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Enhanced resistance to anthracnose disease in chili pepper (*Capsicum annuum* L.) by amendment of the nutrient solution with silicon

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SUMMARY
Resistance to anthracnose disease was investigated in *Capsicum annuum* L. 'Muria F1' by adding silicon (Si) to the nutrient solution in a hydroponic system. Four different concentrations of potassium silicate ($K_2Si_2O_5$): 0 mg l^{-1} (control), 50 mg l^{-1}, 75 mg l^{-1}, or 100 mg l^{-1} were used to find the optimum concentration of Si that suppressed anthracnose disease. Disease resistance in the fruit of plants treated with different concentrations of Si was assessed by artificial inoculation with *Colletotrichum gloeosporioides* or *C. capsici*. Significant reductions in lesion areas were observed in fruit from plants treated with Si compared to fruit from Si-free plants. There were 75% and 64% reductions in disease caused by *C. gloeosporioides* in the fruit from plants treated with 75 mg l^{-1} or 100 mg l^{-1} Si, respecively, and the equivalent reductions were 78% and 84% for *C. capsici*. Potassium silicate at 75 mg l^{-1} was also applied at different stages of plant development (vegetative growth or flowering), or at both stages, as separate treatments to determine the effect of the stage of Si application on the severity of anthracnose disease. Reductions in lesion areas of 76% and 71% were observed on fruit from plants treated with 75 mg l^{-1} Si at both stages, or at the flowering stage, respectively, compared to control fruit (0 mg l^{-1} Si). However, the application of Si during the vegetative growth stage did not reduce lesion areas significantly. Si had no significant effect on plant growth or on fruit quality parameters in *Capsicum*. The mechanisms underlying the effect of Si treatment were investigated by measuring the thickness of the cuticle and by analysing total and cell wall-bound phenolic compounds. The concentrations of cell wall-bound phenolic compounds and cuticle thickness were significantly greater in fruit from plants treated with Si than in fruit from Si-free (control) plants.

Anthracnose disease caused by *Colletotrichum* species, is a major disease of chili pepper (*Capsicum annuum* L.) in tropical and sub-tropical climates and causes severe post-harvest losses (Oanh *et al*., 2004). Two *Colletotrichum* species, *C. capsici* and *C. gloeosporioides* are responsible for anthracnose disease of chili in Sri Lanka (Rajapakse and Ranasighe, 2002). The disease is normally controlled by seed treatment or by foliar sprays with fungicides. However, the use of fungicides has become limited due to environmental and consumer concerns, and the development of fungicide-resistant pathogen populations. Therefore, alternative, more environmentally-friendly control methods need to be investigated.

Silicon (Si) supplements added to hydroponic culture systems have been reported to be beneficial for the growth, yield, and disease resistance of some crops (Epstein, 1994). The severity of powdery mildew in cucumber (Menzies *et al*., 1991), *Fusarium* crown and root rots in tomato (Huang *et al*., 2011) and *Phytophthora* blight disease in bell pepper (French-Monar *et al*., 2010) have all been reduced following the application of Si. However, the extent of disease reduction varied with the growth stage of the plant at which the Si was applied. Identifying the optimum growth stage for the application of Si to optimise disease suppression is essential. Ma *et al*. (1989) studied the effects of Si applied during different growth stages of rice plants and concluded that applying Si during the reproductive stage was most beneficial for plant growth.

Various mechanisms have been proposed to explain the increased level of disease resistance mediated by Si. Ma *et al*. (2006) reported that Si acted as a physical barrier, deposited beneath the cuticle, to impede penetration by fungal appressoria, thereby disrupting the process of infection. On the other hand, Si may signal a biochemical mechanism to suppress fungal infection and disease. Si could induce defence responses such as systemic acquired resistance (Cai *et al*., 2009).

This study reports the effects of treatment with different concentrations of Si, and the effects of the application of Si at different stages of plant growth on enhancing resistance to anthracnose disease in chili pepper (*Capsicum annuum* L.) 'Muria F1' grown in a hydroponic system.

MATERIALS AND METHODS

Plant material

Seeds of *Capsicum annuum* L. 'Muria F1' (East-West Seed International Ltd., Nonthaburi, Thailand) were sown in a 1:1 (v/v) mix of coir dust and compost and maintained in nursery for 6 weeks at 28º – 30ºC and 80 – 85% relative humidity with a 12 h photoperiod. Healthy plants (n = 12)

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were transferred to a non-circulating hydroponic system under the same environmental conditions.

Preparation of potassium silicate

Potassium silicate was prepared according to Brauer (1965). Sodium silicate (40.5 g) was added to 400 ml of 4 M HCl. The precipitate formed was washed thoroughly, then dissolved in 200 ml of 1 M KOH and diluted to 1.0 l with distilled water.

The solution was analysed for its molybdo-reactive silica concentration using a UV visible spectrophotometer (UVD 3000/3200; Labomed Inc., Los Angeles, CA, USA) according to Clesceri *et al*. (1998).

Hydroponic nutrient supplement for plant growth

The cultivation of chili pepper (*C. annuum* L. 'Muria F1') plants was sub-divided into two stages based on the time it took for 50% of the plants to flower. Accordingly, the initial period, from transplanting up to 4 weeks, was considered to be the vegetative stage and the period after 4 weeks from transplanting was considered to be the flowering stage.

Two different nutrient formulations were used during the vegetative stage (NFV) and the flowering stage (NFF), respectively. NFV contained 313 mg l^{-1} N, 80 mg l^{-1} P, 202 mg l^{-1} K, 300 mg l^{-1} Ca, and 78 mg l^{-1} Mg, while NFF contained 366 mg l^{-1} N, 80 mg l^{-1} P, 650 mg l^{-1} K, 169 mg l^{-1} Ca, and 78 mg l^{-1} Mg. Both nutrient formulations included 0.01 mg l^{-1} Cu, 3.90 mg l^{-1} Fe, 0.13 mg l^{-1} Zn, 1.20 mg l^{-1} Mn, 1.00 mg l^{-1} B, and 0.13 mg l^{-1} Mo (Saparamadu, 2008). Adjustments were made to the composition of each nutrient formulation to compensate for the amount of K added when potassium silicate was applied. The pH values of both nutrient solutions were adjusted to 6.3 using 1 M HNO_3. The nutrient solution was renewed once a week.

Pathogen identification and isolation

C. gloeosporioides and *C. capsici*, isolated from anthracnose lesions on diseased *Capsicum* fruit, were cultured on potato dextrose agar (PDA), following surface sterilisation with 1% (v/v) NaOCl for 1 min followed by washing with sterile distilled water. Ten culture plates were incubated at 27º – 30ºC and observed for mycelial growth. The morphology of the cultures and the shapes of the conidia were recorded using a compound microscope (MCX100; Micros, Vienna, Austria). *C. capsici* was identified by its sickle-shaped conidia, the presence of prominent setae (Sutton, 1992), and having a brown colony colour (Rajapakse and Ranasighe, 2002). *C. gloeosporioides* was identified by its orange cotton-like mycelium (Sutton, 1980) and ovoid-shaped conidia (Du *et al*., 2005).

Treatments and experimental design

Application of different concentrations of Si to optimise the treatment (Experiment 1): The nutrient solution was amended using pre-prepared potassium silicate to achieve three final concentrations (50 mg l^{-1}, 75 mg l^{-1}, or 100 mg l^{-1}) in each nutrient solution (NFV or NFF) to be applied as separate treatments. Amendment with Si was applied at each renewal of both nutrient solutions throughout the cultivation period (15 weeks). The nutrient solutions in the control treatment were not amended with Si.

Application of Si during different growth stages (Experiment 2): Plants (n = 12) were treated with 75 mg l^{-1} Si (the optimum Si concentration from Experiment 1) using pre-prepared potassium silicate at the different growth stages as separate treatments (i.e., during the vegetative stage, the flowering stage, or during both stages), while control plants were not treated with Si at any stage.

Experimental design and statistical analysis: The treatments in Experiment 1 and Experiment 2 were arranged in a completely randomised design (CRD) with three replicates and four plants in each replicate of each Si treatment. The Experiments were repeated twice and data were analysed using one-way ANOVA. Means separation was done using Duncan's Multiple Range Test in the SPSS 16.0 statistical package (SPSS Inc., Chicago, IL, USA).

Fruit harvested at the "colour breaker" stage in both Experiments were used to measure reductions in disease severity, fruit quality parameters, and resistance mechanisms, as described below. Plant growth and fruit yield parameters were also recorded.

Fruit inoculation and assessment of disease severity

Conidial suspension (10^5 conidia ml^{-1}) of *C. gloeosporioides* or *C. capsici* were prepared by scraping the mycelium from pure 7 d-old cultures and suspending

TABLE I
Lesion areas on chili pepper fruit inoculated with Colletotrichum *spp.*

Experiment	*Colletotrichum* spp.	Treatment [Si concentration (mg l^{-1}) or growth stage]	Mean lesion area (mm^2)
1	C. *gloeosporioides*	0 (Control)	108.0 a[†]
		50	77.0 b (29%)[‡]
		75	27.5 c (75 %)
		100	38.5 c (64%)
	C. *capsici*	0 (Control)	51.1 a
		50	17.9 b (65%)
		75	11.4 b (78%)
		100	8.3 b (84%)
2	C. *gloeosporioides*	0 (Control)	94.3 a
		75 Vegetative stage	85.33 a
		75 Flowering stage	27.84 b (71%)
		75 Both stages	22.57 b (76%)

[†]Mean values (n = 24) followed by the same lower-case letter for each pathogen in each Experiment are not significantly different at $P \leq 0.05$ as determined by Duncan's Multiple Range Test.
[‡]Values in parentheses represent percentage reductions in the severity of anthracnose disease compared to the Si-free control.

them in sterilised distilled water, followed by filtering through glass wool. Each harvested fruit was challenge-inoculated by placing a 20 µl drop of either conidial suspension at three different points on the fruit surface. Twenty-four fruit were inoculated per treatment. The inoculated fruit were maintained in a moist chamber (95 – 100% RH) at 28º ± 2ºC. The number of days for disease symptoms to appear in each treatment was recorded. Lesion areas were recorded each day for 10 d, and the mean lesion area per fruit was then calculated.

Total soluble phenolic compound (TSPC) and cell wall-bound phenolic compound (CWBPC) concentrations

Twelve fruit from each 75 mg l^{-1} Si-treated plant (n = 12) at both developmental stages, and from Si-free control plants (n = 12) were used for phenolic compound analysis. Harvested fruit were inoculated with *C. gloeosporioides* and tissue samples were taken from each inoculated spot 2, 3, 4, and 5 d after inoculation (DAI). Twelve replicate measurements were carried out each day for each treatment. Fruit tissue (1.0 g) was extracted separately in 10.0 ml of 80% (v/v) methanol and the TSPC concentration in each fruit extract was determined using the Folin-Ciocalteu reagent with ferulic acid as the standard (Ascensao and Dubery, 2003).

Each residue from methanol extraction for TSPC analysis was used to measure CWBPCs. It was dried at 70ºC for 24 h. Ten mg of each dried residue was suspended in 1.0 ml of 0.5 M NaOH for 1 h at 96ºC, then the supernatant was acidified to pH 2 with 0.1 M HCl, followed by centrifugation at 10,625 × *g* for 10 min. The mixture was extracted with 10.0 ml of 99% (v/v) diethyl ether, evaporated to dryness, and re-suspended in 10 ml of 80% (v/v) methanol. CWBPC values were determined using the Folin-Ciocalteu reagent (Ascensao and Dubery, 2003).

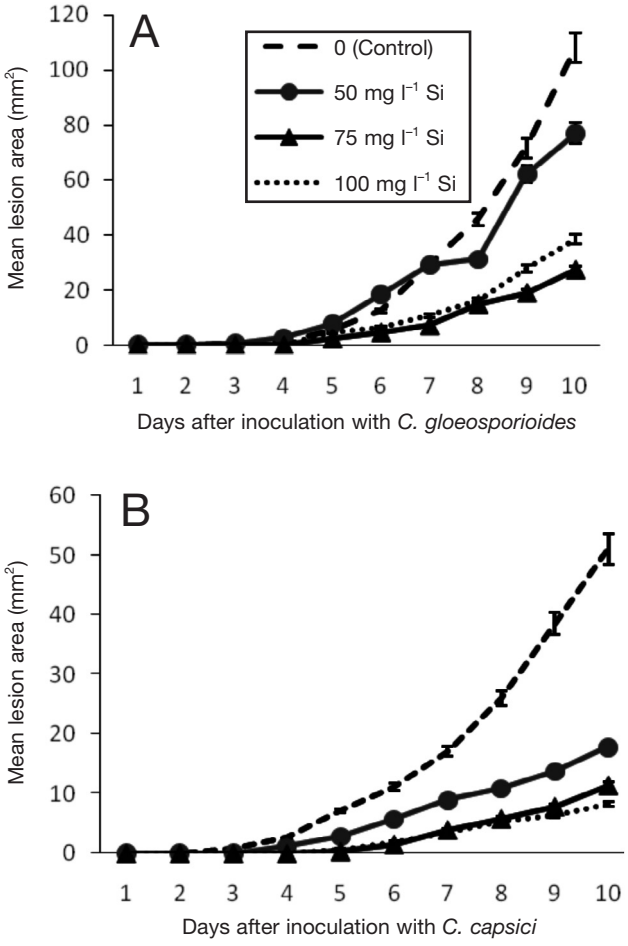

FIG. 1
Lesion development on chili pepper fruit challenge-inoculated with *Colletotrichum gloeosporioides* (Panel A) or *C. capsici* (Panel B). Fruit (n = 24) were harvested from plants treated with different concentrations of Si (0 mg l^{-1}, 50 mg l^{-1}, 75 mg l^{-1}, or 100 mg l^{-1}) in the hydroponic nutrient solution. Bars represent ± standard errors.

Cuticle thickness

Twenty-four fruit from each 75 mg l^{-1} Si-treated plant (n = 12) at both developmental stages and from Si-free control plants were used to measure cuticle thickness. Three cross-sections (0.1 mm-thick) of each fresh fruit were mounted on a glass slide and cuticle thickness was measured using a calibrated ocular micrometer (Pyser; SGI Ltd., Prabano, P. R. China) at a magnification 400X.

Assessment of selected fruit quality, growth and yield parameters in Si-treated chili pepper plants

Ten fruit from each treatment were used to measure the parameters described below. Fruit firmness (in N) was measured using a penetrometer (FT 40; Wagner Instruments, Greenwich, CT, USA).

An extract was prepared by crushing each fruit separately in a grinder (Butterfly Emerald; Gandhi Appliances Ltd., Mumbai, India) and squeezing the pulp through a two layers of muslin cloth. The total soluble solids content (TSSC) of each fruit extract was then measured using a Brix refractometer (WZ-103; Zhejiang Top Instruments Co. Ltd., Zhejiang, P. R. China). The pH of each fruit extract was measured using an IQ150 pH meter (Spectrum Technologies Inc., Aurora, IL, USA).

Growth and fruit yield parameters such as shoot lengths, root lengths, numbers of leaves per plant, fruit lengths, and fruit weights from each chili pepper plant were also recorded.

RESULTS AND DISCUSSION

Lesion areas on chili pepper fruit from plants supplied with different concentrations of Si (Experiment 1)

Resistance to anthracnose disease in chili pepper was investigated by challenge-inoculation of fruit with either of two *Colletotrichum* spp. Significant reductions in anthracnose lesion areas caused by either fungus were observed on fruit from plants treated with 50 – 100 mg l^{-1} Si compared to Si-free control plants. Fruit challenge-inoculated with *C. capsici* showed a greater reduction in lesion areas compared to fruit inoculated with *C. gloeosporioides* (Table I). The greatest reduction in lesion area caused by *C. gloeosporioides* (75%) was observed in fruit from plants treated at 75 mg l^{-1} Si, whereas against *C. capsici* it was 84% in fruit from plants treated at 100 mg l^{-1} Si (Table I). Similarly, the severity of anthracnose disease has been shown to be reduced by Si in crops such as cucumber (Kanto, 2002), Chinese cabbage (Yang *et al*., 2008), bean (Polanco, 2012), and tomato (David and Weerahewa, 2012).

C. gloeosporioides lesion areas were larger than those of *C. capsici* (Table I), suggesting a higher virulence of *C. gloeosporioides*, irrespective of the application of Si. It

TABLE II
Total soluble phenolic compound (TSPC) and cell wall-bound phenolic compound (CWBPC) concentrations in fruit from Si-treated or Si-free chili pepper plants

	Phenolic compound concentrations in fruit (mg g^{-1} FW)			
	TSPC		CWBPC	
DAI‡	Si-free plants	Si-treated plants	Si-free plants	Si-treated plants
2	328.4 a†	348.3 a	121.1 a	131.0 a
3	502.5 a	485.9 a	126.4 a	344.7 b
4	597.1 a	552.4 a	112.8 a	172.5 b
5	517.5 a	563.3 a	102.9 a	146.0 b

†Pairwise mean values (n = 12) followed by the same lower-case letter for each category of phenolic compound at each DAI are not significantly different at $P \leq 0.05$ as determined by one-way ANOVA and Duncan's multiple range test.
‡DAI, days after inoculation with *Colletotrichum gloeosporoides*.

has been shown that *C. gloeosporioides* caused anthracnose disease on both green and ripe pepper fruit, while *C. capsici* caused lesions only on ripe red pepper fruit (Kim *et al*., 1989). Rajapaksa and Ranasighe (2002) reported that lesion diameters and the rate of lesion development caused by inoculation with *C. capsici* were highest at the red ripe stage of chili fruit than at other stages of maturity. In the current study, challenge inoculation was applied to mature green fruit (harvested at the colour breaker stage) before they ripened. This could be the reason for the lower lesion areas caused by *C. capsici*.

The appearance of anthracnose lesions on chili pepper fruit following challenge inoculation with either *C. gloeosporioides* or *C. capsici* was delayed in fruit from Si-treated plants compared to fruit from Si-free plants (Figure 1). Anthracnose lesions appeared early (3 – 4 DAI) on fruit from plants treated with 50 mg l^{-1} Si and on control fruit. However, lesions were delayed by 2 d on fruit from plants treated with either 75 or 100 mg l^{-1} Si (Figure 1). Similarly, a delay in the appearance of anthracnose lesions has been reported on bean pods from Si-treated plants compared to Si-free plants (Polanco *et al*., 2012).

The rate of lesion development was slower on fruit harvested from plants treated with 75 or 100 mg l^{-1} Si, irrespective of the causal organism. However, rates were higher on fruit from 50 mg l^{-1} Si-treated or control fruit inoculated with *C. gloeosporioides* (Figure 1). Seebold *et al*. (2001) showed that the rate of lesion expansion on the leaves of rice plants decreased as the concentration of Si increased.

The application of 75 mg l^{-1} or 100 mg l^{-1} of Si significantly reduced lesion areas caused by either *Colletotrichum* species, therefore 75 mg l^{-1} Si was selected as the optimum concentration for Experiment 2.

Lesion areas on chili pepper fruit from plants treated with 75 mg l^{-1} Si at different stages of growth (Experiment 2)

Significant reductions in lesion areas caused by *C. gloeosporioides* were observed in chili pepper fruit from plants treated with 75 mg l^{-1} of Si at both stages (76%) or at the flowering stage (71%), compared to fruit from Si-free plants. However, there was no difference in lesion areas on fruit from plants treated with 75 mg l^{-1} Si at the vegetative stage or from Si-free plants (Table I). Treating plants with Si before the formation of fruit appeared to have no positive effect on resistance against anthracnose disease. In the current study, the vegetative stage (4 weeks) was shorter than the flowering stage (10 weeks). Hence, the application of Si to plants for a longer period during the flowering stage may have contributed to the higher resistance observed in fruit from Si-treated plants.

The greatest reductions in lesion areas were observed on fruit from plants treated with 75 mg l^{-1} Si during both stages. Therefore, possible mechanisms responsible for reductions in the development of anthracnose lesions were investigated further using fruit from plants treated with 75 mg l^{-1} Si during both developmental stages, or from Si-free control plants.

Total soluble phenolic compound (TSPC) and cell wall-bound phenolic compond (CWBPC) concentrations

TSPC concentrations in chili pepper fruit from 75 mg l^{-1} Si-treated or Si-free plants fluctuated between 400 – 600 mg g^{-1} during the period tested (Table II). Thus, there was no significant effect of Si treatment on TSPC concentrations in chili pepper fruit. However, higher CWBPC concentrations were detected in fruit from 75 mg l^{-1} Si-treated plants compared to Si-free control fruit. The mean CWBPC concentration was 344.7 mg g^{-1} in *Capsicum* fruit from Si-treated plants, while it was significantly lower (126.4 mg g^{-1}) in control fruit 3 DAI. Similarly, CWBPC concentrations were significantly higher in fruit from Si-treated plants than from control plants 4 DAI and 5 DAI (Table II).

C. gloeosporioides directly penetrates host cells. The penetration and infection processes can be inhibited by

TABLE III
Fruit quality parameters and some growth and yield parameters of chili pepper plants treated with various concentrations of Si (Experiment 1) or with 75 mg l^{-1} Si at different growth stages (Experiment 2)

Experiment	Treatment (Si concn. and/or growth stage)	Fruit firmness (N)	TSSC‡ (°Brix)	pH value	Shoot length (cm)	Root length (cm)	No. of leaves per plant	Fruit length (cm)	Fruit fresh weight (g)
1	0 (Control)	18.63†	7.40	5.5	49.3	19.4	55	12.3	24.0
	50	19.61	7.47	5.3	51.1	18.7	59	12.5	26.2
	75	21.28	7.53	5.5	56.0	19.3	51	12.0	23.7
	100	22.55	7.50	5.4	52.4	20.0	50	12.8	25.0
2	0 (Control)	19.60	7.53	5.7	50.6	20.4	60	12.3	25.4
	75 Vegetative stage	20.90	7.50	5.7	48.2	19.0	57	13.0	24.8
	75 Flowering stage	24.20	7.43	5.6	53.1	20.8	55	12.7	27.3
	75 Both stages	23.50	7.50	5.5	55.0	19.6	64	13.1	28.2

†There was no significant difference in mean values (n = 12) for any fruit quality, or plant growth or yield parmeter among treatments in either Experiment at $P \leq 0.05$ as determined by Duncan's multiple range test.
‡TSSC, total soluble solids content.

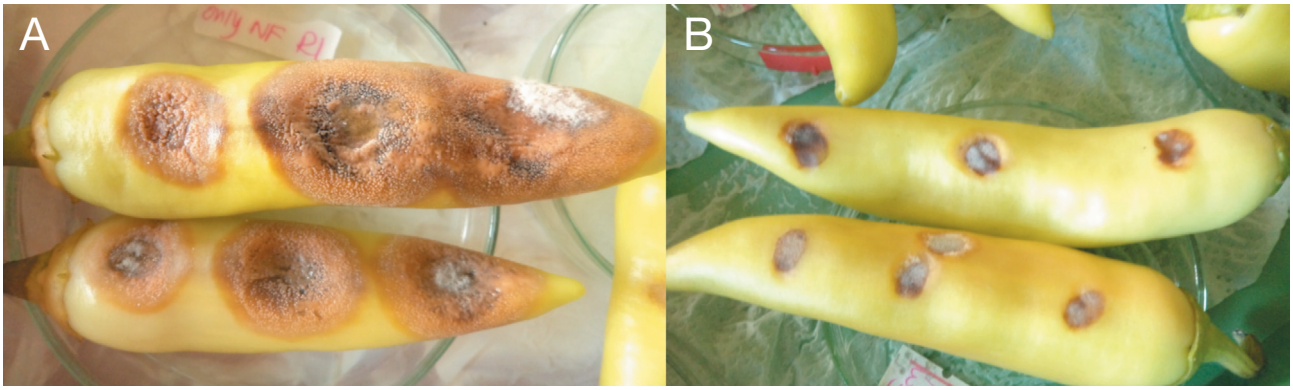

Fig. 2
Lesion development on chili pepper fruit challenge-inoculated with *C. gloeosporioides* 10 d after inoculation. Panel A, fruit from Si-free (Control) plants. Panel B, fruit from plants treated with 75 mg l^{-1} Si at the flowering stage.

preformed or induced chemical inhibitors in plant cells (Pruskey, 1998). Phenolic polymers in cell walls act as a barrier, rendering cell walls more resistant to mechanical and/or enzymatic disruption by a pathogen (Kolattukudy, 1987). In cucumber, Menzies *et al.* (1991) observed extensive deposition of phenolic compounds and polymerisation of Si surrounding the site of fungal penetration following Si treatment. Cherif *et al.* (1994) also reported that nutrient solutions amended with Si stimulated the accumulation of electron-dense phenolic-like material in host tissue infected with *Pythium ultimum*.

Cuticle thickness

The mean cuticle thickness (34.3 µm) was significantly greater in fruit from 75 mg l^{-1} Si-treated plants during both stages than in fruit from Si-free plants (19.6 µm), as determined by one-way ANOVA at $P \leq 0.05$. A thicker cuticle may have contributed to the increased resistance of fruit by mechanically impeding pathogen invasion and the process of disease development.

Kim *et al.* (2002) concluded that the deposition and polymerisation of Si below the cuticle prevented or delayed fungal penetration. It has been shown that there was a negative correlation between cuticle thickness and the incidence of disease caused by *C. gloeosporioides* in pepper fruit (Oh *et al.*, 1999). Biles *et al.* (1993) suggested that the cuticle acted as a physical barrier which inhibited infection by *Phytophthora capsici* which causes fruit rot disease in *C. annuum*. Biles *et al.* (1993) also suggested that peroxidases appeared to play an important role in cuticle development in pepper fruit. The application of Si to cucumber plants increased the activity of peroxidases (Cherif *et al.*, 1994).

Further studies are needed to determine the precise mechanism (or mechanisms) responsible for the suppression of anthracnose disease by Si in chili pepper.

Fruit quality parameters and selected growth and yield parameters in Si-treated chili pepper plants

TSSC values in chili pepper fruit were in the range of 7.40 – 7.53 ºBrix and pH values were in the range of 5.3 – 5.7 in Experiments 1 and 2. The highest firmness values (22.55 N) were observed in fruit from 100 mg l^{-1} Si-treated plants, while the lowest firmness (18.63 N) was observed in fruit from Si-free plants (Experiment 1). Moreover, the highest firmness value (24.2 N) was observed in fruit from the flowering stage treatment (Experiment 2; Table III). However, there were no statistically significant differences in fruit quality parameters such as fruit firmness, TSSC and pH values between treatments.

There were also no significant differences in plant growth and fruit yield parameters (i.e., shoot length, root length, number of leaves, fruit length, and fruit weight) in Si-treated or Si-free chili pepper plants (Table III). This may indicate that the reduction in anthracnose lesion area development in Si-treated fruit was not caused by any improvement in plant growth or fruit quality.

CONCLUSION

Supplementing the hydroponic nutrient solution with 75 mg l^{-1} Si or 100 mg l^{-1} Si significantly reduced the severity of anthracnose disease caused by *C. capsici* or *C. gloeosporioides* in *Capsicum annuum* L. 'Muria F1'. The application of Si during the flowering stage resulted in the maximum reduction in severity of anthracnose disease.

Cell-wall bound phenolic compound concentrations and cuticle thickness increased in fruit from Si-treated chili pepper plants, which also showed reduced development of anthracnose lesions. This could be related to a direct role of Si in the development of disease resistance in plants, which is a subject for further research.

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