14). Solutions were replaced every week.

Experiments with cv. Williams 82. Repeated experiments ($r = 2$) were initially conducted with plants of Williams 82. The two treatments (control and 0.4 mM Si) contained six replications (one plant each) that were arranged in two completely randomized blocks (three replications per block) ($n = 24$).

Experiments with different cultivars. The second series of experiments consisted of two treatments (control and 1.7 mM Si) and four soybean cultivars (Cristalina, Hikmok sorip, Majesta, and

Williams 82) that were arranged in two completely randomized blocks with six replications per cultivar (three replications per block). This experiment was repeated over two seasons: fall and winter ($n = 192$).

Inoculation with *P. pachyrhizi*. The inoculum was prepared with spores obtained from plants grown in a greenhouse previously inoculated with a fungal isolate obtained from the North Florida Research and Education Center (Quincy, FL). One day prior to inoculation, urediniospores stored at -80°C were heat shocked for 5 min at 40°C and hydrated for 16 h at 100% relative humidity in a dew chamber. Urediniospores were then suspended in sterile distilled water containing 0.01% Tween 20 (vol/vol), and a concentration of 2×10^4 urediniospores per ml was determined with the aide of a hemacytometer. A volume of 2.5 ml per plant was sprayed onto soybean leaves with an atomizer bottle (Magasin Central d'Énergie). The leaves were covered with 12-lb plastic bags (Bertrand & Fils Inc.) overnight (16 h) in the greenhouse at 20°C and 90% relative humidity to maintain leaf wetness. After 16 h, the plastic bags were removed and the inoculated plants were grown at $25/20^{\circ}\text{C}$ with a relative humidity of 65%. These experiments were conducted under research permit number P-2009-02974 of the Canadian Food Inspection Agency (CFIA).

X-ray microanalysis of Si deposition. Si deposition within soybean leaves was determined by scanning electron microscopy (SEM) coupled with X-ray microanalysis mapping. For each cultivar, two leaf samples per treatment were collected the third week after commencing silicon treatment (day 35). For each treatment, the middle leaflet of the third trifoliate leaf from the growing tip of the plant was collected. Leaf samples were prepared as described by Guével et al. (15), and SEM analyses were performed using a CAMECA SX-100 Universal EPMT microscope (Cameca Instruments Inc.) operating at a voltage of 15 kV and a current of 20 nA.

Silicon content in plant leaves. Inductively coupled plasma optical emission spectrometry (ICP-OES, Jobin-Yvon Horiba JY2000-2) was used to measure silicon content of soybean plants. The third trifoliate leaves from the growing tip of the plant were collected 3 weeks after the beginning of silicon amendment (day 35). For each cultivar, three leaf samples per treatment were obtained. Leaves were freeze-dried, ground to a powder using mortar and pestle, and prepared as described by Côté-Beaulieu et al. (4).

Disease assessment. Observations were made every other day on the first inoculated trifoliate leaves starting 2 days after inoculation. Rust severity was based on the percentage of the leaf area covered by lesions with pustules, using an evaluation scale from 0 to 8 in which a rating of 0 = no disease, 1 = up to 2.5% of leaf area affected, 2 = 2.5 to 5%, 3 = 5 to 10%, 4 = 10 to 15%, 5 = 15 to 25%, 6 = 25 to 35%, 7 = 35 to 67.5%, and 8 = 67.5 to 100% (Bayer CropScience, 2006). The disease scores for the two plants from each pot were averaged to give one mean score per pot per day. Midpoint values of the disease rating scale were used to plot disease progress curves. In order to compare disease development among treatments, the area under the disease progress curve (AUDPC) was calculated as:

$$\text{AUDPC} = \sum_{i=1}^{n-1} [(y_i + y_{i+1}) \div 2] (t_{i+1} - t_i)$$

where y_i = rust severity at the i th observation, t_i = time (days), and n = total number of observations (32).

Statistical analysis. Analysis of variance was used to determine significant treatment effects using JMP8 (SAS Institute Inc.). Silicon content in plant leaves and disease assessment (soybean rust development over time and AUDPC) were compared ($P \leq 0.05$) using Fisher's test.

Results

Experiments with cv. Williams 82. X-ray microanalysis showed very little Si deposition in leaves of control soybean plants, thus confirming that the hydroponic system was efficient at minimizing external Si contamination (Fig. 1A). In plants treated with 0.4 mM Si, Si deposition was visually more apparent and mostly

Fig. 1. X-ray microanalysis mapping images from soybean leaves (cv. Williams 82) treated with **A**, control and **B**, 0.4 mM silicon (Si). Silicon deposits are indicated by colors. Red represents the highest concentration of Si and black indicates no Si.

concentrated at the base of trichomes and within cells surrounding them (Fig. 1B).

ICP-OES measurements revealed no significant differences in the overall concentration of Si between plants treated or not treated with Si (1,031 versus 1,002 ppm). On average, the presence of Si accounted for ca. 0.1% on a dry weight basis, which would classify soybean Williams 82 as a low accumulator.

For disease assessment, first symptoms for both treatments were observed on soybean leaves 9 days after inoculation with *P. pachyrhizi*. Regardless of the treatments, all plants produced tan-colored lesions (designated Tan reactions), indicating a highly susceptible reaction, as opposed to reddish brown lesions (designated RB reaction), indicating a form of resistance according to the scale used in this study. The disease progressed slowly over the next 2 to 3 weeks and reached a severity plateau between 3 and 3.5 on our rating scale. Area under disease progress curve (AUDPC) showed no significant difference between the treatments. Based on our results, Si amendments did not appear to influence the severity of rust incidence on Williams 82.

Experiments with different cultivars. X-ray microanalysis of soybean plants grown in a standard nutrient solution without Si amendments showed no visible Si deposition (results not shown). For the four cultivars treated with 1.7 mM, our observations revealed the same pattern of Si deposition noted in Figure 1 whereby Si was mostly concentrated in and around the trichomes (Fig. 2).

However, differences in Si accumulation were observed among the cultivars tested, and in particular, Hikmok sorip (Fig. 2A) appeared to have a denser and more regular pattern of Si presence in leaf cells.

Considering that Si concentration *in planta* cannot always be estimated accurately using X-ray microanalysis, shoot content of Si was quantified by ICP-OES analysis (Fig. 3). ANOVA on the Si concentration data revealed a significant effect of treatment ($F = 160.19$, $P < 0.0001$) for the four cultivars tested. Hikmok sorip accumulated significantly more Si than all the other cultivars, a result that did concur with the X-ray microanalysis (Fig. 2A to D). Cristalina had an intermediate level of Si accumulation. Williams 82 and Majesta were the lowest accumulators, but Williams 82 did absorb more Si in this experiment when treated with the 1.7 mM Si solution. This result was attributed to the higher Si concentration in the treatment solution and to warmer conditions in the greenhouse that favored evapo-transpiration and thus translocation of Si.

Initial assessment of rust severity was done 10 days after inoculation, when the first symptoms were observable on plants (Fig. 4). In control plants, all cultivars produced tan-colored lesions typical of a susceptible reaction. From the onset, Hikmok sorip was the most susceptible and maintained a significantly higher severity of disease throughout the evaluation period (Fig. 4A). For its part, Williams 82 was the least infected and showed considerably less disease symptoms than in the first experiment. AUDPC for each

Fig. 2. X-ray microanalysis mapping images from soybean treated with 1.7 mM silicon (Si). Images **A** to **D** represent different soybean cultivars: **A**, Hikmok sorip; **B**, Cristalina; **C**, Majesta; and **D**, Williams 82. Si deposits are indicated by colors. Red represents the highest concentration of Si and black indicates no Si.

season and each cultivar is shown in Table 1. Disease severity was more pronounced in the winter than in the fall season on two of the tested cultivars, as higher condensation always prevails on the leaves of greenhouse plants in the colder winter months. However, the trends were very similar in both seasons in terms of the effect of Si on disease severity.

When plants were treated with 1.7 mM Si, Hikmok sorip displayed a strong resistance to the disease by producing symptoms typical of a hypersensitive reaction (HR) (Fig. 4B and Fig. 5A). Pustules (uredinia) were not found on any of the inoculated plants (Fig. 5B). In the case of Williams 82, Cristalina, and Majesta, all showed tan-colored lesions with pustules, and their AUDPCs were similar to those observed on plants treated with the Si-free solution.

Discussion

Despite the urgent need to find alternative methods to fungicides for reducing soybean rust infections, no single strategy has yielded successful control. In this work, the use of silicon amendments has highlighted new and interesting phenomena that could be exploited in efforts to fend off *P. pachyrhizi*.

The application of Si as a prophylactic measure against plant diseases has proven efficient in a number of plant-pathogen interactions (8). In the case of soybean rust, very few studies have investigated the potential of Si. In most instances, Si was applied to the plant using foliar applications (5,26,28,36). Foliar applications of Si have been found to be much less effective than Si applied as fertilizer because Si is not absorbed by the leaves and any control effects are probably not related to the intrinsic properties of Si *in planta* (15). Lemes et al. (21) reported better results against soybean rust with Si-rich soil amendments than with foliar applications, and their data showed no additional accumulation of Si in plants receiving foliar applications. Under their experimental conditions, plants grown in Si-enriched soil did absorb more Si, but Si amendments raised soil pH significantly, which affected the soil-extractable Ca and Mg concentrations. In addition, plants from one cultivar appeared to vary in their ability to absorb Si whether they were grown in the field or in a greenhouse (21). For these reasons, in our experiments, Si was fed to the plant through a hydroponic solution amended with Si and buffered at pH 6.2, in an attempt to determine with precision if soybean cultivars varied in their ability to absorb Si and if and how Si could have an effect on rust suppression.

As a first approach, we used X-ray microanalysis images to detect the presence of Si in leaves of Williams 82. Si deposition within the leaves followed a particular pattern and tended to accumulate in silicaphile cells, as proposed by Blackman (2). Most of

these cells are concentrated around the trichomes. This accumulation around the trichomes is coherent with the concept that Si is translocated to the plant shoots following the evapo-transpiration stream and that water is mostly lost through the stomata and trichomes (18,33).

While X-ray microanalysis images gave a qualitative description of Si deposition, ICP-OES analysis has become the most reliable method to measure Si concentrations in biological material, as the data obtained present less variance than colorimetric methods for instance (11). The results of the ICP-OES analysis revealed that in spite of the apparent differences in Si accumulation indicated by X-ray microanalysis (Fig. 1), the total Si content in plant shoots was not significantly different, whether the plants were treated with Si or not. This result was somewhat surprising considering that soybean has been described previously as a moderate absorber of Si (17). On the other hand, because there is a direct link between the ability of a plant to absorb Si and the benefits derived from it, it stands to reason that we found no significant difference in disease severity between controls and plants treated with Si. It is also quite possible that the concentration used, 0.4 mM, albeit representative of natural conditions (7), was not sufficient to make a difference in suppressing soybean rust under our test conditions.

The results with Williams 82 raised a concern about the Si uptake capacity of soybean plants. It is recognized that each plant species has its own Si uptake capacity, which would be dictated by the presence of specific Si transporters (24). While no such transporters have been identified yet in soybean, the plant is reported to accumulate moderate levels of Si. Because of the divergence between our results and previous reports, we decided to investigate potential differences in Si absorption among various soybean cultivars, using 1.7 mM Si, which is the maximal solubility of Si in water (20). Our analyses did reveal intrinsic differences among the

Fig. 3. Silicon (Si) concentration in shoots of soybean plants grown in Hoagland solution (control) or potassium silicate-amended Hoagland solution (1.7 mM Si). Si content was determined by inductively coupled plasma-optical emission spectrometry (ICP-OES). Values with the same letter did not differ significantly at $P \leq 0.05$ as determined by Fisher's test.

Fig. 4. Soybean rust development over time on soybean plants from different cultivars grown in A, Hoagland solution (control) or B, silicon-amended Hoagland solution. Rust severity was scored on the first trifoliate leaf following the cotyledonal leaves, using an evaluation scale from Bayer CropScience (2006). Score 0 to 8, based on percent leaf area covered by lesions associated with pustules.

Table 1. Area under disease progress curve (AUDPC) for soybean rust on different soybean cultivars treated with control or silicon (Si)-amended solutions^x

Cultivars	AUDPC ^y			
	Fall		Winter	
	Control	1.7 mM Si	Control	1.7 mM Si
Williams 82	13.05 ab	7.1 ab	62.4 b	60.17 b
Majesta	52.67 c	45.08 c	68.67 bc	60.67 b
Cristalina	19.38 b	14.25 b	70.17 bc	81.83 c
Hikmok Sorip	74.79 d	0.0 a	68.2 bc	0.0 a
Factorial analysis ^z (Sum of squares)	Silicon: 11136.99 ** Cultivar: 18737.28 **		Silicon: 3110.08 * Cultivar: 10059.2 **	

^x Values with the same letter within each season did not differ significantly at $P \leq 0.05$ as determined by Fisher's test.

^y Each value is based on pooled data from repeated experiments ($n = 12$).

^z * $P < 0.001$, ** $P < 0.0001$.

Fig. 5. Hypersensitive reaction caused by *Phakopsora pachyrhizi* on soybean cultivar Hikmok sorip grown in a 1.7 mM silicon solution. **A**, Brown specks surrounded by chlorosis, typical of a hypersensitive reaction (HR), on the upper leaf. **B**, Absence of pustules on the lower leaf.

cultivars tested and highlighted interesting phenomena with one cultivar in particular, Hikmok sorip from Korea. Indeed, unlike the three other cultivars that seemed to accumulate Si in typical silicaphile cells, Hikmok sorip accumulated Si in a more homogenous pattern over the whole surface of the leaf. ICP-OES analyses confirmed that Hikmok sorip was the highest Si accumulator among all cultivars tested. It is also noteworthy that plants from Williams 82 did absorb more Si when fed 1.7 mM, a result supporting the notion that 0.4 mM was not high enough in the first set of experiments to make a difference within our experimental conditions. Our results thus show that there exists innate variability within soybean germplasm in terms of Si absorption. Whether this variability is attributable to the presence or efficacy of Si transporters remains to be elucidated.

The most striking observation in line with this differential Si absorption is arguably the fact that Hikmok sorip displayed the highest resistance to *P. pachyrhizi* when fed with soluble Si. This brings further concrete evidence that Si accumulation can indeed have a strong prophylactic role against soybean rust in soybean. Perhaps even more convincing is the fact that plants of Hikmok sorip, following Si treatment and inoculation with urediniospores of *P. pachyrhizi*, displayed symptoms typical of a hypersensitive reaction. This is particularly relevant since Si feeding has been hypothesized to be associated with induced resistance in recent years and results obtained here tend to confirm, at least in part, this phenomenon. Fawe et al. (10) speculated that Si, in the form of silicic acid, could act as an activator of systemic acquired resistance (SAR) in a manner reminiscent to that of salicylic acid. In additional support to that association, the prophylactic role of Si has been particularly manifest against biotrophs such as powdery mildews, a class of pathogens known to be affected by defense responses regulated by the salicylic acid-dependent pathway (13). It thus seems logical, as shown here and previously (21), that a

biotrophic pathogen such as soybean rust would be affected by Si nutrition in plants, and yet this is the first report of the HR phenomenon with Si not only for soybean rust but also for any rust pathogen. The implications of our findings could potentially be manifold, not only in providing an additional control approach against soybean rust but also in opening up new possibilities of exploiting the prophylactic properties of Si.

In conclusion, this study provides a thorough description of Si uptake in soybean plants by relying on the latest techniques of qualitative and quantitative Si analyses; SEM-X-ray microanalysis and ICP-OES. Consequently, our results have highlighted that there exist significant differences in Si absorption within soybean genotypes. These results find their relevance in the observation that Hikmok sorip, the highest Si accumulator, displayed resistance to soybean rust, through clear manifestation of HR when it was fed with Si prior to inoculation. This provides further proof that Si absorption in soybean plants can alleviate infection by soybean rust.

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