Silicon induced resistance against powdery mildew of roses caused by *Podosphaera pannosa*

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Powdery mildew, caused by *Podosphaera pannosa*, is a very common disease in greenhouse potted roses, resulting in poor marketing value and hence economic losses. Alternatives to chemical control are necessary, and therefore the ability of silicon (Si) applied to roots to control the disease was investigated, as well as the mechanisms behind the observed disease reductions. Four genotypes of miniature potted roses representing different genetic backgrounds and susceptibility to disease were studied. Plants were watered with a nutrient solution containing either 3·6 mM Si (100 ppm) supplied as K_2SiO_3 (Si+) or no Si (Si−) before inoculation with *P. pannosa*. Si application increased leaf Si content two- to four-fold compared to control plants. Confocal microscopy showed that Si deposition was larger in Si+ than in Si− plants and that deposition mainly occurred in the apoplast, particularly in epidermal cell walls. Si application delayed the onset of disease symptoms by 1–2 days and disease severity was reduced by up to 48·9%. The largest reduction was found in the two most resistant genotypes, which also had the highest increase in Si uptake. The Si-induced disease protection was accompanied by increased formation of papillae and fluorescent epidermal cells (FEC) as well as deposition of callose and H_2O_2, especially at the sites of penetration and in FEC, which are believed to represent the hypersensitive response. Si treatment reduced powdery mildew development by inducing host defence responses and can therefore be used as an effective eco-friendly disease control measure.

Keywords: induced resistance, miniature potted roses, *Podosphaera pannosa*, powdery mildew, silicon

Introduction

Miniature potted roses are a very important ornamental crop for the floriculture industry. The annual world production is estimated to be more than 100 million plants, and Denmark is the largest producer with about 50% of the world's production (Pemberton *et al.*, 2003). Powdery mildew caused by *Podosphaera pannosa* [syn: *Sphaerotheca pannosa* var. *rosae*] is one of the most widespread diseases of potted roses (Leus *et al.*, 2006). The disease results in poor marketing value for the roses because of the presence of greyish white powdery fungal growth on leaf surfaces causing leaf distortion, curling and premature defoliation. So far, powdery mildew has been managed mainly by synthetic fungicides. However, environmental considerations have necessitated increasing restrictions on the use of pesticides, and therefore environmentally friendly production methods for plant disease suppression need to be developed.

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Silicon is regarded as an essential element for many plant species and Si application has been demonstrated to inhibit various plant pathogens, e.g. *Magnaporthe oryzae* infecting rice (Rodrigues *et al.*, 2004), *Blumeria graminis* f.sp. *tritici* infecting wheat (Bélanger *et al.*, 2003; Rémus-Borel *et al.*, 2005) and *Podosphaera fuliginea* infecting cucumber (Menzies *et al.*, 1991).

Plants take up Si as soluble silicic acid (H_4SiO_4), which is transported via the transpiration stream and subsequently accumulates as insoluble amorphous Si compounds primarily in the apoplast (Bélanger *et al.*, 2003). There are several hypotheses concerning the role of Si in inhibiting fungal infection. Si has been thought to protect through mechanical strengthening of the plant (Fauteux *et al.*, 2005). In accordance with this, the enhanced resistance of Si-treated host plants to pathogenic fungi has been suggested to result from more efficient resistance to pathogen penetration of host tissue, e.g. due to the specific deposition of Si compounds in cell walls as suggested for rice blast (Kim *et al.*, 2002). Recent work also indicates that Si may play a more active role in plant–pathogen interactions by stimulating other plant defence responses such as deposition of phenolic compounds/phytoalexins with antifungal properties and enhanced production of defence-related enzymes

(Bélanger *et al.*, 2003; Rodrigues *et al.*, 2004; Rémus-Borel *et al.*, 2005).

There appear to be no detailed previous reports on Si-induced protection of roses against powdery mildew or other diseases and the current investigation was therefore undertaken to study the ability of Si to control powdery mildew in miniature potted roses. This was combined with studies of quantitative and temporal differences in the infection processes and defence responses against *P. pannosa* with and without amendment with Si in rose genotypes varying in resistance to the pathogen.

Materials and methods

Plants

Four genotypes of miniature potted roses, 99/9496-19, 98/8285-1 (moderately resistant), 95/5166-1 and Smart (highly susceptible), representing different genetic backgrounds and different levels of susceptibility to powdery mildew, were obtained from Poulsen Roser A/S, Fredensborg, Denmark.

Roses were propagated and maintained in a greenhouse at 23°C and 60% RH under natural daylight conditions, with supplementary light (E-Papillon, SON T 600 W). Rose cuttings were placed in 10 cm pots containing the soil mix Pindstrup Substrate A1-389 for roses (Pindstrup Mosebrug A/S), watered and covered with polyacryl and polythene sheets for 2 weeks to maintain high humidity until rooting. Subsequently, the covers were removed and the plants maintained for another 2 weeks in the greenhouse. The plants were pruned for uniformity and moved to a growth chamber with cycles of 16 h light (200 $\mu Em^{-2}s^{-1}$, Philips Master IL-D 36 w/865)/23°C/60% RH and 8 h darkness/20°C/80% RH.

Treatment with silicon

All experiments comprised 32 plants of each of the tested genotypes. For each genotype, 16 plants received 3·6 mM Si (Si+) or no Si (Si−) as a control. Soluble Si was supplied in a nutrient solution containing 3·6 mM Si (Sikal™, 9·1% Si and 25·5% K in the form of potassium metasilicate, K_2SiO_3; Yara Industries). The nutrient content for the 3·6 mM solution was: N (16 mM), P (0·8 mM), K (7·2 mM), Mg (0·8 mM), Ca (3·6 mM), S (0·8 mM), Fe (0·042 mM), Mn (0·021 mM), Zn (0·004 mM), B (0·021 mM), Cu (0·001 mM) and Mo (0·001 mM) (Yara Industries). For the Si− solution (0 mM Si), the nutrient content was essentially the same, except for the following elements: N (13 mM) and K (4·7 mM). The 3·6 mM Si+ solution had a higher K content due to the high K concentration in Sikal™. Since Sikal™ is alkaline, extra nitric acid was added to the Si+ solution to obtain a pH of 5·5–5·7 which slightly increased the N content of the solution. The pH of the Si− solution was adjusted to the same value. The electrical conductivity was 1·9 S m^{-1} for the Si+ solution and 1·6 S m^{-1} for the Si− solution.

After the propagation period (4 weeks), plants were moved to the growth chamber and watered for the first time with the Si+ and Si− solutions, respectively. Plants were subsequently watered for 10 min with the two nutrient solutions every 72 h until disease scoring, a total of 10 times. After 3 weeks in the growth chamber, the plants were inoculated with the pathogen.

Pathogen, inoculation and disease scoring

A single spore isolate of *Podosphaera pannosa* was maintained on the susceptible host genotype Smart in a growth chamber under cycles of 16 h light (200 μE m^{-2} s^{-1}, Philips Master IL-D 36 w/865)/23°C/60% RH and 8 h darkness/20°C/80% RH. The fifth developed leaf from every branch of 7-week-old plants was labelled and inoculated by shaking rose plants infected by *P. pannosa* above the test plants in a closed chamber to reach a defined density of 1–2 conidia mm^{-2}.

Plants were examined daily for onset of the first microscopic symptoms of powdery mildew. Disease severity was determined visually *in planta*, using a magnifying glass, at 4, 5, 6, 7 and 8 days after inoculation (dai) and finally, using a stereo microscope, at 9 dai on detached leaves. At each time point and for each treatment of each rose genotype, disease onset (appearance of the first visible symptoms) was recorded. Furthermore, percent coverage with powdery mildew on the surfaces of 32 individual leaflets was determined and the mean disease severity calculated as the average of the 32 leaflets.

Measurement of Si content in rose leaves

Si content was determined in uninoculated 7-week-old plants of all four rose genotypes (99/9496-19, 95/5166-1, 98/8285-1 and Smart). The fifth leaf from 10 Si+ and 10 Si− plants was oven dried at 70°C for 24 h. Dried tissue was finely ground in a sample mill (Cyclotec 1093; Foss Analytical) to pass through a 40-mesh screen. Si content was determined by a modification of the autoclave-induced digestion procedure of Elliott & Snyder (1991). Briefly, 100 mg of dried leaf sample was placed in a 100 mL polyethylene tube and wetted with 2 mL 50% H_2O_2 and 3 mL 50% NaOH. Each tube was vortexed and covered with a loose fitting plastic cap. The tubes were placed in an autoclave at 138 kPa for 60 min at 126°C. Subsequently, tubes were removed and the total volume equilibrated to 50 mL with distilled water. The Si content was then determined calorimetrically as follows: 1 mL was taken from the digested plant tissue and mixed with 10 mL distilled water. Then, 9 mL acetic acid (20% v/v), 2·5 mL ammonium molybdate (Sigma-Aldrich) solution (100 g L^{-1}, pH 7·0), 1·25 mL tartaric acid (20% v/v) (VWR International) and 0·25 mL of a reducing solution were added. The reducing solution was prepared by dissolving 4 g sodium sulphite, 0·8 g 1-amino-2-napthol-4-sulphonic acid (Merck) and 50 g sodium bi-sulphite in 500 mL water. The tartaric acid was added 5 min after the ammonium molybdate, and 10 min later, the

absorbance was measured in a spectrophotometer (Milton Roy, Spectronic 401) at 650 nm. A standard curve relating the amount of Si to the absorbance at 650 nm was used to determine the Si content in the plant material. The standard curve was made by dilutions of a 1000 ppm Si standard (VWR International) using deionised water. Subsequently, the Si content was calculated, adjusted for weight of plant material used and expressed as ppm silicon in dry matter.

Confocal microscopy for localization of Si

Confocal microscopy was used to study the spatial Si deposition in the fifth developed leaf of genotype Smart both inoculated and uninoculated with the pathogen (7-week-old plants), and treated or untreated with Si. For each treatment combination, three leaves were examined. Prior to examination, epicuticular wax was removed from the rose leaves using the cellulose acetate stripping method (Silcox & Holloway, 1986). A 5% (w/v) solution of cellulose acetate in acetone was applied to the adaxial leaf surface with a small paint brush and allowed to dry, and the resulting film was gently peeled off to remove the surface wax. After wax removal, leaves were stained with the weakly basic amine LysoTracker Yellow HCK-123, a fluorescence dye (Invitrogen, final concentration 1 μM) (Descles *et al.*, 2007). Prior to examination, leaves were stained for 2 h with a 200 nM working solution of the stain after which excess stain was removed. The stained samples were examined in a confocal laser scanning microscope (Leica TCS SP/MP; Leica Microsystems) equipped with an argon laser. The wavelength for excitation was 488 nm. The 'green' fluorescence of the stain was detected using an emission filter of 505–545 nm. A 660–800 nm emission filter was used for the detection of the 'red' autofluorescence of the chloroplasts. The objective used was HC PL APO × 20/0·70 W. For each recording, one stack of 25 images (60 μm) was made. This volume included the epidermis and uppermost mesophyll cell layer. The stack started at the level of the outer periclinal epidermal cell wall after removal of epicuticular wax and image number 3 (epidermis level) and 20 (mesophyll level) were selected for comparison of treatments.

Infection biology of *P. pannosa* and structural responses in rose primed with Si

Infection biology and defence responses were compared between Si-treated and untreated plants of the moderately resistant genotype 99/9496-19 and the highly susceptible genotype Smart. Leaves were collected for microscopy at 24 and 72 h after inoculation (hai) and cleared as described by Shetty *et al.* (2003). The fifth developed leaves were studied using bright field and epifluorescence microscopy (excitation maximum 330–385 nm, dichroic mirror DM 400 nm, barrier filter >420 nm) after staining with 0·1% Evans blue (Sigma-Aldrich) in lactoglycerol (lactic acid:glycerol:water,

1:1:1, v/v) for localization of fungal structures. Three leaves were studied at each time point in each genotype. The number of nongerminated conidia was recorded on each leaf, and subsequently the development of 50 randomly chosen germinated conidia was studied on each leaf (total of 150 germinated conidia per genotype and time point). For each conidium, it was recorded whether it formed appressoria, whether it caused penetration, whether it caused formation of single or multiple fluorescent epidermal cells (FEC) and papillae at penetration sites and whether elongating secondary hyphae (ESH) formed. Penetration was considered to occur when haustoria developed from appressoria or FEC were formed. FEC and papillae were considered to stop infection when no ESH developed from germlings where these responses occurred.

For scanning electron microscopy of *P. pannosa* structures, 10 leaf pieces, 4×7 mm^2, were cut at 5 dai and prepared for electron microscopy as described by Shetty *et al.* (2003). Specimens were examined in a FEI Quanta 200 ESEM Scanning Microscope.

Accumulation of H_2O_2

Accumulation of H_2O_2 was studied by staining with 3,3′-diaminobenzidine (DAB, Sigma-Aldrich) (Shetty *et al.*, 2003). The inoculated leaves of genotype Smart were cut from the plant at 14 and 62 hai and the cut end immediately placed in a DAB solution for 10 h. At 24 and 72 hai, the leaves were cleared as described by Shetty *et al.* (2003). The leaves were examined by bright field microscopy and stained with 0·1% Evans blue in lactoglycerol for identification of fungal structures. A reddish-brown staining at penetration sites indicated accumulation of H_2O_2.

Callose deposition

Inoculated leaves of genotype Smart were harvested from Si-treated and control plants at 72 hai and cleared as described above. Callose deposition was studied by staining with 0·005% aniline blue in 0·15 M K$_2$HPO$_4$ (pH 8·2) for 90 min as described by Shetty *et al.* (2009). In addition, callose was visualized in cleared leaves after staining with aniline blue in a SP5 confocal microscope (Leica Microsystems) using excitation and emission wavelengths of 405 nm and 460–500 nm, respectively.

Statistical analysis

Data from studies of Si content of rose leaves, onset of disease and disease severity represent continuous variables and were analysed by analyses of variance assuming a normal distribution. Variances were stabilized by appropriate transformations when necessary. Data for Si content and disease severity were analysed separately for each genotype as well as in a model with the two factors genotype and treatment. Likewise, data for onset of disease were analysed separately for each time point and

in a model with the three factors time point, genotype and treatment. Data from studies of infection biology represent discrete variables since it was recorded whether or not a certain event took place (e.g. whether a conidium germinated or not, whether a germinated conidium formed appressoria and whether appressoria with successful penetration formed haustoria or not). Consequently, these data were analysed by logistic regression, assuming a binomial distribution (corrected for over-dispersion when present) as described by Shetty *et al.* (2003). For comparison of variables (percentages), odds ratios were calculated using Si− plants (controls) as a reference (odds ratio = 1·00).

Data were analysed by PC-SAS (release 9·2; SAS Institute) and hypotheses were rejected at $P < 0.05$. All experiments were performed at least twice with similar results. Only representative data are presented.

Results

Si deposition in rose leaves

Treatment of the four rose genotypes 99/9496-19, 95/5166-1, 98/8285-1 and Smart with 3·6 mM Si resulted in significantly ($P < 0.001$) higher Si content than in the control plants (Fig. 1). The Si content in untreated control plants of all the genotypes was similar (3–4 ppm). The four genotypes differed significantly ($P < 0.001$) from each other in their ability to take up Si, with the moderately resistant genotypes 99/9496-19 and 98/8285-1 taking up more compared to the highly susceptible genotypes 95/5166-1 and Smart. There was a
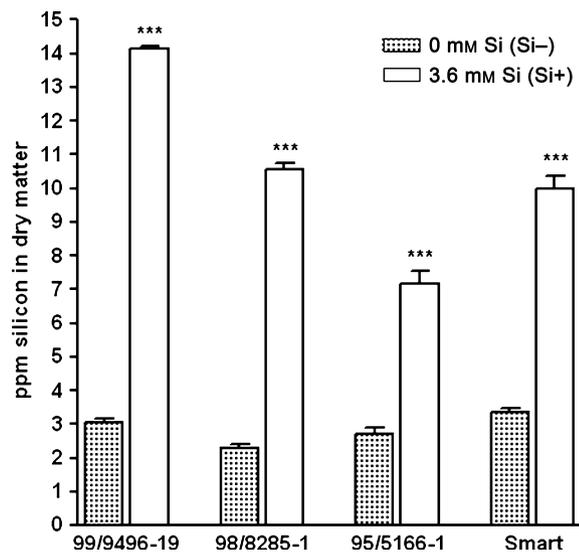

Figure 1 Silicon content in the fifth developed leaf (of every branch) of the four rose genotypes 99/9496-19 and 98/8285-1 (moderately resistant) and 95/5166-1 and Smart (highly susceptible) either treated with 3·6 mM (Si+) or 0 mM (Si−) silicon. Bars represent standard errors and within each genotype, three asterisks on a column denote a significant difference at $P < 0.001$.

significant statistical interaction between the factors genotype and Si amendment ($P < 0.05$). Whereas application of 3·6 mM Si increased the Si uptake in all genotypes, the level of uptake varied and were 365·3%, 358·2%, 165·8% and 198·9% for 99/9496-19, 98/8285-1, 95/5166-1 and Smart, respectively, compared to the Si− control plants.

Microscope examination of leaves after staining with the fluorescence dye LysoTracker Yellow HCK-123 revealed that in the epidermis, deposition of Si occurred in both Si+ and Si− plants (Fig. 2a–d). In uninoculated leaves, Si deposition occurred to a higher degree in Si+ compared to Si− plants, as seen by the more intense fluorescence here. Si was mainly localized in the apoplast, more specifically in the cell walls (Fig. 2a,b). In the uninoculated leaves (Fig. 2a,b), Si deposition was evenly distributed and more faint than in the inoculated leaves, where Si tended to accumulate in small aggregates in patches, which were unevenly distributed in the cells (Fig. 2c,d). The tendency was most obvious in the Si amended plants (Fig. 2d).

In the mesophyll (Fig. 2e–h), Si deposition was much lower than in the epidermis and was observed only in the upper part of a few cells, just beneath the epidermis. Deposition was much lower in Si− than in Si+ plants in both uninoculated (Fig. 2e,f) and inoculated (Fig. 2g,h) leaves, with no differences between inoculated and uninoculated leaves. In non-stained leaves, no signal for Si was observed in the epidermis or mesophyll in Si+ or Si− plants (data not shown).

Disease reduction after Si uptake

Treatment of rose with a 3·6 mM Si solution resulted in a significant ($P < 0.001$) overall reduction of powdery mildew severity in all four genotypes compared to their respective controls at 9 dai (Fig. 3). In the Si− plants, genotype Smart displayed the highest disease severity and genotype 99/9496-19 the lowest, and the same general trend was seen after Si treatment. There was a significant statistical interaction between the factors genotype and Si amendment. Whereas application of 3·6 mM Si decreased the disease severity in all genotypes, the level of reduction varied and was 48·9%, 48·5%, 38·2% and 39·8% for 99/9496-19, 98/8285-1, 95/5166-1 and Smart, respectively.

The incubation period for development of powdery mildew (period until onset of visible symptoms) was delayed in Si+ compared to Si− plants of both genotypes 99/9496-19 and Smart. Control (Si−) plants of genotype Smart showed disease symptoms already at 4 dai whereas the first symptoms in Si+ plants only occurred at 6 dai. Likewise, in 99/9496-19, symptom expression was delayed from 5 dai in Si− plants to 6 dai in Si+ plants. Furthermore, disease severity after Si treatment was significantly lower in both genotypes 99/9496-19 and Smart compared to their respective controls (0·9% and 5·6%, respectively) from 5 dai (data not shown), the largest reduction being observed for 99/9496-19.
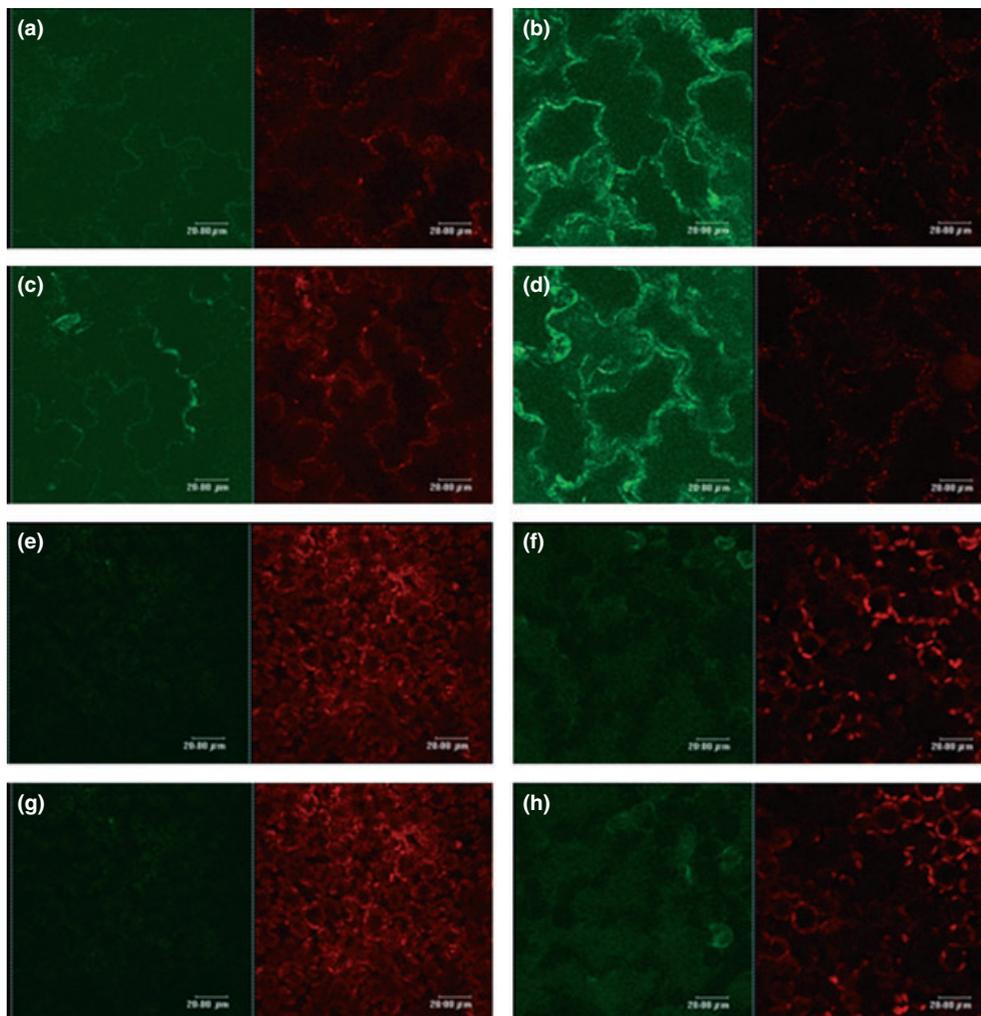
Figure 2 Confocal microscopy of silicon deposition in epidermis (a–d) and mesophyll (e–h) in leaves of rose genotype Smart after staining with LysoTracker Yellow HCK-123 at 3 dai (52-day-old plants). Each pair of images are recorded from the same optical section representing emission wavelengths at 505–545 nm (green) and 600–800 nm (red), respectively. Green represents silicon stained with LysoTracker Yellow HCK-123 and red represents autofluorescence from chlorophyll. (a) Control leaf (Si−), not inoculated with *Podosphaera pannosa*. (b) Leaf treated with 3·6 mM Si (Si+), not inoculated with *P. pannosa*. (c) Control leaf (Si−), inoculated with *P. pannosa*. (d) Leaf treated with 3·6 mM Si (Si+), inoculated with *P. pannosa*. (e) Control leaf (Si−), not inoculated with *P. pannosa*. (f) Leaf treated with 3·6 mM Si (Si+), not inoculated with *P. pannosa*. (g) Control (Si−), inoculated with *P. pannosa*. (h) Leaf treated with 3·6 mM Si (Si+), inoculated with *P. pannosa*.

Effect of Si uptake on infection biology and host responses

Podosphaera pannosa conidia germinated with a short primary germ tube, appearing approximately 6 hai and establishing the first contact with the host (Fig. 4a). Subsequently one or two, occasionally up to four, appressorial or secondary germ tubes developed (Fig. 4a) and appressoria formed within 24 hai. Direct penetration of the epidermal cell wall took place from an appressorium and resulted in formation of a haustorium in the epidermal cell (Fig. 4b,c). In the case of successful establishment of the pathogen, it developed elongating secondary hyphae (ESH) on the leaf surface to spread the fungal colony (Fig. 4b–d) and from these, new haustoria formed (Fig. 4b,c). Around 5 dai, *P. pannosa* started sporulating

by producing conidial chains from the superficial mycelium (Fig. 4e,f). As a response to host penetration or penetration attempts, the rose plant responded with formation of fluorescent papillae just beneath the appressorium (Fig. 4b,c) as well as formation of fluorescent epidermal cells (FEC). The latter response was seen as brightly fluorescing whole cells by epifluorescence microscopy where the pathogen had penetrated (Fig. 4c).

Results of quantitative bright field and epifluorescence microscopy of Si− and Si+ plants of the genotypes 99/9496-19 (moderately resistant) and Smart (highly susceptible) are shown in Tables 1 and 2. In Table 1, the different infection steps are analysed, based on the number of germinated conidia. None of the infection events examined was significantly affected by Si amendment in
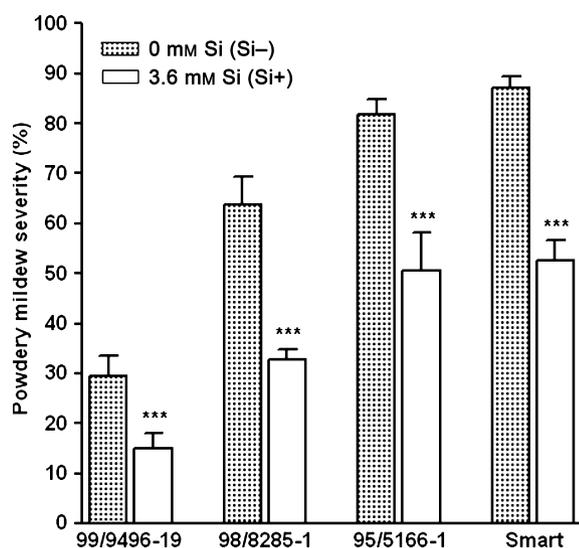
Figure 3 Comparison of powdery mildew severity at 9 days after inoculation in the fifth developed leaf (of every branch) of the four rose genotypes 99/9496-19 and 98/8285-1 (moderately resistant) and 95/5166-1 and Smart (highly susceptible) either treated with 3·6 mM Si (Si+) or 0 mM (Si–) silicon. Bars represent standard errors and within each genotype, three asterisks on a column denotes a significant difference at $P < 0.001$.

either genotype at 24 hai. However, growth after penetration (formation of haustoria and ESH) was only observed at 24 hai for Si– plants of genotype Smart. At 72 hai, the percentage of germinated conidia did not differ between Si+ and Si– plants in either genotype. Likewise, the percentage of conidia forming appressoria in genotype Smart did not differ between the Si+ and Si– treatments, but was significantly reduced (12·5%) in Si+ compared to Si– leaves in 99/9496-19. On the other hand, penetration was not significantly altered in 99/9496-19 whereas it was significantly reduced (19·1%) in genotype Smart. At 72 hai, formation of haustoria and ESH were significantly reduced by 28·7% and 26·5% in Si+ leaves in 99/9496-19 and Smart, respectively. Host defence responses (formation of papillae and FEC) were significantly increased in Si+ compared to Si– plants in both genotypes. Thus, FEC increased by 24·4% and 23·4% and papillae by 25·9% and 23·5% in 99/9496-19 and Smart, respectively. Furthermore, the percentage of FEC and papillae stopping penetration was significantly increased (233·3% and 200·0%, respectively) by Si treatment in 99/9496-19, but not in Smart.

Data for the developmental steps in the host–pathogen interaction are presented relative to the formation of appressoria in Table 2. This was done in order to determine whether the efficiency of the appressoria was inhibited or whether inhibition of *P. pannosa* as seen in Table 1 was merely the consequence of the reduction in the number of germinating and developing conidia. For both genotypes, there were no significant differences in either parameter examined at 24 hai. At 72 hai, the percentage

of appressoria causing penetration was significantly reduced (15·5%) in genotype Smart whereas there was no significant difference in 99/9496-19. Formation of haustoria and ESH were significantly lower in Si+ than in Si– plants (18·7% and 22·9%) in 99/9496-19 and Smart, respectively. Conversely, the percentages of appressoria associated with papillae and FEC was increased in 99/9496-19 (37·5% and 43·6%, respectively) and in Smart (28·6% and 28·9%, respectively). Likewise, the proportion of papillae and FEC stopping infection were significantly higher in Si+ than in Si– plants in 99/9496-19 (285·1% and 242·0%, respectively) and Smart (260·9% and 137·3%, respectively).

Association of infection structures of *P. pannosa* with H_2O_2 and callose accumulation

Staining with DAB for localization of H_2O_2 revealed a close relationship with infection structures (Fig. 5). Thus, H_2O_2 production was often observed in the vicinity of penetration attempts and infections, often around papillae (Fig. 5a). Furthermore, H_2O_2 production occurred in association with FEC and these cells often displayed a granulated cytoplasm (Fig. 5b). Arrest of fungal growth was often associated with H_2O_2 accumulation and this was seen more often and with a higher intensity in Si-treated plants than in control plants. In addition, H_2O_2 accumulated as early as 24 hai (Fig. 5b), with a higher intensity after Si treatment than in the control plants.

Callose deposition in cleared leaves was difficult to study due to a faint signal and intense autofluorescence (data not shown). However, confocal microscopy after staining with aniline blue revealed deposition of callose at penetration sites, especially associated with papillae and in the cell wall region (Fig. 6). Callose deposition was also observed in the haustorial region of the pathogen (Fig. 6).

Discussion

Rose powdery mildew caused by *Podosphaera pannosa* is an economically significant problem all over the world, particularly in greenhouse potted roses. This disease is difficult to control in a sustainable way. Here, it is shown that application of 3·6 mM Si through watering significantly reduced powdery mildew severity up to 48·9%, depending on the host genotype.

Silicon mediated protection against plant pathogenic fungi has been demonstrated in several pathosystems, with special emphasis on crops like rice (Rodrigues *et al.*, 2003) and wheat (Bélanger *et al.*, 2003; Guével *et al.*, 2007). This effect has also been demonstrated with horticultural crops, including cucumber (Schuerger & Hammer, 2003) and tomato (Dannon & Wydra, 2004). Si amendment has even been tested in rose production under varying conditions, e.g. in rockwool cultures for cut flower production (Hwang *et al.*, 2005), but the effect on powdery mildew was not assessed. However, Si amendment does not always result in reduced disease severity.
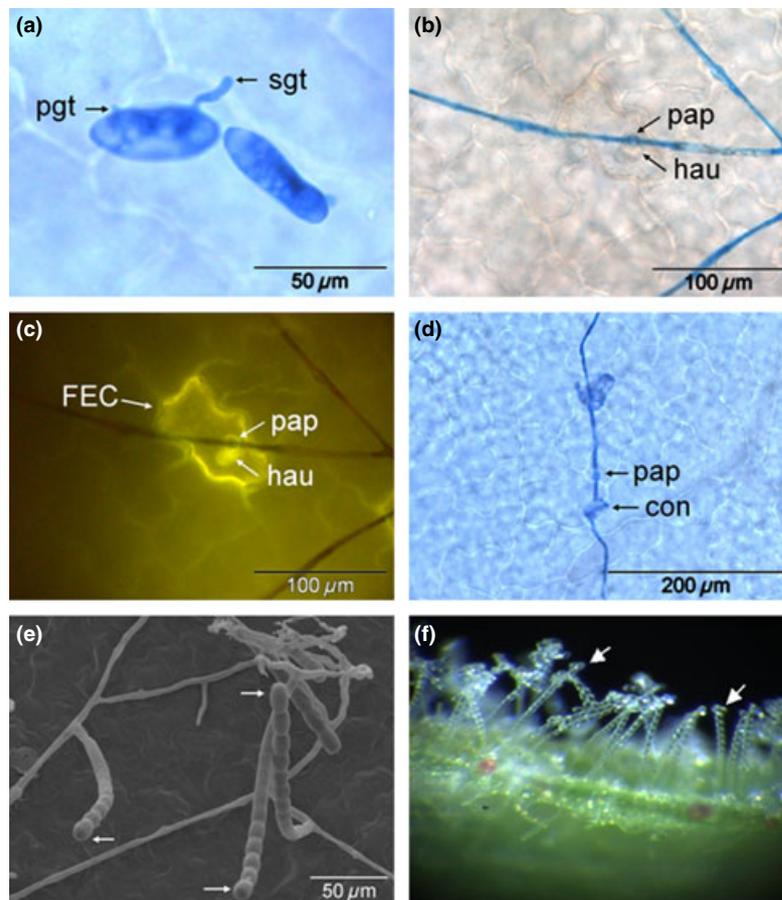
Figure 4 Infection structure formation of *Podosphaera pannosa* in rose genotype Smart (a–f). (a) Germinating conidia at 24 h after inoculation (hai). (b–c) Bright field and epifluorescence microscopy, respectively, showing formation of papilla with haustorium and FEC at 72 hai. (d) Penetration and formation of papillae and elongating secondary hyphae at 48 hai. (e) Scanning electron micrograph showing elongating secondary hyphae and conidial chains at 5 dai. (f) Macroscopic symptoms of infection with numerous conidial chains at 9 dai. pgt, primary germ tube; sgt, secondary germ tube; con, conidium; pap, papilla; hau, haustorium; FEC, fluorescent epidermal cell. Arrows show conidial chains in e–f.

Moyer *et al.* (2008) found that potassium and calcium silicate application to the growth medium of gerbera daisy was not able to reduce severity of powdery mildew caused by *Erysiphe cichoracearum/Podosphaera fusca*. It was suggested that the reason for this lack of effect could be that the disease pressure was very high and that infection took place before Si had a time to exert sufficient protection. Furthermore, the final Si concentration in the gerbera daisy plants was rather low compared to, for example, the concentrations observed in roses here.

Results here show a difference in the ability of Si to protect roses of different genetic backgrounds, the highest protection being observed in the most resistant genotypes. Interestingly, after application of 3·6 mM Si, the two genotypes with the highest Si deposition also showed the highest protection against disease, whereas such a correlation was not seen in the control plants (Si−). A similar situation has been reported in rice where Si amendment of a resistant cultivar resulted in greater Si uptake and protection against rice blast compared to a suscepti-

ble cultivar (Cai *et al.*, 2008) and could reflect the greater potential for Si uptake and defence by some host genotypes.

Si was localized almost exclusively in the epidermal cells in rose leaves, more precisely in the cell walls and extracellular spaces. These findings are in accordance with the Si deposition pattern found in various monocot species (Fauteux *et al.*, 2005; Ma & Yamaji, 2006). In other dicots, silicon application has resulted in deposition of Si in root cell walls (Heine *et al.*, 2007) and has been observed as a uniform pattern in Si+ leaves with the strongest deposition on and around trichomes (Samuels *et al.*, 1991; Ghanmi *et al.*, 2004). Previous studies on Si localization in leaves used X-ray microanalysis and transmission electron microscopy (Ghanmi *et al.*, 2004). The preparation of material for these studies is quite laborious and time-consuming. Therefore, this study used confocal microscopy and specific staining for Si, applying the stain LysoTracker Yellow HCK-123, with the epicuticular wax initially being removed to avoid unspecific

Table 1 Quantitative studies of the infection biology of *Podosphaera pannosa* and defence responses in the fifth developed leaf (of every branch) of rose plants of the moderately resistant genotype 99∕9496-19 and the highly susceptible Smart either treated with 3·6 mᴍ Si (Si+) or untreated (Si−). Values are assessed on the basis of the number of germinated conidia

Percent conidia	24 hai			72 hai		
	Si+	Si−	Odds ratio[a]	Si+	Si−	Odds ratio[a]
Genotype 99∕9496-19						
Germinated	16·9	16·2	1·05[NS]	15·9	16·5	0·95[NS]
With appressoria	72·0	72·0	1·00[NS]	70·0	80·0	0·58*
Epidermal penetration	2·0	0·7	3·04[NS]	64·7	68·7	0·31[NS]
With haustoria	0·0	0·0	1·00[NS]	44·7	62·7	0·48***
With ESH	0·0	0·0	1·00[NS]	44·7	62·7	0·48***
With FEC	2·0	0·7	3·04[NS]	64·7	52·0	1·68*
With papillae	8·0	6·7	1·22[NS]	68·0	54·0	1·81*
With FEC stopping infection	2·0	0·7	3·04[NS]	20·0	6·0	3·92***
With papillae stopping infection	8·0	6·7	1·22[NS]	24·0	8·0	3·63***
Genotype Smart						
Germinated	18·1	17·0	1·08[NS]	17·8	17·2	1·04[NS]
With appressoria	88·7	92·0	0·68[NS]	88·7	92·7	0·62[NS]
Epidermal penetration	1·3	1·3	1·00[NS]	64·7	80·0	0·46**
With haustoria	0·0	1·3	5·58[NS]	57·3	78·0	0·38***
With ESH	0·0	1·3	5·58[NS]	57·3	78·0	0·38***
With FEC	1·3	0·7	2·01[NS]	63·3	51·3	0·24*
With papillae	6·7	4·7	1·46[NS]	66·7	54·0	1·70*
With FEC stopping infection	1·3	0·0	1·86[NS]	7·3	2·0	3·88*
With papillae stopping infection	6·6	3·3	2·07[NS]	10·7	4·7	2·44[NS]

[a]Odds ratio for comparison of treatments (control used as reference, odds ratio = 1·00).
The number of asterisks indicates the degree of significance. *Significant at $0·05 < P < 0·01$, **significant at $0·01 < P < 0·001$, ***significant at $P < 0·001$, NS: nonsignificant $P > 0·05$.

Table 2 Quantitative studies of the infection biology of *Podosphaera pannosa* and defence responses in the fifth developed leaf (of every branch) of rose plants of the moderately resistant genotype 99∕9496-19 and the highly susceptible Smart either treated with 3·6 mᴍ Si (Si+) or untreated (Si−). Infection processes were assessed based on the number of appressoria producing infection structures

Percent appressoria	24 hai			72 hai		
	Si+	Si−	Odds ratio[a]	Si+	Si−	Odds ratio[a]
Genotype 99∕9496-19						
With penetration	2·8	0·9	3·06[NS]	92·1	85·9	2·00[NS]
With haustoria	0·0	0·0	1·00[NS]	63·9	78·6	0·49*
With ESH	0·0	0·0	1·00[NS]	63·9	78·6	0·49*
With FEC	2·8	0·9	3·06[NS]	89·5	65·1	4·60***
With papillae	11·0	9·3	1·22[NS]	97·1	67·6	16·4***
With FEC stopping infection	2·8	0·9	3·05[NS]	28·5	7·4	4·93***
With papillae stopping infection	11·0	9·3	1·22[NS]	34·2	10·0	4·70***
Genotype Smart						
With penetration	1·5	1·5	1·00[NS]	72·9	86·3	0·43*
With haustoria	0·0	1·4	5·96[NS]	64·7	84·1	0·34***
With ESH	0·0	1·4	5·96[NS]	64·7	84·1	0·34***
With FEC	1·5	0·7	2·09[NS]	71·4	55·5	2·01*
With papillae	7·5	5·1	1·52[NS]	75·3	58·4	2·17**
With FEC stopping infection	1·5	0·0	1·86[NS]	8·3	2·3	4·09*
With papillae stopping infection	7·5	3·6	2·16[NS]	12·1	5·1	2·58*

[a]Odds ratio for comparison of treatments (control used as reference, odds ratio = 1·00).
The number of asterisks indicates the degree of significance: *Significant at $0·05 < P < 0·01$, **significant at $0·01 < P < 0·001$, ***significant at $P < 0·001$, NS: nonsignificant $P > 0·05$.

staining. This technique is new for higher plants, but has previously been applied for studies of Si in diatoms (Descles *et al.*, 2007) and in mammalian systems. The method was found to be quite sensitive and rapid, and furthermore it was possible to work with living plants. This technique may be applicable when there is a need to
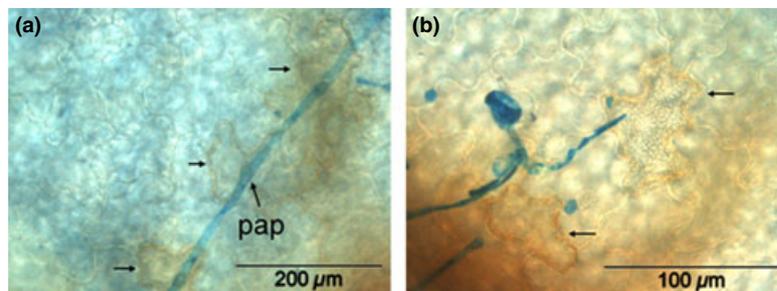
Figure 5 Association of infection structures of *Podosphaera pannosa* with H_2O_2 within rose genotype Smart. (a) Leaves treated with 0 mM Si (Si−) at 3 days after inoculation. (b) Leaves treated with 3·6 mM Si (Si+) at 24 h after inoculation. Arrows show H_2O_2 accumulation in whole cells, some with granulated cytoplasm.
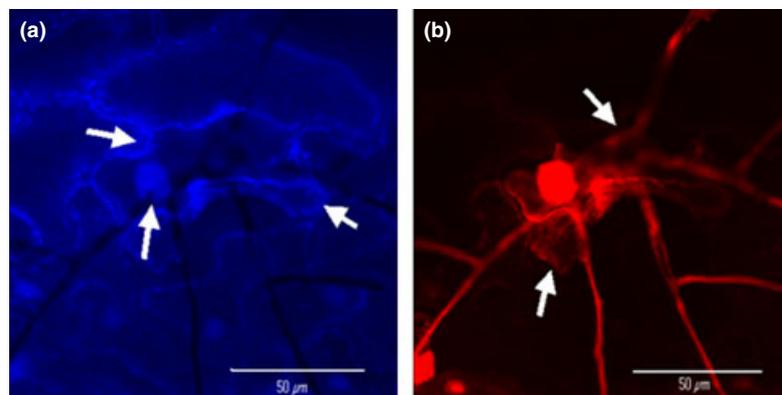

Figure 6 Confocal microscopy of leaves of rose genotype Smart inoculated with *Podosphaera pannosa* at 72 h after inoculation (52-day-old plants) from plants treated with 3·6 mM Si (Si+) and stained with aniline blue. Each pair of images is recorded from the same optical section using excitation and emission wavelengths of (a) 405 nm and 460–500 nm. The blue colour represents callose stained with aniline blue (arrows). (b) 600–800 nm. The red colour represents autofluorescence (arrows) from host and pathogen.

obtain fast, but reliable results on the exact location of Si deposition in plants and when there is a need to study the dynamics of Si deposition in an easy way. Hence, it was observed that inoculation induced Si deposition especially in the Si+ plants and also that the appearance of the deposits changed.

There are different hypotheses concerning the role of Si in inhibition of fungal pathogens. Initially, enhanced protection of Si-treated plants against pathogenic fungi was suggested to result from increased resistance to pathogen penetration of host tissue, e.g. due to the specific deposition of Si-containing compounds in cell walls (Kim *et al.*, 2002). Indeed, it has been reported that Si was deposited in cell walls at the site of fungal penetration (Samuels *et al.*, 1991; Epstein, 1994; Fauteux *et al.*, 2005). Similarly, Rodrigues *et al.* (2004) suggested a mechanical role of Si in protecting rice against *Magnaporthe oryzae*. The hypothesis that an enhanced physical strengthening is responsible for inhibition of pathogens may be quite complicated to demonstrate. Furthermore, this hypothesis has been recently questioned as it appears that amendment of plants with Si may result in enhancement of defence responses (reviewed by Ghanmi *et al.*, 2004; Fauteux *et al.*, 2005). Therefore, in order to further elucidate

the mechanisms by which Si reduces disease severity, it is necessary to study if defence responses in the host are enhanced, i.e. whether induced resistance (*sensu* Kloepper *et al.*, 1992) is involved. Various host responses have been reported in rose against infection by pathogens, including accumulation of phenolics, lignin and callose (Conti *et al.*, 1986; Dewitte *et al.*, 2007), PR-proteins and papillae (Dewitte *et al.*, 2007) and reactive oxygen species (Gachomo & Kotchoni, 2008). Conti *et al.* (1985) reported that infected rose cells became necrotic and this resembled the hypersensitive reaction (HR). Similarly, in this study infected rose cells often fluoresced brightly when using epifluorescence microscopy and this phenomenon was termed FEC. In other host–pathogen interactions, such as the interaction between barley and *Blumeria graminis* f.sp. *hordei*, such fluorescent cells have been interpreted as an indication of the hypersensitive response (Wei *et al.*, 1994). It is highly likely that FEC in rose also represent cells undergoing HR, as this is quite a common and efficient response to stop infection by biotrophic pathogens (Shetty *et al.*, 2008). A correlation was observed between elicitation of FEC and arrest of fungal growth, but a formal connection between FEC and HR remains to be shown.

The ability of *P. pannosa* to produce appressoria on the two rose genotypes at 72 hai was differently affected by Si application. Thus, in 99/9496-19, Si+ plants had a significantly lower proportion of germinated conidia forming appressoria than Si− plants, whereas no difference was seen in genotype Smart. On the other hand, the proportion of appressoria causing penetration in genotype Smart was significantly reduced in Si+ compared to Si−plants, whereas this was not seen in 99/9496-19. A possible reason for these differences could be found in the nature of the cuticle and epicuticular wax, which is known to vary between cultivars of certain plant species and thus affect the ability of pathogens to infect (Jørgensen *et al.*, 1995). Although Conti *et al.* (1985) failed to detect any apparent differences in the wax layer of the two rose cultivars they studied, the chemical composition may have varied. Furthermore, the composition of cuticle and wax may be altered by applications of Si. Wang & Galletta (1998) showed that foliar application of potassium silicate to strawberry enhanced the leaf content of fatty acids such as oleic, linoleic and linolenic acids. These fatty acids have previously been shown to be efficient inducers of resistance against another biotrophic pathogen, *Sclerospora graminicola* infecting pearl millet (Amruthesh *et al.*, 2005). Si application in roses might also act by eliciting endogenous elicitors of defence in the host.

Si was found to effectively reduce formation of haustoria and elongating secondary hyphae by the pathogen and this was correlated to an increase in papilla formation and frequency of FEC as well as in the ability of these defence responses to reduce infection. The timing and magnitude of these responses varied between Si-treated and control plants for both genotypes, with the moderately resistant 99/9496-19 giving the highest degree of reduction in accordance with its higher level of resistance. The results indicate that the reductions in formation of haustoria and ESH correspond well to the increase in formation of FEC and papillae. However, more important is the efficiency of these responses in stopping the pathogen and here the data indicate that Si amendment resulted in a considerable reduction in fungal growth. On the other hand, fungal growth was not completely stopped, indicating that neither response completely stops fungal growth.

The induced formation of papillae and FEC in response to infection attempts by *P. pannosa* was associated with the accumulation of H_2O_2 and deposition of callose, with a more prominent occurrence in Si+ plants. H_2O_2 accumulation is a host response effective in preventing development and establishment of biotrophic pathogens (Borden & Higgins, 2002; Shetty *et al.*, 2003, 2008). Accumulation of callose was observed at the penetration sites of *P. pannosa* and around the haustorial neck of the pathogen in infected cells. A similar observation was made by Kuzuya *et al.* (2006) in melon. In addition to callose fluorescence, infected rose leaves showed considerable autofluorescence. Therefore, in order to clearly verify the presence of callose, it was necessary to use confocal microscopy which can specifically detect fluorescence from callose. On the other hand, the presence of unspecific fluorescence indicates the involvement of phenolics and flavonoids as host responses to infection (Conti *et al.*, 1986). Currently, detailed analyses of metabolites induced in rose after Si application are therefore being performed, with special emphasis on flavonoids and phenolics and their role in disease resistance. This may help in identifying key metabolic intermediates in disease resistance against rose powdery mildew and subsequently in finding effective target systems for more site-directed disease control strategies.

In the present studies, root application of Si resulted in significant reductions in powdery mildew infection and growth, but did not prevent the disease completely. Nevertheless, the protection offered might still have important practical implications in commercial rose production. Si is relatively easy to apply in greenhouse systems and the reduction in disease severity and delay in onset of symptom expression are important in themselves as this may help to improve product quality, as well as reduce the disease pressure in the crop and thereby the need for disease control. In addition, boosting of the inherent ability of rose to defend itself against *P. pannosa* will result in a reduced need for pesticides and/or possibility for the use of alternative control agents, which are not effective when used alone. Therefore, use of Si as a supplement to disease control, especially in genotypes with a relatively high inherent resistance against powdery mildew, has a strong potential in integrated pest management and might help to make production of miniature potted roses more sustainable.

References

Amruthesh KN, Geetha NP, Jørgensen HJL, de Neergaard E, Shetty HS, 2005. Unsaturated fatty acids from zoospores of *Sclerospora graminicola* induce resistance in pearl millet. *European Journal of Plant Pathology* **111**, 125–37.

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*). *Journal of Phytopathology* **93**, 402–12.

Borden S, Higgins VJ, 2002. Hydrogen peroxide plays a critical role in the defense response to tomato to *Cladosporium fulvum*. *Physiological and Molecular Plant Pathology* **61**, 227–36.

Cai K, Gao D, Luo S, Zeng R, Yang J, Zhu X, 2008. Physiological and cytological mechanisms of silicon-induced resistance in rice against blast disease. *Physiologia Plantarum* **134**, 324–33.

Conti GG, Bassi M, Maffi D, Bonecchi R, 1985. Host-parasite relationship in a susceptible and a resistant rose cultivar inoculated with *Sphaerotheca pannosa* I. Fungal growth, mechanical barriers and hypersensitive reaction. *Journal of Phytopathology* **113**, 71–80.

Conti GG, Bassi M, Maffi D, Bocci AM, 1986. Host-parasite relationship in a susceptible and a resistant rose cultivar inoculated with *Sphaerotheca pannosa* II. Deposition rates of callose, lignin and phenolics in infected or wounded cells and their possible role in resistance. *Journal of Phytopathology* **117**, 312–20.

Dannon EA, Wydra K, 2004. Interaction between silicon amendment, bacterial wilt development and phenotype of *Ralstonia solanacearum* in tomato genotypes. *Physiological and Molecular Plant Pathology* **64**, 233–43.

Descles J, Vartanian M, El Harrak A *et al.*, 2007. New tools for labeling silica in living diatoms. *New Phytologist* **177**, 822–9.

Dewitte A, Leus L, Huylenbroeck VJ, Bockstaele V, Ho MH, 2007. Characterization of reactions to powdery mildew (*Podosphaera pannosa*) in resistant and susceptible rose genotypes. *Journal of Phytopathology* **155**, 264–72.

Elliott CL, Snyder GH, 1991. Autoclave-induced digestion for the colorimetric determination of silicon in rice straw. *Journal of Agricultural and Food Chemistry* **39**, 1118–9.

Epstein E, 1994. The anomaly of silicon in plant biology. *Proceedings of the National Academy of Sciences, USA* **91**, 11–7.

Fauteux F, Remus-Borel W, Menzies JG, Bélanger RR, 2005. Silicon and plant disease resistance against pathogenic fungi. *FEMS Microbiology Letters* **249**, 1–6.

Gachomo EW, Kotchoni SO, 2008. Extract from drought-stress leaves enhances disease resistance through induction of pathogenesis related proteins and accumulation of reactive molecules. *Biotechnology* **7**, 273–9.

Ghanmi D, McNally DJ, Benhamou N, Menzies JG, Bélanger RR, 2004. Powdery mildew of *Arabidopsis thaliana*: a pathosystem for exploring the role of silicon in plant-microbe interactions. *Physiological and Molecular Plant Pathology* **64**, 189–9.

Guével 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**, 429–36.

Heine G, Tikum G, Horst WJ, 2007. The effect of silicon on the infection by and spread of *Pythium aphanidermatum* in single roots of tomato and bitter gourd. *Journal of Experimental Botany* **58**, 569–77.

Hwang SJ, Park HM, Jeong BR, 2005. Effects of potassium silicate on the growth of miniature rose 'Pinocchio' grown on rockwool and its cut flower quality. *Journal of the Japanese Society for Horticultural Science* **74**, 242–7.

Jørgensen HJL, Lyshede OB, Allerup S, 1995. Epicuticular wax of the first leaves of two barley cultivars studied by cryo scanning electron microscopy. *Journal of Agronomy and Crop Science* **174**, 217–24.

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**, 1095–103.

Kloepper JW, Tuzun S, Kuć JA, 1992. Proposed definitions related to induced disease resistance. *Biocontrol Science and Technology* **2**, 349–51.

Kuzuya M, Yashiro K, Tomita K, Ezura H, 2006. Powdery mildew (*Podosphaera xanthii*) resistance in melon is categorized into two types based on inhibition of the infection processes. *Journal of Experimental Botany* **9**, 2093–100.

Leus L, Dewitte A, Van Huylenbroeck J, Vanhoutte N, Van Bockstaele E, Höfte M, 2006. *Podosphaera pannosa* (syn. *Sphaerotheca pannosa*) on *Rosa* and *Prunus* spp.: characterization of pathotypes by differential plant reactions and ITS sequences. *Journal of Phytopathology* **154**, 23–8.

Ma JF, Yamaji N, 2006. Silicon uptake and accumulation in higher plants. *Trends in Plant Science* **11**, 392–7.

Menzies JG, Ehret DL, Glass ADM, Helmer T, Koch C, Seywerd F, 1991. The effects of soluble silicon on the parasitic fitness of *Sphaerotheca fuliginea* on *Cucumis sativus*. *Phytopathology* **81**, 84–8.

Moyer C, Peres NA, Datnoff LE, Simonne EH, Deng Z, 2008. Evaluation of silicon for managing powdery mildew on gerbera daisy. *Journal of Plant Nutrition* **31**, 2131–44.

Pemberton HB, Kelly JW, Ferare J, 2003. Pot rose production. In: Roberts A, Debener T, Gudin S, eds. *Encyclopedia of Rose Science*. Oxford, UK: Elsevier Science, 587–93.

Rémus-Borel W, Menzies JG, Bélanger RR, 2005. Silicon induces antifungal compounds in powdery mildew-infected wheat. *Physiological and Molecular Plant Pathology* **66**, 108–15.

Rodrigues FÁ, Vale FXR, Datnoff LE, Prabhu AS, Korndörfer GH, 2003. Effect of rice growth stages and silicon on sheath blight development. *Phytopathology* **93**, 256–61.

Rodrigues FÁ, McNally DJ, Datnoff LE *et al.*, 2004. Silicon enhances the accumulation of diterpenoid phytoalexins in rice: a potential mechanism for blast resistance. *Phytopathology* **94**, 177–83.

Samuels AL, Glass ADM, Ehret DL, Menziers JG, 1991. Mobility and deposition of silicon in cucumber plants. *Plant Cell & Environment* **14**, 485–92.

Schuerger AC, Hammer W, 2003. Suppression of powdery mildew on greenhouse-grown cucumber by addition of silicon to hydroponic nutrient solution is inhibited at high temperature. *Plant Disease* **87**, 177–85.

Shetty NP, Kristensen BK, Newman M-A, Møller K, Gregersen PL, Jørgensen HJL, 2003. Association of hydrogen peroxide with restriction of *Septoria tritici* in resistant wheat. *Physiological and Molecular Plant Pathology* **62**, 333–46.

Shetty NP, Jørgensen HJL, Jensen JD, Collinge DB, Shetty HS, 2008. Roles of reactive oxygen species in interactions between plants and pathogens. *European Journal of Plant Pathology* **121**, 267–80.

Shetty NP, Jensen JD, Knudsen A *et al.*, 2009. Effects of *β*-1,3-glucan from *Septoria tritici* on structural defence responses in wheat. *Journal of Experimental Botany* **60**, 4287–300.

Silcox S, Holloway PJ, 1986. A simple method for removal and assessment of foliar deposits of agrochemicals using cellulose acetate film stripping. *Aspects of Applied Biology* **11**, 13–7.

Wang SY, Galletta GJ, 1998. Foliar application of potassium silicate induces metabolic changes in strawberry plants. *Journal of Plant Nutrition* **21**, 157–67.

Wei YD, de Neergaard E, Thordal-Christensen H, Collinge DB, Smedegaard-Petersen V, 1994. Accumulation of a putative guanidine compound in relation to other early defense reactions in epidermal cells of barley and wheat exhibiting resistance to *Erysiphe graminis* f.sp. *hordei*. *Physiological and Molecular Plant Pathology* **45**, 469–84.