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**Damage to *Trichothecium roseum* caused by sodium silicate
is independent from pH**

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1 **Damage to *Trichothecium roseum* caused by**
2 **sodium silicate is independent from pH**

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ABSTRACT: *Trichothecium roseum* is one of the most important postharvest pathogens in arid and semi-arid regions. Sodium silicate (NaSi) and environmental pH have significant inhibitory effects on fungal growth. However, no study has addressed the relationship of the combined NaSi/pH effects on *T.roseum*. In this work, we showed that spore germination, germ tube elongation and mycelial growth of *T. roseum* were significantly inhibited by various NaSi concentrations, which had correspondingly increasing pHs. Furthermore, these NaSi solutions showed a much higher effect than pH treatments alone. The pathogenicity of NaSi-treated conidia on a model assay (conidia inoculated apple fruit) was dramatically reduced while no changes of pathogenicity were evident in the corresponding pH (various NaOH solutions) treatments. Fluorescent microscopy, using propidium iodide staining, showed damage of the plasma membranes of *T. roseum* conidia treated with both NaSi and NaOH, although the damage was more severe with NaSi. Leakage of proteins and sugars were significantly higher in NaSi-treated and NaOH-treated conidia when compared to untreated controls. In addition, serious damage was observed in the conidia exposed to NaSi for longer exposure periods of time. Ultrastructural observations showed that treatment with either NaSi or NaOH caused a plasmolysis state and disorganized organelles. Taken together the results show that NaSi has inhibitory effects on *T. roseum* and that inherent higher pH of NaSi solutions of higher concentrations simply act as an enhancer of the inhibitory effects of NaSi.

KEY WORDS: Sodium silicate, Inhibition, Pathogenicity, Conidia ultrastructure

44 Introduction

45 *Trichothecium roseum* is an important postharvest pathogen, especially causing core rot of apples
46 (Hu et al. 1995) and pink rot of muskmelons (Bi et al. 2003; Huang et al. 2000). Moreover, the
47 pathogen has been isolated from many fruits such as pears (Sommer et al. 2002), grapes (Serra et al.
48 2005), and stone fruits (Hong and Michailides 1997). Besides economic losses, the pathogen also
49 possesses a strong ability to produce trichothecenes, which are mycotoxins harmful to humans and
50 animals (Tang et al. 2014; Ueno 1980). Current management to control the diseases caused by *T.*
51 *roseum* still relies heavily on synthetic chemical fungicides, such as iprodione and azoxystrobin (Bi et
52 al. 2005). However, the increase concerns of the impacts of fungicides on human health and the
53 environment as well as resistance development has prompted the search to develop alternatives to the
54 use of fungicides (Tripathi and Dubey 2004).

55 Reports indicated that the application of soluble silicon was effective in controlling fungal
56 diseases such as powdery mildews, root rots, and rice blast (Fauteux et al. 2006; Ishiguro 2001; Liang
57 et al. 2005; Moscoso-Ramírez and Palou 2014), as well as postharvest diseases such as green mold of
58 citrus, pink rot of muskmelons, and rots of Hami melons (Bi et al. 2006; Guo et al. 2007; Li et al.
59 2009b; Liu et al. 2010). The mechanisms of disease control by soluble silicon mainly attributes to the
60 direct inhibition of the growth of the pathogens (Bi et al. 2006; Li et al. 2009b; Qin and Tian 2005),
61 and by induced host resistance (Chérif et al. 1992; Rémus-Borel et al. 2005). Similarly sodium silicate
62 (NaSi) has been generally recognized as safe by the Food and Drug Administration (FDA) of the
63 United States (21CFR 182.90 and 21CFR 182.1711). NaSi has been shown to induce broad-spectrum
64 antimicrobial properties against postharvest pathogens including *Penicillium digitatum* (Liu et al.
65 2010), *Fusarium sulphureum* (Li et al. 2009b), *Alternaria alternata*, *F. semitectum*, and *T.roseum* (Bi
66 et al. 2006).

67 However, the specific inhibitory mechanisms of NaSi remains unknown. Liu et al. (2010)
68 suggested NaSi could damage the plasma membrane of *P. digitatum* spores and increase the leakage of
69 proteins and sugars from mycelia. Since solutions of NaSi are inherently alkaline, an increase in pH
70 might enhance antifungal activity (Guo et al. 2007; Liu et al. 2010). In fact, it was reported that for *P.*
71 *expansum* spore germination was inhibited, the content of soluble protein in spores was decreased, and
72 the levels of aggregated proteins increased under pH stress (Li et al. 2009a). To our knowledge, little

information concerning the relationship between inhibition effects of NaSi and corresponding pH is available.

In an attempt to study the relationship between NaSi and pH during pathogenicity of *T. roseum* conidia on an apple fruit model assay, we quantified the conidial germination and mycelial growth, after treatment with NaSi and corresponding pH. Membrane integrity and permeability, as well as ultrastructural alterations in conidia were also observed.

Materials and methods

Pathogen, fruits and chemicals

An isolate of *T.roseum*, the pathogen of core rot of apple, was provided by the Institute of Plant Protection, Gansu Academy of Agricultural Sciences, China. Prior to experimentation, the isolate was inoculated into apple fruit and re-isolated onto PDA potato dextrose agar (PDA) (Oxoid, UK) at 28 °C. A conidial suspension was obtained from 10-day-old PDA cultures by flooding the surface of the culture with sterile distilled water containing 0.05% (v/v) Tween-80. The conidial suspension was filtered through four layers of sterile cheesecloth and the concentration was adjusted with the aid of a hemocytometer prior to use.

Fruit of apple cv. Delicious was harvested at commercial maturity from Tiaoshan Farm, in Jingtai County of Gansu province, China, on September 23, 2012. The fruit was packed individually, placed in a corrugated box (80 apples/box) and transported to the laboratory within 24 h. Apples of the same size and without physical injuries or visible infection were disinfected by immersion in 2% (v/v) sodium hypochlorite for 2 min, then rinsed with sterile water, air dried and used for inoculation.

Sodium silicate ($\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$), and sodium hydroxide (NaOH) were obtained from Guangfu Fine Chemical Research Institute (Tianjin, China). Propidium iodide (PI) and low gelling temperature agarose were obtained from Sigma-Aldrich (Shanghai, China). Epon 812 was from EMS (Hatfield, Pennsylvania, USA). Glutaraldehyde, sodium cacodylate, osmium tetroxide, uranyl acetate, and lead citrate were from SPI chemical companies (Westchester, Pennsylvania, USA).

Preparation of NaSi solutions at different concentrations in potato dextrose broth

NaSi was added to 100 mL sterile potato dextrose broth (PDB) (Oxoid, UK) to make solutions at concentrations of 0, 20, 40, 60, 80, and 100 mM and was dissolved completely by shaking. NaSi solutions were kept in incubator at 28 °C for 1 h and the pHs of the solutions were determined. To

make control solutions having the same pH as the NaSi solutions, a stock solution of 10 M sodium hydroxide was added to distilled water until the corresponding pHs of the NaSi solutions was obtained. The pH of all solutions was measured using a PHS-3C digital pH-meter (Leici, Shanghai, China).

Determination of the effects of NaSi and pH on germination, germ tube elongation, and mycelial growth

Ten μL aliquots of a conidial suspensions (1×10^6 conidia mL^{-1}) were plated on 1% agar flakes (5 mm in diameter) prepared with: a) different concentrations of NaSi (20, 40, 60, 80, and 100 mM); b) different concentrations of NaOH, corresponding to each of the different pHs of the NaSi; and c) sterile distilled water as a control respectively according to Li et al. (2012). Conidial germination and germ tube length were assessed by observing approximately 200 conidia per treatment under a light microscope after 15 h of incubation at 28 °C. Conidia were considered germinated if the germ tube was equal to or greater than the diameter of the conidia. Germinated conidia were expressed as percentage of the total number of evaluated conidia. Germ tube length was measured with an ocular micrometer. There were three replicates for each treatment, and the experiment was repeated.

Mycelial growth was assayed according to Yao and Tian (2005). Briefly, mycelial disks (5 mm in diameter) from two-week-old cultures were placed in the center of 90 mm Petri dishes containing 20 mL of PDA prepared with: a) different concentrations of NaSi (20, 40, 60, 80, and 100 mM); b) different concentrations of NaOH, corresponding to each of the different pHs of the NaSi solutions; and c) sterile distilled water as a control. The mycelial growth was determined by measuring colony diameter after 5 d at 28 °C. Each treatment contained three replicates and the entire experiment was repeated.

Determination of the effects of conidia pretreated with NaSi and pH on lesion diameter in inoculated apples

The conidial suspensions (1×10^6 conidia mL^{-1}) of *T. roseum* were prepared with: a) different concentrations of NaSi (20, 40, 60, 80, and 100 mM), b) different concentrations of NaOH, corresponding to each of the different pHs of the NaSi solutions; and c) sterile distilled water as a control. Then the suspensions were shaken at 200 rpm for 3, 6, and 9 h at 28 °C. Four wounds (3 mm deep and 3 mm wide) were made with a sterile nail on the equator of each surface-sterilized apple fruit, and 20 μL of pretreated conidial suspensions was inoculated into each wound. Inoculated apples were

then stored in low density polyethylene bags at room temperature ($20^{\circ}\text{C}\pm 2^{\circ}\text{C}$) with a relative humidity (RH) of 75-80% for 15 days. Lesion diameters of inoculated fruit were then determined in two directions and averaged. Each treatment contained three replicates with 10 fruits per replicate. The experiment was conducted three times.

Determination of effects of NaSi and pH on conidial plasma membrane integrity

A modification of the method by Avis et al. (2009) was used. Briefly, aliquots of 100 μL of conidial suspensions (1×10^7 conidia mL^{-1}) were transferred to a 2 mL sterile Eppendorf tube and centrifuged at $8000\times g$ for 5 min. The supernatant was decanted and replaced with three different solutions: a) 1 mL of 100 mM NaSi; b) 1 mL of NaOH solution with pH of 12.6; and c) 1 mL of sterile distilled water. Conidial suspensions were shaken at 200 rpm at 28°C for 0, 3, 6, and 9 h, collected by centrifugation at $8,000\times g$ for 5 min at 4°C , then washed with sterile distilled water twice. Propidium iodide (PI, a compound that penetrates damaged membranes) was added to evaluate the integrity of the plasma membrane in the fungi exposed to these three solutions. According to the method of Liu et al. (2007), the conidial suspensions were stained with $10\text{ }\mu\text{g mL}^{-1}$ of PI for 5 min at 30°C , then collected by centrifugation, and washed twice with sterile distilled water to remove residual dye. The conidia were observed with a microscope (Zeiss Axioskop 40, Carl Zeiss, Oberkochen, Germany) equipped with an individual fluorescein rhodamine filter set (Zeiss no.15: excitation BP 546/12 nm, emission LP 590 nm). Three fields of view from each cover slip were chosen randomly and the number of conidia in a bright-field was counted and defined as the total number, and membrane integrity (MI) was calculated according to the formula:

$$\text{MI}(\%) = [1 - (\text{number of stained conidia} / \text{number of total conidia})] \times 100.$$

The experiment was repeated.

Determination of the effects of NaSi and pH on conidial protein and sugar leakage

A modification of the method by Avis et al. (2009) was used. Briefly, 100 μL of each conidial suspension (1×10^7 conidia mL^{-1}) was transferred to a 2mL sterile eppendorf tube and centrifuged at $8000\times g$ for 5min. The supernatant was decanted and replaced with: a) 1mL of NaSi at 0, 20, 40, 60, 80, and 100mM; b) 1mL of NaOH solutions, corresponding to each of the different pHs of the NaSi solutions; and c) 1mL of sterile distilled water as a control. Conidial suspensions were shaken at 200 rpm for either 3, 6, or 9 h at 28°C , centrifuged at $8000\times g$ for 5 min. Supernatants were collected for determining the leakage of intracellular content by assays of total soluble proteins and total soluble

sugars. The content of soluble protein was determined according to the method by Bradford (1976) and the content of soluble sugar was estimated by the phenol-sulfuric acid method (Dubois et al., 1956). The protein and sugar leakage were expressed as 1×10^6 conidial suspension per mL. Each treatment was replicated three times and the experiment was repeated.

Determination of the effects of NaSi and pH on ultrastructure of conidia

Conidia treated with either NaSi at 100 mM, the NaOH solution with pH of 12.60, or distilled sterile water (control) for 3, 6, and 9 h were fixed with a mixture of 3% glutaraldehyde and 3% acrolein (pH 7.4) in 0.1 M sodium cacodylate buffer (SCB) under vacuum for 2 h at 4°C, and centrifuged ($16,000 \times g$ for 5 min at 4 °C). Fixed conidia were analyzed by the transmission electron microscopy (TEM) according to the method of Avis et al. (Avis et al. 2009). Briefly, gels of 1 to 2 mm³ were prepared by adding 3% low gelling temperature agarose in SCB to the pellet. After thorough rinsing with 0.1 M SCB, the gels were post-fixed with 2% osmium tetroxide in 0.1 M SCB for 1 h at 20 °C, dehydrated using a series of increasing ethanol concentrations, and embedded in Epon 812. Ultrathin sections were obtained using a diamond knife and stained by soaking in 2% uranyl acetate for 15 min, and then post-stained in lead citrate for 1 min. Structure alterations in the conidia were analyzed using an electron microscope (JEM-1230, Japan) operating at 80kV. For each treatment, the test was repeated twice and at least two blocks were examined for each replicate.

Statistical analysis

All statistical analyses were performed using SPSS version 17.0 (SPSS, Inc., Chicago, IL). Data from assays of plasma membrane integrity were compared in independent-samples *t*-test. Others were analyzed by one-way ANOVA. Mean separations were performed by Duncan's multiple range tests. Differences at $P < 0.05$ were considered to be significant.

Results

The pHs of NaSi solution at different concentrations in different solvents

The pH value of NaSi solutions at different concentrations of 20, 40, 60, 80, and 100 mM in PDB changed between 10.4 and 12.3. However, the pH value of NaSi solutions at the different

187 concentrations in sterile distilled water changed between 12.0 and 12.6 in sterile (Table 1). The pH of
188 NaSi in the PDB was slightly lower than in sterile distilled water.

189 **NaSi solutions have greater effects than pH on germination, germ tube elongation and mycelial**
190 **growth:**

191 The conidial germination and germ tube elongation were significantly inhibited by the NaSi
192 solution at different concentrations and NaOH solutions. However, NaSi was more effective under
193 higher NaSi concentrations than NaOH only solutions (Fig. 1A, B). The conidial germination and germ
194 tube elongation in NaSi reduced by 89.7% and 86.4%, respectively, when compared with the NaOH pH
195 solution of 12.6. The conidial germination and germ tube elongation in NaSi at 100 mM were reduced
196 by 94% and 90.8%, respectively, when compared with the control of sterile distilled water.

197 Treatments with NaSi at all concentrations and NaOH solutions inhibited colony diameter.
198 However, differences were observed between NaSi and NaOH treatments: the colony diameter of the
199 NaOH treatment at pH 10.44 was reduced greater than that NaSi at 20 mM (Fig. 1C). However, when
200 NaSi concentration was more than 40 mM, the inhibition effect on colony diameter was significantly
201 greater ($P < 0.05$) than those of NaOH solutions. For example, NaSi at 80 mM and the NaOH pH
202 solution of 11.9 decreased colony diameter by 83% and 68%, respectively, compared with the control;
203 the effect of NaSi on colony diameter was 1.9 times greater than the NaOH. The colony diameter was
204 completely inhibited by NaSi at 100 mM.

205 **Pretreated conidia with NaSi has significant effects on lesion diameter in inoculated apples**

206 The lesion diameters in inoculated apples with NaSi-treatment conidia at 60, 80 and 100 mM were
207 significantly reduced compared with the NaOH treatment and the control. There was no significant

208 difference among NaSi treatment at 20, 40 mM, NaOH treatments and the control (Fig. 2). The lesion
209 diameters decreased in inoculated fruit treated with NaSi at 60 mM for 9 h (Fig. 2D). The lesion
210 diameters decreased with an increase in concentrations of NaSi and treatment length (Fig. 2D, E, F).
211 The NaSi concentration at 100 mM with an incubation time of 3 h, resulted in lesion diameters which
212 were reduced by 64.2% and 61.3%, respectively, compared to those of the control and the NaOH
213 solution pH of 12.6. Moreover, the lesion development was completely inhibited with NaSi-treatment
214 conidia at 80 mM for 9 h (Fig. 2E), and at 100 mM for 6 h and 9 h (Fig. 2F).

215 **NaSi treatment damages plasma membrane integrity of conidia**

216 The membrane integrity (MI) with NaSi at 100 mM and NaOH solution pH of 12.6 was
217 compromised; the NaSi treatment caused more severe damage than NaOH treatment (Fig. 3A, B).
218 However, the MI of the control conidia remained stable during incubation (Fig. 3B). The MI of conidia
219 NaSi and NaOH treatment declined significantly with the increase incubation time. The MI of conidia
220 treated with NaSi was 5.8 times greater than that of the NaOH (Fig. 3B). Similar results were also
221 observed with PI staining, with the NaSi treatment producing a greater number of dyed conidia than the
222 NaOH after 9 h of treatment. However, dyeing in the control conidia indicated little changes (Fig 3A).

223 **NaSi treatment caused leakage of sugar and protein of conidia**

224 With the increase in incubation time, the sugar leakage from conidia treated with NaSi and pH
225 increased, however, the leakage of sugar in the control conidia was only increased slightly. A
226 difference in leakage was observed between NaSi and pH treatments, and NaSi was more effective at
227 the higher mM concentrations (Fig. 4A). When NaSi concentration was 100 mM, the sugar leakage
228 was 1.6 times and 1.2 times greater than the control and the corresponding pH, respectively, after 9 h of

incubation (Fig.4F). Similar results were observed for NaSi-treatment at 40, 60 and 80 mM (Fig. 4C, D, E). On the other hand, the sugar leakage in NaSi-treatment at 20 mM was significantly lower than the NaOH treatment pH of 12.0 (Fig. 4B) ($p<0.05$).

Similarly, with the increase in incubation time, the protein leakage of conidia treated with NaSi and pH increased while the leakage of protein in the control conidia was increased slightly (Fig. 5A). NaSi treatment caused more severe protein leakage than corresponding pH. Protein leakage increased slowly with the treatment of NaSi at 3 h and 6 h, then increased rapidly at 9 h of incubation. For example, when NaSi concentration was 100 mM, the protein leakage at 9 h incubation was 1.6 times greater than that at 6 h of incubation. No significant difference was observed between 3 h and 6 h of incubation. On the other hand, protein leakage increased rapidly with the corresponding pH treatment at 3 h then increased slowly at 6 h and 9 h of incubation. For corresponding pH of 12.6, the protein leakage after incubation for 6 h and 9 h was 1.4 times greater than that incubated at 3 h. No significant difference was observed between 6 h and 9 h incubation. The leakage of NaSi-treatment conidia at 100 mM was 2.9 times and 1.6 times higher than those of the control and the corresponding pH, respectively, after 9 h of incubation (Fig. 5F).

NaSi treatment causes severe damage on ultrastructure of conidia

Typical conidial ultrastructure of *T. roseum* was shown in Figure 6A, B, and C. After 3 h and 6 h in sterile distilled water (pH 6.4), the conidia generally displayed smooth visible cell wall and cell membrane, integrity of the plasma membrane, organized cytoplasm and visible nuclei and other organs with well-defined envelopes (Fig 6A, B). With prolonged incubation in sterile water (9 h), the thickening of cell wall tissue appeared, although the plasma membrane and cytoplasm remained intact

and organized (Fig 6C). For NaSi treatment at 100 mM and 3 h incubation, *T. roseum* conidia showed irregular cell-wall thickening, indistinct organelles and a plasmolysis state (Fig 6D). With prolonged incubation for 6 h, the cell-wall layers were not obvious, the cytoplasm appeared condensed and most conidia were greatly altered and organelles were barely distinguished (Fig 6E). A longer exposure period (9 h) with 100 mM NaSi induced a more severe plasmolysis state as revealed by the retraction and/or rupture of the plasma membrane, and disorganization and lysis of the cytoplasm content, as well as extensive fragmentation of the plasma membrane (Fig 6F). For NaOH treatment at pH 12.6 and 3 h incubation, the conidia showed normal cell-wall, normal cell-membrane and cytoplasm, clearly visible organelles, and was overall similar to the structure of the control (Fig. 7G). With longer incubation time, cell-wall became thickened, a plasmolysis state was clearly observed, the cytoplasm had disintegrated, and organelles appeared degraded (Fig. 7H, I). However, the cell-wall was visible, the membrane was still intact, and no exudates were observed. Overall, treatment with NaSi caused more severe ultrastructural changes to conidia than pH.

Discussion

The results of this study indicate that NaSi and pH treatments cause damage to *T. roseum* but that NaSi treatment caused damages more severe than those of pH treatments alone (using various NaOH solution as a proxy for pH). NaSi was effective in inhibiting conidial germination, germ tube elongation and mycelial growth of *T. roseum* and there also was a concentration-dependent manner in which NaSi treatment with the 100 mM showed a complete inhibition on germination, germ tube elongation and mycelial growth. Our results were consistent with other findings that NaSi showed more antifungal activity against several postharvest fungal pathogens (Bi et al. 2006; Halasz et al. 2010; Liu

et al. 2010; Wang et al. 2011). It is well known the pH values of NaSi solutions rise with the increased concentrations, therefore it was proposed that the effect of inhibition of NaSi was due to the increase in pH (Guo et al. 2007; Liu et al. 2010). In the present study, we found that the NaOH solution could also inhibit conidial germination, germ tube elongation and mycelial growth. However, the inhibitory effect showed little change within the range tested of NaOH solutions pHs. Thus, the higher concentrations of NaSi demonstrated a much stronger inhibitory effect than those of the NaOH. To our knowledge, this is the first study contrasting the effect between NaSi and NaOH treatment on conidial germination, germ tube elongation and mycelial growth. NaSi is a base salt and aqueous solutions of NaSi dissociate into Na^+ and silicate anions at $\text{pH} > 11$ (Halasz et al. 2010). According to our results, we can hypothesize that the silicate anions probably played a more important role in inhibition than the hydroxide ions.

In vivo results suggested that the pathogenicity of NaSi-treated conidia decreased, while no evidence for change in pathogenicity was found for the NaOH treatment compared with the control. Although, *in vitro*, NaOH pH solution could inhibit the conidial germination, germ tube elongation and mycelial growth, the germination in NaOH-treated conidia did not affect fungal growth when inoculated on the apple host. Apple flesh is acidic and watery, which could neutralize the hydroxide ions and restore the osmotic pressure around the NaOH-treated conidia. It is possible that the germination of NaOH treated conidia was only delayed and reversed in the right environment. The pathogenicity of NaSi-treated conidia decreased markedly with the increased time when NaSi was more than 40 mM. The NaSi-treated conidia at 100 mM for 6 h and 9 h lost the pathogenicity completely. It is possible the conidia were irreversible damaged or dead under this conditions. Our

291 previous results also confirmed the viability of conidia treated with NaSi rapidly decreased (Zhao et al.
292 2015).

293 The plasma membrane plays a vital role in maintaining a homeostatic environment, exchanging
294 materials, and transferring energy and information in the cell. Liu et al. (2010) reported antifungal
295 activity of silicon was attributed to the fact that NaSi treatment resulted in damage of the plasma
296 membrane, and leakage of proteins and sugars of *P. digitatum* spores. In our experiments, reduced
297 membrane integrity was observed with NaSi-treated and corresponding pH-treated conidia shown by PI
298 staining and determination of the leakage of cytoplasmic materials; these results confirmed that release
299 of intracellular components is characteristic of NaSi antifungal effects. Conidial ultrastructural changes
300 by NaSi and NaOH treatment were observed by TEM. Results also confirmed that NaSi induced even
301 greater damage than the NaOH treatment. Longer exposure periods to NaSi caused rupture of the
302 plasma membrane, caused disorganization and lysis of the cytoplasm content, and extensive
303 fragmentation of the plasma membrane, as well as exudates. Similar results have been recently reported
304 by Qin (2010), who suggested that borate disrupted the cell membrane of *B. cinerea*, eventually leading
305 to leakage of cytoplasmic materials and the death of the fungal pathogen. Shi et al. (2011; 2012) found
306 that borate altered the ultrastructure of *Colletotrichum gloeosporioides* conidia, leading to
307 disintegration of the cytoplasm and degradation of mitochondria. Avis et al. (2009) documented
308 membrane retraction, undulation, and invagination, loss of membrane integrity, cytoplasmic leakage,
309 and cell rupture in *Fusarium sambucinum* after exposure to AlCl_3 and $\text{Na}_2\text{S}_2\text{O}_5$. In NaOH treatment,
310 the membrane was still intact and no substances were exuded, although disorganized cytoplasm and
311 organelles and a plasmolysis state were observed. It has been reported that ambient pH inhibits growth

of microorganisms by affecting enzyme activities, nutrient availability and the proton gradient across the plasma membrane, as well as cell wall remodeling (Pia Schmidt et al. 2008).

In the present research, the pH of NaSi from 20 mM to 100 mM in aqueous solution only changed from 12.0 to 12.6. NaSi is the salt of a very weak acid, so $[H^+] = ([acid]/[salt]) * K_{acid}$, and does not change much when the solution is diluted (Iler, 1979); thus, NaSi is a characteristic buffer solution. NaOH solutions have the same pH as the NaSi solutions. We have assumed the mechanism of the antifungal activity of NaSi was likely caused by the silicate anions. However, the reasons for the inhibition of conidial germination are still not well understood. Although our findings showed that the plasma membrane was greatly damaged by NaSi treatment, the mechanism behind plasma membrane damage appeared to be complex. There are several reasons for that. On the one hand, NaSi affects lipoxygenase (LOX) activity, which is the key enzyme in the fatty acid metabolism and catalyzes polyunsaturated fatty containing a cis-1,4-pentadiene structure (linoleic acid, linolenic acid) to produce hydrogen-peroxide. This led to cell wall thickening (O'Brien et al. 2012) and activates membrane lipid peroxidation to produce a series of free radicals such as ROO, RO. These radicals will eventually damage cell membrane structure (Reverberi et al. 2007). On the other hand, salt stress could induce plant oxidative stress and antioxidant defense (Mandhanian et al. 2006; Rodriguez-Rosales et al. 1999; Shi et al. 2007). So, we have deduced that NaSi may be a stress factor to induce fungal oxidative stress and cause the impairment of intracellular redox status, as a result of an increase in generation of reactive oxygen (ROS) beyond the cellular capacity to neutralize, which could lead to a hyperoxidation state (Heller and Tudzynski 2011). Higher ROS is responsible for oxidation of proteins, lipids, and nucleic acids and may cause cell death (Gessler et al. 2007). We also found that the application of

exogenous antioxidants, such as ascorbic acid and reduced glutathione, increased the viability of NaSi-treated conidia (data not shown). Thus the mechanism of membrane damage of NaSi was mainly related to the higher ROS. But further research should focus on the relationship between ROS production in *T. roseum* and NaSi treatment.

In conclusion, we found that NaSi and NaOH treatments could cause damage to *T. roseum* conidia, and NaSi showed more damage than corresponding pH. Although, *in vitro*, NaOH could inhibit the conidial germination, germ tube elongation and mycelial growth, *in vivo*, NaOH treatment did not reduce the pathogenicity of *T. roseum* conidia. Moreover, the membrane integrity, the leakage and the structure of NaSi-treated conidia were also changed considerably more than those treated by NaOH. Thus, we speculated that NaOH delayed the germination of conidia resulting in dormancy while application of NaSi caused the degradation of the organs in the conidia and eventual death.

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Draft

Table 1 The pHs of NaSi at different concentration in potato dextrose broth (PDB) solution and in sterile distilled water ($\pm$ SE)

NaSi (mM)	0	20	40	60	80	100
pH in PDB	7.0	10.4 $\pm$ 0.03	11.0 $\pm$ 0.05	11.3 $\pm$ 0.03	11.9 $\pm$ 0.06	12.3 $\pm$ 0.03
pH in water	6.4	12.0 $\pm$ 0.01	12.3 $\pm$ 0.02	12.4 $\pm$ 0.01	12.5 $\pm$ 0.02	12.6 $\pm$ 0.02

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Fig. 1 Effect of sodium silicate (NaSi) at different concentrations and NaOH solutions pH (adjusted to achieve the proper pHs found in the NaSi solutions) on conidial germination (A), germ tube length (B), and mycelial growth (C) of *T. roseum*. Treatments followed by different letters are statistically different by the Duncan's multiple range test ($p < 0.05$). Conidial germination and germ tube length were measured microscopically after 15 h of incubation at 28 °C in agar flake and sterile distilled water as a control (0). Mycelial growth was determined by measuring colony diameter after 5 d of inoculation incubated at 28 °C and PDA as a control (0).

Fig.2 Effect of wound-inoculated with sodium silicate (NaSi) -treated and NaOH solutions pH (adjusted to achieve the proper pHs found in the NaSi solutions) -treated conidia of *T. roseum* on pathogenicity in apple fruits. Conidia (1×10^6 conidia ml⁻¹) were pretreated with NaSi at 20, 40, 60, 80 and 100 mM, and NaOH solutions for 3 h, 6 h and 9 h at 28 °C, and sterile distilled water as control. Inoculated fruits were stored in low density polyethylene bags at room temperature (20 °C $\pm$ 2 °C) with a relative humidity (RH) of 75-80% for 15 days. Bars represent standard deviations of the means. Treatments followed by different letters are statistically different according to the Duncan's multiple range test ($p < 0.05$).

Fig.3 Effects of 100 mM sodium silicate (NaSi) and NaOH solutions pH of 12.6 on plasma membrane integrity of *T. roseum* conidia. The conidia were treated at 28 °C with NaSi at 100 mM and NaOH solutions pH of 12.6 for 3, 6 and 9 h, sterile water as the control. A: Microscopy images of *T. roseum* conidia after 9 h of incubation, B: percentage of plasma membrane integrity of *T. roseum* conidia. Data presented are averages of pooled data. Bars represent standard deviations of the means. PI (propidium iodide), a compound that penetrates damaged membranes was assayed to evaluate the integrity of the plasma membrane in the fungi exposed to the solutions.

Fig.4 Effects of sodium silicate (NaSi) at different concentrations and NaOH solutions pH (adjusted to achieve the proper pHs found in the NaSi solutions) on sugar leakage of *T. roseum*. Conidia were incubated NaSi at 20, 40, 60, 80 and 100 mM and NaOH solutions for 3 h, 6 h and 9 h at 28 °C, and sterile distilled water as control. Supernatants were used to assay the sugar leakage. A: Control; B: NaSi at 20 mM, pH at 12.0; C: NaSi at 40 mM, pH at 12.3; D: NaSi at 60 mM, pH at 12.4; E: NaSi at 80 mM, pH at 12.5; F: NaSi at 100 mM, pH at 12.6. Bars represent standard deviations of the means. Treatments followed by different letters are statistically different according to the Duncan's multiple range test ($p < 0.05$).

Fig.5 Effects of sodium silicate (NaSi) at different concentrations and NaOH solutions pH (adjusted to achieve the proper pHs found in the NaSi solutions) on protein leakage of *T. roseum* conidia. Conidia were incubated NaSi at 20, 40, 60, 80 and 100 mM and NaOH solutions for 3 h, 6 h and 9 h at 28 °C, and sterile distilled water as control. Supernatants were used to assay the protein leakage. A: Control; B: NaSi at 20 mM, pH at 12.0; C: NaSi at 40 mM, pH at 12.3; D: NaSi at 60 mM, pH at 12.4; E: NaSi at 80 mM, pH at 12.5; F: NaSi at 100 mM, pH at 12.6. Bars represent standard deviations of the means. Treatments followed by different letters are statistically different according to the Duncan's multiple range test ($p < 0.05$).

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Fig. 6 Effects of 100 mM sodium silicate (NaSi), NaOH solution pH of 12.6 and distilled sterile water (control) on ultrastructure of *T. roseum* conidia. A to C, After exposure to distilled sterile water for 3 h, 6 h, and 9 h, intact cell wall, membrane and other organelles, although the thickening of the cell wall at 9 h. D to F, After exposure to 100 mM NaSi for 3 h, 6 h, and 9 h, irregularly thickened cell wall, indistinct organelles, a plasmolysis state (black arrow), degradation of cytoplasm content, cell membrane blebbing (white arrow), leakage of cell wall and cell membrane (double arrow), the cell membrane is fragmented (arrowheads) and excludes were observed. G to I, After exposure to NaOH solution pH of 12.6 for 3 h, 6 h, and 9 h, irregularly thickened cell wall, indistinct organelles, a plasmolysis state (black arrow), degradation of cytoplasm content, cell membrane blebbing (white arrow), intact cell wall and membrane. CW: cell wall, N: cell nucleus, CP: cytoplasm, CM: cell membrane, L: lomasome. EX: excludes.