Journal of
Plant Pathology & Microbiology
ISSN: 2157-7471

Huang et al., J Plant Pathol Microbiol 2016, 7:4
http://dx.doi.org/10.4172/2157-7471.1000345

Research Article Open Access

Effect of Nanoscale Silicate Platelets on Azoxystrobin-resistant Isolates of *Botrytis cinerea* from Strawberry *In Vitro* and *In Vivo*

Ying-Jie Huang[1], Pi-Fang Linda Chang[1], Jenn-Wen Huang[1], Jiang-Jen Lin[2] and Wen-Hsin Chung[1]*

[1]*Department of Plant Pathology, National Chung Hsing University, No. 145, Xingda Rd., Taichung 40227, Taiwan*
[2]*Institute of Polymer Science and Engineering, National Taiwan University, No. 1, Sec. 4, Roosevelt Rd., Taipei 10617, Taiwan*

Abstract

The inhibitory effect of nanoscale silicate platelets modified with different surfactants, i.e., NSS1450 and NSS3150 was evaluated for spore germination and mycelial growth of azoxystrobin-resistant (AR) and -sensitive (AS) *Botrytis cinerea* isolates from strawberry. The treatments with NSS1450 at the concentration of more than 50 mg/L significantly reduced spore germination by 99.2-100.0% for AR isolates and by 100.0% for AS isolates. In contrast, NSS3150 failed to show an inhibition for the spore germination of AR *B. cinerea* isolates. In another test, NSS1450 at concentrations higher than 500 mg/L could reduce mycelial growth by 60.2-100.0% and 93.8-100.0% for AR and AS isolates, respectively, while NSS3150 showed similar inhibition on the spore germination. Further, NSS1450 at the concentrations between 500 to 1000 mg/L and accompanied with a fungicide, azoxystrobin, demonstrated high inhibitory rate 93.8 to 100.0% inhibition) in AR and AS isolates of *B. cinerea*. Under scanning electron microscope, the NSS1450 at the concentration of 1000 mg/L affected the morphologies of spores and mycelia in shrinking. The disease severity on strawberry leaves was 8.3% after 13 days incubation when treated with NSS1450 at 24 h after the pathogen inoculation. Moreover, spraying the mixture solution of 100 mg/L NSS1450 and 100 mg a.i./L azoxystrobin at 24 h after or before pathogen inoculation showed significantly efficacy compared with inoculation pathogen only. The results indicated that the silicate nanoplatelets after modification by a proper surfactant or NSS1450 had the potentials for treating gray mold disease although further studies are still needed under greenhouse and field conditions.

Keywords: Azoxystrobin-resistant; *Botrytis cinerea;* Nanoscale silicate platelet

Introduction

Gray mold caused by *B. cinerea* is the common crop disease while more than 235 plants have been identified as the host, including strawberry, orchid, legume, *Brassica* spp., tomato and grape [1]. Strawberry gray mold is considered the most important diseases due to the severity of affecting strawberry production [2]. Currently, the major treatment for gray mold is by chemical methods [3]. In Taiwan, several chemical agents, including anilinopyrimidines, benzimidazoles, dicarboxidimides, demethylation inhibitors and strobilurins are practiced currently for controlling strawberry diseases [4]. However, several researchers have indicated that *B. cinerea* showed a low sensitivity or even resistance to the chemicals, anilinopyrimidine [2,5-8], dicarboxidimide [2,9-14] and strobilurins [15-18]. Among these fungicides, strobilurin fungicides are quinone outside inhibitors (QoIs) to interrupt the electron transport chain in mitochondria [19]. Except *B. cinerea*, more than fifty plant fungal pathogens have been reported to show resistance to strobilurin fungicides due to the single-site action mechanism (Fungicide Resistance Action Committee, www.frac.info). In Taiwan, *B. cinerea* from strawberry also had been demonstrated to be resistant to azoxystrobin [16]. Therefore, finding an alternative other than chemicals for controlling gray mold in strawberry is urgent.

In recent years, nanomaterials have been actively studied for various applications [20]. Different species and geometric shapes of nanomaterials, such as nanospheres, nanorods, nanowires, nanocubes and nanoprisms of inorganic materials are developed and synthesized [21]. Some of the examples include silver nanoparticle, nanoscale silicate platelets, carbon nanotubes, and the nanoparticles of TiO_2, MgO, ZnO, copper and gold [22,23]. Several applications and new functions for antimicrobial ability against human and plant pathogens have been revealed [24]. Silver nanoparticles, nanoscale silicate platelets, ZnO nanoparticles and carbon nanotubes have been well documented for the functions of inhibiting the growth of human pathogens including *Bacillus subtilis* [25], *Escherichia coli* [26,27], *Pseudomonas aeruginosa* [28], *Staphylococcus aureus* [25-28], *Streptococcus pyrogens* [28]. The inhibitory effects on plant pathogens including *Bipolaris sorokiniana* and *Magnaporthe oryzae* [29], *Raffaele* sp. [30], *Colletotrichum* spp. [31,32], *Botrytis cinerea* [33], green mold in postharvest [34], *Xanthomonas hortorum* pv. *pelargonii* and *X. axonopodis* pv. *poinsettiicola* [35] have been reported.

In the present study, we report the controls of plant pathogens or diseases by nanoscale silicate platelets aiming at achieving the following objectives; 1) growth effect in spore and mycelia of azoxystrobin-resistant and azoxystrobin-sensitive *B. cinerea* isolates, 2) the involved mechanism or morphological change on the spores and mycelia under the direct observation of scanning electron microscope (SEM), and 3) quantitative measurement of the control efficacy of on strawberry gray mold disease. The overall purpose is to establish the efficacy and the mechanism of the silicate platelets on plant pathogen controls.

Materials and Methods

Fungal isolates and nanoscale silicate platelets

Table 1 shows the four *B. cinerea* isolates, two azoxystrobin-resistant (AR) isolates (GBS1-81 and GBS1-104) and two azoxystrobin-

***Corresponding author:** Wen-Hsin Chung, Department of Plant Pathology, National Chung Hsing University, No. 145, Xingda Rd., Taichung 40227, Taiwan, Tel: +886-422840780; E-mail: wenchung@nchu.edu.tw

Received April 10, 2016; **Accepted** April 25, 2016; **Published** April 28, 2016

Citation: Huang YJ, Chang PFL, Huang JW, Lin JJ, Chung WH (2016) Effect of Nanoscale Silicate Platelets on Azoxystrobin-resistant Isolates of *Botrytis cinerea* from Strawberry *In Vitro* and *In Vivo*. J Plant Pathol Microbiol 7: 345. doi:10.4172/2157-7471.1000345

Concentration (mg a.i./L)	Inhibition rate of mycelia growth (%)			
	GBS1-81	GBS1-104	GBS3-93	GBS0-15
1	0.0	0.0	4.7	7.0
10	9.7	0.0	57.8	4.7
100	8.7	2.3	81.3	39.8
500	5.5	0.0	100.0	61.7
1000	8.0	12.5	100.0	67.2

Table 1: Inhibition rate of mycelial growth of *Botrytis cinerea* treated with different concentrations of azoxystrobin.

sensitive (AS) isolates (GBS3-93 and GBS0-15), obtained from diseased tender leaves of strawberry to azoxystrobin sensitivity. The four isolates were cultured in PDA (potato dextrose agar, Difco, USA) at 20℃ in dark for mycelial growth and sporulation. Two different species of nanoscale silicate platelets (NSP), NSS1450 and NSS3150 were prepared according to the previously Lin et al. (US patents 8 629 200 (2014) and 13/933,913 (2015); ROC patents I435695 (2011)). The average dimension for nanoscale silicate platelets was ca. 80-100 nm × 80-100 nm × 1 nm with the chemical functionalities of Si-OH and Si-ONa ionic charges. NSS1450 and NSS3150 were the cationic and nonionic surfactant-modified NSP, respectively.

Tests of nanoscale silicate platelets on spore germination of AR and AS *B. cinerea*

To assay the inhibitory effects of NSS1450 and NSS3150 on the spore germination of four *B. cinerea* isolates, spore suspensions (10^6 spore/ml) of AR and AS *B. cinerea* isolates prepared from 5 days old fungal culture were mixed with NSS1450 and NSS3150, respectively. The concentration of the mixed solution was 10, 50, 100, 500, 1000 and 2000 mg/L. After incubation at 20℃ for 1, 6, 12 and 24 h, 100 µL of the treated spore suspension (10^4 spore/mL) was spread on PDA medium and incubated at 20℃ in dark. The number of spore germination was counted under a microscope after 24 h of incubation. The spore suspension mixed with sterile water (SW) was used as the control. The experiment was repeated twice with three replicates for each treatment. Inhibition rate of spore germination was calculated by the following expression:

Inhibition (%) = 【(control-treatment)/control 】× 100

Effect of nanoscale silicate platelets on mycelial growth of AR and AS *B. cinerea*

To assay the inhibitory effects of NSS1450 and NSS3150 on the mycelial growth of four *B. cinerea* isolates, the nanoscale silicate platelets stock (10^5 mg/L) solution was prepared and added to the PDA medium to give final concentrations of 10, 50, 100, 500, 1000 and 2000 mg/L. Amended PDA plates were inoculated with 3 mm mycelium disk of AR and AS *B. cinerea* from 4-day old fungal cultures. Plates were incubated at 20℃ in dark, and the colony diameter was measured after 3 days of incubation. The PDA plate without NSS was the control. The experiment was repeated twice with three replicates for each treatment. To assess the difference in the mycelial growth of *B. cinerea* due to treatments, the percentage inhibition was calculated by the following expression:

Inhibition (%) = 【(control-treatment)/control】× 100

Effect of azoxystrobin combined with nanoscale silicate platelets on mycelial growth of AR and AS *B. cinerea*

The effect of fungicide azoxystrobin in combination with nanoscale silicate platelets on mycelia growth of AR and AS *B. cinerea* isolates was evaluated on PDA plates. The fungicide stock (5000 mg a.i./L) and the silicates stock (10^5 mg/L) solutions were prepared and added to the PDA medium to give the final concentrations of fungicide azoxystrobin 100, 500 and 1000 mg a.i./L (10% SC, Syngenta, Taiwan) and the NSS concentrations of 1, 10, 100, 500 and 1000 mg/L. Amended PDA plates were inoculated with 3-mm mycelium disk of AR and AS *B. cinerea* from 4 days old fungal cultures. Plates were incubated at 20℃ in dark, and the colony diameters were measured after 3 days of incubation. The PDA plate without any of NSS or azoxystrobin was used as the control. The experiment was repeated twice with three replicates for each treatment. To assess the difference in the mycelial growth of *B. cinerea* among the treatments, the percentage inhibition was calculated by the following expression:

Inhibition (%) = 【(control-treatment)/control】× 100

Morphological observation of AR isolate treated with NSS1450 by scanning electron microscope

Scanning electron microscope (SEM) was used to study the morphological change of AR GBS1-104 isolate after treating with NSS1450, both spore and mycelia of GBS1-104. GBS1-104 isolate (10^7 conidia/mL) treated with 0, 100 and 1000 mg/L NSS1450 at 20℃ for 12 and 24 h, respectively, were loaded on 0.22 µmm Millipore (GS, Millipore Co., USA) to observe the spores by SEM (ABT-150S, TOPCON, Japan) based on SEM Cryo-Transfer system E7400 (Bio-read, USA). For mycelia observation, 3-day-old cultures of the GBS1-104 isolate (3 days old) was grown on PDA at 20℃ in dark and sprayed with 0, 100 and 1000 mg/L of NSS1450. After incubation for 12 and 24 h, mycelia discs (3 mm in diameter) from PDA plates, both treated and untreated with NSS1450, were observed.

Efficacy of NSS1450 only and its combination with azoxystrobin on controlling gray mold of strawberry

The NSS1450 was used in the control experiment of strawberry gray mold based on the detached leaf inoculation method. Conidia of *B. cinerea* (AR isolate GBS1-104) were harvested from 7 day old cultures grown on PDA medium and incubated at 20℃ in dark, by scraping the mycelium with a glass rod using 10 mL of sterile distilled water. Spore concentration was then adjusted using the haemocytometer to 10^6 conidia/mL for inoculation. The 2 month old strawberry (*Fragaria* × *ananassa* Duch) plants cv. Feng Xiang for inoculation was obtained from Taiwan Seed Improvement and Propagation Station, Hsinhse, Taichung. Treatments included: a) spraying the 100 mg/L NSS1450 solution at 24 h after or before pathogen inoculation; b) spraying the 100 mg a.i./L azoxystrobin solution at 24 h after or before pathogen inoculation; c) spraying the mixture solution of 100 mg/L NSS1450 and 100 mg a.i./L azoxystrobin at 24 h after or before pathogen inoculation; d) spraying 100 mg/L NSS1450 solution at 48 h after or before pathogen inoculation; e) spraying 100 mg/L NSS1450 solution at 72 h after or before pathogen inoculation; f) spraying 100 mg/L NSS1450 solution only; g) inoculating the pathogen suspension only; h) spraying distilled water only. All inoculated strawberry leaves in 15 cm petri dish were kept in a dark growth chamber with relative humidity of 90 to 95% at 20°C for 7, 10 and 13 incubation days. Evaluation of gray mold development was carried out based on appearance of disease symptoms. The disease index is separated into four rankings, 0: no symptom; 1: symptom area under 25%; 2: symptom area between 26 to 50%; 3: symptom area between 50 to 75%; 4: symptom area over 75%. The measurement of disease severity (%) is as the following: [(Σni x i)/(N x 4)] × 100 (i: disease index; ni: number of the ith plant; N:

total number of plant). The experiment was run in triplicates, with each replicate representing a petri-dish with inoculated detached leaves.

Statistical analysis

Data from mycelial growth, and gray mold disease severity were analyzed using analysis of variance (ANOVA). Fisher LSD test at the 5% significant level was used to compare the means of the treatment in each experiment. All statistical analyses were conducted using SAS/STAT software (SAS Institute, 1989).

Results

Effect of nanoscale silicate platelets on spore germination of AR and AS *B. cinerea*

Table 2 shows the inhibition of the spore germination of AR and AS isolates *B. cinerea* exposed to NSS1450 and NSS3150 at different concentrations. By comparison, NSS1450 inhibited the germination of AR and AS isolates *B. cinerea* more significantly than NSS3150 (Table 2) at all concentrations except 10 mg/L, when a weak inhibitory effect (only 30.8% inhibition) in AR isolate GBS1-104 was observed. NSS1450 exhibited high inhibition rate of spore germination in AR isolate GBS1-81 (99.2% to 100%) and AR isolate GBS1-104 (100%) at all other concentrations. Furthermore, the high inhibition of nearly 100% in AS isolates GBS3-93 and GBS0-15 was achieved. In contrast, NSS3150 showed a variation of inhibitory effect in AR or AS isolates at various concentrations and failed to show a significant inhibition against AR or AS isolates.

Inhibition of nanoscale silicate platelets on mycelial growth of AR and AS *B. cinerea*

Table 3 shows the inhibition of mycelial growth in the presence of NSS. The inhibition of mycelial growth of AR and AS *B. cinerea* isolates was in the range of 60.2 to 100.0% in the concentrations of 500 to 2000 mg/L In the case of NSS1450 at 50-100 mg/L concentrations, the inhibition of 65.5% to 90.8% in AS isolate GBS3-93, and a moderate degree of inhibition on the two AR isolates (GBS1-81 and GBS1-104) and one AS isolate (GBS0-15) were observed. It was noticed that NSS1450 at a low concentration of 10 mg/L did not affect AR and AS *B. cinerea* isolates. In contrast, NSS3150 at 10 to 100 mg/L, all of *B. cinerea* isolates were not significantly affected. The most sensitive isolate to NSS315 was AS isolate of GBS3-93 (62.1% to 96.6% inhibition), followed by two AR isolates and AS isolate of GBS0-15 under the tests at concentrations of 500 to 2000 mg/L.

Treatment	Concentration (mg/L)	Inhibition rate of spore germination (%)															
		GBS1-81[a]				GBS1-104[a]				GBS3-93[b]				GBS0-15[b]			
		1 hr	6 hr	12 hr	24 hr	1 hr	6 hr	12 hr	24 hr	1 hr	6 hr	12 hr	24 hr	1 hr	6 hr	12 hr	24 hr
NSS1450	10	100.0	100.0	100.0	100.0	30.8	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	50	99.4	99.5	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	10sc0	99.3	99.5	99.3	99.2	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	500	99.7	99.9	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	1000	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	2000	99.6	99.9	99.8	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
NSS3150	10	26.2	31.2	20.2	25.9	0.0	0.0	0.0	0.0	0.0	2.6	0.0	0.0	44.4	53.0	5.8	7.8
	50	27.7	36.0	0.0	0.0	0.0	0.0	0.0	0.0	1.8	1.6	2.7	0.0	44.3	45.8	0.0	0.6
	100	42.8	51.9	3.0	0.1	0.0	0.0	0.0	0.0	16.0	8.5	12.2	0.0	32.3	21.2	1.9	5.0
	500	73.5	74.7	7.2	11.6	0.0	0.0	31.9	30.3	19.2	33.9	29.6	0.0	20.0	26.9	39.6	29.6
	1000	80.4	83.9	30.8	30.3	59.9	0.0	22.1	21.6	98.1	45.4	42.2	30.7	30.0	38.5	39.7	39.3
	2000	93.1	93.3	53.4	53.5	91.4	23.7	36.2	40.3	98.1	100.0	92.7	43.8	47.8	52.3	51.2	63.3
CK		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[a]Azoxystrobin-resistant isolates
[b]Azoxysrobin-sensitive isolates

Table 2: Inhibition rate of spore germination of *Botrytis cinerea* isolates treated with nanoscale silicate platelets NSS1450 and NSS3150.

Treatment	Concentration (mg/L)	Inhibition rate of mycelial growth (%)			
		GBS1-81[a]	GBS1-104[a]	GBS3-93[b]	GBS0-15[b]
NSS1450	10	0.0 a[c]	0.0 a	0.0 a	0.0 a
	50	3.9 a	15.7 b	65.5 b	3.1 a
	100	32.8 b	14.9 b	90.8 c	18.0 a
	500	60.2 c	89.7 c	100.0 c	95.3 c
	1000	98.4 d	100.0 c	100.0 c	93.8 c
	2000	100.0 d	100.0 c	100.0 c	100.0 c
NSS3150	10	0.0 a	0.0 a	0.0 a	0.0 a
	50	0.0 a	0.0 a	0.0 a	0.0 a
	100	0.0 a	0.0 a	0.0 a	0.0 a
	500	43.0 b	46.7 b	62.1 b	54.7 b
	1000	64.8 c	65.1 c	96.6 c	66.4 b
	2000	77.3 d	68.2 c	100.0 c	69.5 b
CK		0.0 a	0.0 a	0.0 a	0.0 a

[a]Azoxystrobin-resistant isolates
[b]Azoxystrobin-sensitive isolates
[c]Means followed by the same letter are not significantly different based on Fisher's least significant difference (LSD) test (p=0.05).

Table 3: Inhibition rate of mycelial growth of *Botrytis cinerea* treated with nanoscale silicate platelets NSS1450 and NSS3150.

Efficacy of azoxystrobin with nanoscale silicate platelets on inhibition of mycelial growth

The combinations of NSS1450 or NSS3150 at the concentration ranging from 100 to 1000 mg/L and the fungicide azoxystrobin at 1 to 1000 mg a.i/L significantly reduced the mycelium growth rate of AR and AS *B. cinerea* isolates (Tables 4 and 5). Among these tests, it was noted a dose effect for NSS1450. At the concentrations from 500 to 1000 mg/L in the presence of, azoxystrobin at all concentrations tests showed the strongest inhibitory effect (93.8% to 100.0% inhibition) in AR and AS isolates f *B. cinerea*. However, NSS1450 at 100 mg/L in combination with azoxystrobin only showed marked inhibition in AS isolates (100% inhibition), but not in AR isolates (39.8% to 72.7%). By comparison, NSS3150 at the concentration of 500-1000 mg/L and in combination with azoxystrobin at all concentrations tested had 100% inhibition rate of mycelial growth for one AR isolate GBS1-81 and all AS isolates (Table 5). However, at the low concentration of 100 mg/L, the results showed no effect on AR isolates but 92.2% to 100.0% inhibition rate on AS isolates.

Morphological observation of AR isolate treated with NSS1450 by scanning electron microscope

The spore morphology of AR isolate GBS1-104 treated by NSS1450 indicated that some of GBS1-104 spores surface had slightly shrinking its abnormal shape at 12 h and 24 h after 100 mg/L of NSS1450 treatment (Figures 1b and 1c) in comparison with the control (Figure 1a), and some germ tube showing the shrunk spores (Figure 1d). On the other hand, most of GBs1-104 spores surface appeared to be shrunk after 12 and 24 h after the treatment of 1000 mg/L NSS1450 (Figures 1e-1g). For mycelia observation, the mycelia of GBS1-104 isolate did not have significantly changed at the concentration of 100 mg/L treatment (Figures 2a and 2b). Contrary to 100 mg/L, the 1000 mg/L of NSS1450 caused mycelia seriously shrunk at 12 h and 24 h treatment (Figures 2c and 2d) in relative to the control (Figure 2e).

Efficacy of NSS1450 alone and in combination with azoxystrobin on the control of gray mold of strawberry

The effect on gray mold of strawberry was examined by using NSS1450 and its combination with azoxystrobin. Table 6 shows the reduction of disease severity after the pathogen inoculation. At day 13th, gray mold severity was the lowest when spraying NSS1450 at 24 h after pathogen inoculation (8.3%) and the highest when spraying NSS1450 at 48 h (66.7%). In this study, the NSS1450 with azoxystrobin had no synergistic effect on reducing disease of gray mold on strawberry. Before the pathogen inoculation, the spraying of NSS1450 at 24, 48 and 72 h allowed the control of disease severity at 50.0%, 83.3% and 91.7% at 13 days of inoculation, respectively. Further, the combination of NSS1450 and azoxystrobin showed significantly lowering of the disease severity of 33.3% at the 13th day.

NSS1450 (mg/L)	Az (mg a.i./L)	Inhibition rate of mycelia growth (%)			
		GBS1-81[a]	GBS1-104[a]	GBS3-93[b]	GBS0-15[b]
100	1	39.8	61.7	100.0	100.0
	10	54.7	72.7	100.0	100.0
	100	53.1	60.9	100.0	100.0
	500	49.2	44.5	100.0	100.0
	1000	64.1	64.1	100.0	100.0
500	1	100.0	94.5	100.0	100.0
	10	100.0	96.9	93.8	100.0
	100	100.0	95.3	100.0	100.0
	500	100.0	98.4	100.0	100.0
	1000	100.0	97.7	100.0	100.0
1000	1	100.0	100.0	100.0	100.0
	10	100.0	100.0	100.0	100.0
	100	100.0	100.0	100.0	100.0
	500	100.0	100.0	100.0	100.0
	1000	100.0	100.0	100.0	100.0
CK	0	0.0	0.0	0.0	0.0

[a]Azoxystrobin-resistant isolates.
[b]Azoxystrobin-sensitive isolates

Table 4: Inhibition rate of mycelia growth of *Botrytis cinerea* treated different concentration of NSS1450 mixed with azoxystrobin (Az).

NSS3150 (mg/L)	Az (mg a.i./L)	Inhibition rate of mycelial growth (%)			
		GBS1-81[a]	GBS1-104[a]	GBS3-93[b]	GBS0-15[b]
100	1	0.0	0.0	100.0	100.0
	10	0.0	32.2	100.0	100.0
	100	0.0	0.0	100.0	100.0
	500	0.0	15.6	100.0	100.0
	1000	9.4	0.0	100.0	100.0
500	1	100.0	31.3	100.0	100.0
	10	100.0	42.2	100.0	100.0
	100	100.0	57.8	100.0	100.0
	500	100.0	51.6	100.0	100.0
	1000	100.0	59.4	100.0	100.0
1000	1	100.0	100.0	100.0	100.0
	10	100.0	100.0	100.0	100.0
	100	100.0	100.0	100.0	100.0
	500	100.0	100.0	100.0	100.0
	1000	100.0	100.0	100.0	100.0
CK	0	0.0	0.0	0.0	0.0

[a]Azoxystrobin-resistant isolates.
[b]Azoxystrobin-sensitive isolates

Table 5: Inhibition rate of mycelial growth of *Botrytis cinerea* treated with different concentrations of NSS3150 combined with azoxystrobin (Az).

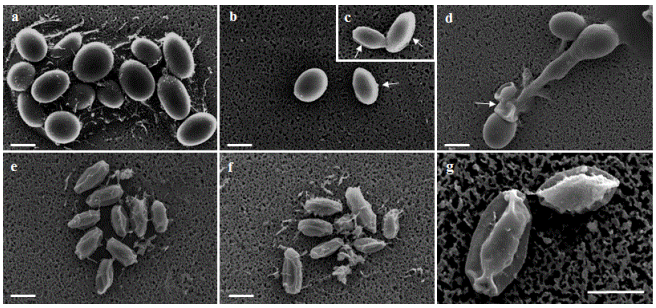

Figure 1: SEM pictures of azoxystrobin-resistant isolate GBS1-104 of *Botrytis cinerea* spores treated with 100 and 1,000 mg/L nanosilicate platelets NSS1450 under different timing courses. (a): spores showed normal size after treated with water; (b) and (c) spores showed slightly shrinking after treated with 100 mg/L NSS1450 at 12 h (arrow point); (d): germ tube showed shrinking after treated with 100 mg/L NSS1450 at 24 h (arrow point); (e): spores showed shrinking after treated with 1,000 mg/L NSS1450 at 12 h; (f) and (g): spores showed shrinking after treated with 1,000 mg/L NSS1450 at 24 hr. Bar=5 μm.

Discussion

Results showed that nanoscale silicate platelets decorated with NSS1450 had high efficacy in reducing the spore germination and mycelial growth of azoxystrobin-resistant and -sensitive *Botrytis cinerea* isolates. By comparison, NSS3150 had less effectiveness in controlling the disease. It was indicated that the two silicate platelets

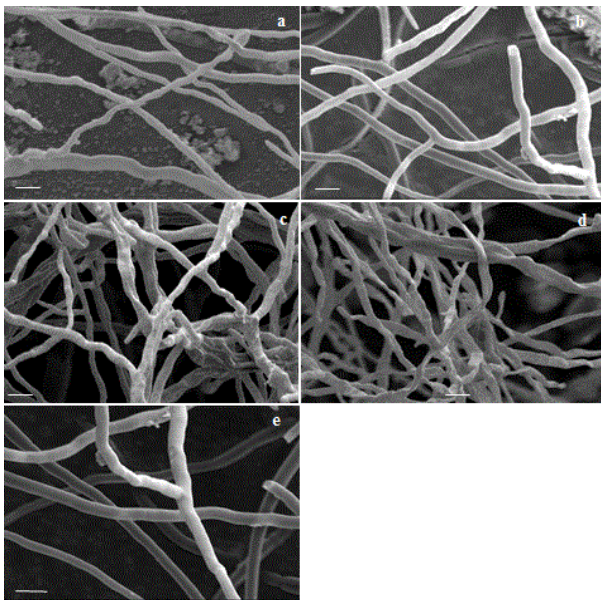

Figure 2: SEM pictures of azoxystrobin-resistant isolate GBS1-104 of *Botrytis cinerea* mycelia treated with NSS1450. (a): mycelia showed normal size after treated with 100 mg/L NSS1450 at 12 h; (b): mycelia showed normal size after treated with 100 mg/L NSS1450 at 24 h; (c): mycelia showed shrinking after treated with 1,000 mg/L NSS1450 at 12 h; (d): mycelia showed shrinking after treated with 1,000 mg/L NSS1450 at 24 h; (e): mycelia treated with water. Bar = 5 µm.

Treatment[a]	Disease severity (%)					
	P-T[b]			T-P[b]		
	7 d	10 d	13 d	7 d	10 d	13 d
A	0.0 a[c]	0.0 a	8.3 b	16.7 b	25.0 b	50.0 d
B	8.3 b	33.3 c	83.3 e	16.7 b	25.0 b	41.7 c
C	25.0 d	50.0 d	58.3 c	16.7 b	33.3 c	33.3 b
D	16.7 c	58.3 e	66.7 d	41.7 d	75.0 e	83.3 e
E	8.3 b	25.0 b	58.3 c	33.3 c	66.7 d	91.7 f
F	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
G	100.0 e	100.0 f	100.0 f	100.0 e	100.0 f	100.0 g
H	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a

[a]A) to spray the 100 mg/L NSS1450 solution at 24 hr after or before the pathogen inoculation; B) to spray the 100 mg a.i./L azoxystrobin solution at 24 hr after or before the pathogen inoculation; C) to spray the mixture solution of 100 mg/L NSS1450 and 100 mg a.i./L azoxystrobin at 24 hr after or before the pathogen inoculation; D) to spray 100 mg/L NSS1450 solution at 48 hr after or before the pathogen inoculation; E) to spray 100 mg/L NSS1450 solution at 72 hr after or before the pathogen inoculation; F) to spray 100 mg/L NSS1450 solution only; G) to inoculate the pathogen suspension only; H)to spray distilled water only .
[b]P-T: treated with NSS1450 or azoxystrobin after the pathogen inoculation; T-P: treated with NSS150 or azoxystrobin before the pathogen inoculation.
[c]Means followed by the same letter are not significantly different based on Fisher's least significant difference (LSD) test (p=0.05).

Table 6: Control of gray mold caused by azoxystrobin-resistant GBS1-104 isolate on strawberry leaves by different treatments.

had different zeta potential. The zeta potential values of NSS1450 and NSS3150 were measured to be 42 mv and -30 mv, respectively. Benefield et al. [36] reported when the zeta potential value was greater than 30 mv, the nanomaterials could distribute uniformly and remain stable in the solution. Hence, it was speculated that NSS1450 could distribute more uniformly and remain in solution for longer time than NSS3150 thereby facilitation its interaction with *B. cinerea* isolates and enhancing the inhibitory efficacy.

Results also showed that spores were more sensitivity to NSS1450 than mycelia. In this study, the spores were suspended with NSS1450 solution directly, and the mycelia were grew on the PDA mixed with NSS1450. It was possible that NSS1450 could be absorbed by subglobse or ellipsoid spores of *B. conidia* completely when the spores were suspended with NSS1450 solution. On the contrary, the mycelia were grew on solid PDA medium. Although the mycelia discs were transferred to the PDA mixed with NSS1450, the mycelia might not attach NSS1450 uniformly. Thus, spores suspended with the silicate solution might be a suitable method for evaluation of the efficacy of nanoscale silicate platelet.

The combination of NSS1450 with azoxystrobin demonstrated good efficacy for inhibiting the mycelial growth. Previous studies revealed that a nanomaterial might function as a delivery system for fungicides to reach pathogens in more efficient manner in the case of silver nanoparticles mixing with fluconazole [37] and with tebuconaole or chlorotalonil [38]. Thus, a synergistic ability could be achieved. In this study, the mycelial inhibition rate of AR isolates was increased when mixing NSS1450 with azoxystrobin. It was demonstrated that NSS1450 had synergistically induce the efficacy of azoxystrobin on inhibition of resistant isolates. The carrier effect was observed for NSS and might be applicable to other fungicides.

The direct observation by SEM indicated that the high concentration of NSS1450 (1000 mg/L) could cause spore and mycelia shrinkages Previous study revealed that the cell of *Staphylococcus aureus* and *Escherichia coli* could be surface wrinkled or shrunk after the treatment by nanoscale silicate platelets [26]. Silver nanoparticles damaged the mycelia of *Raffaelea* sp. (causing quercus wilting) [30] or *Colletotrichum acutatum* [32]. ZnO nanoparticles caused the abnormalities for mycelia of *B. cinerea* and conidiophore of *Penicillium italicum* based on SEM observation [34]. According to these observations, our results showed that NSS1450 could also be absorbed by spores and mycelia of *B. cinerea* and caused surface changes. Moreover, it was also known that silver nanoparticle could affect the transport systems of bacterial cells [39]. Results of control experiments demonstrated that the NSS1450 alone or combined with azoxystrobin could decrease the disease severity of gray mold caused by azoxystrobin-resistant (AR) isolate on strawberry. Several nanomaterials had been reported to control plant diseases, such as nanosized silver-silicate [40] and silver nanoparticle [29,32]. Nanosized silver-silicate could control the powdery mildew of pumpkin in greenhouse and field [40], and silver nanoparticles could reduce the ryegrass diseases caused by *Bipolaris sorokiniana* and *Magnaporthe grisea* [29] and pepper anthracnose caused by *C. acutatum* [32]. According to our best knowledge, this is the first report that nanosilicate platelet could control gray mold caused by AR isolate on strawberry. Consequently, the NSS1450 could be an alternative material to control gray mold in crops, especially caused by AR isolates. However, more crops gray mold caused by *B. cinerea* need to be examined. The treatment time of applying NSS1450 could influence the inhibitory efficacy of gray mold on strawberry leaves. Previous studies indicated that spraying silver nanoparticles before pathogens inoculation had significant prevention on diseases control [29,32]. However, spraying NSS1450 before pathogen inoculation did not show significant control efficacy. The mechanism of silver nanoparticles in antifungal activity has been demonstrated to associate with the germination and infection process of different fungi, and the silver nanoparticles could rapidly generate toxic Ag^+ ion on leaves surface first and attached with spores later [29]. Comparing with silver nanoparticles, there was no concern of release toxic chemical such as silver ion from the nanoscale silicate platelets of NSS1450. We consider that *B. cinerea* inoculation first and keeping high moisture within 24

h could assist NSS1450 to be absorbed by *B. cinerea* (negative charge). The efficacy decreased following the NSS1450 treatment time due to penetration of leaf tissue by *B. cinerea*. On the other hand, spraying NSS1450 first could not keep high moisture continually and caused the later inoculation of *B. cinerea* difficultly to attach the NSS1450 on leaf surface. The detailed experimental procedures regarding the moisture effect remains an important topic for future studies in greenhouse and field trials.

Acknowledgments

We wish to thank the Ministry of Science and Technology of Taiwan for the financial supports.

References

1. Prins TW, Tudzynski P, Von Tiedemann A, Tudzynski B, Ten Have A, et al. (2000) Infection strategies of *Botrytis cinerea* and related necrotrophic pathogens. Fungal Pathology pp: 33-64.
2. Elad Y, Shpialter L, Korolev N, Mamiev M, Rav David D, et al. (2008) Integrated chemical and cultural control for grey mould (*Botrytis cinerea*) management in Lisianthus. In: Modern Fungicides and Antifungal Compounds IV. Proceedings of the 15th International Reinhardsbrunn Symposium.
3. Rabølle M, Spliid NH, Kristensen K, Kudsk P (2006) Determination of fungicide residues in field-grown strawberries following different fungicide strategies against gray mold (*Botrytis cinerea*). J Agric Food Chem 54: 900-908.
4. Fei WC, Wang YM (2004) Plant protection manual. Taiwan Agriculture Chemicals and Toxic Substance Research Institute, Wufeng, Taichung pp: 835.
5. Baroffio CA, Siegfried W, Hilber UW (2003) Long-term monitoring for resistance of *Botryotinia fuckeliana* to anilinopyrimidine, phenylpyrrole and hydroxyanilide fungicides in Switzerland. Plant Disease 87: 662-666.
6. Chapeland F, Fritz R, Lanen C, Gredt M, Leroux P (1999) Inheritance and mechanisms of resistance to anilinopyrimidine fungicides in *Botrytis cinerea* (*Botryotinia fuckeliana*). Pestic Biochem Physiol 64: 85-100.
7. Forster B, Staub T (1996) Basis for use strategies of anilinopyrimidine and phenylpyrrole fungicides against *Botrytis cinerea*. Crop Prot 15: 529-537.
8. Sergeva V, Nair NG, Verdana JR, Shen C, Barchia I, et al. (2002) First report of anilinopyrimidine resistant phenotypes in *Botrytis cinerea* on grapevines in Australia. Australas Plant Pathol 31: 299-300.
9. Davis RP, Dennis C (1979) Use of dicarboximide fungicides on strawberries and potential problems of resistance in *Botrytis cinerea*. Proceedings of the British Crop Protection Conference, Pests & Diseases 193-201.
10. Holz B (1979) Uber eine resistenzersheinung von *Botrytis cinerea* an Reben gegen die neuen Kontaktbotrytizide im Gebeit der Mittelmosel. Weinberg und Keller 26: 18-25.
11. Hsiang T, Chastagner GA (1990) Parasitic fitness of benzimidazole and dicarboximide resistant isolates of *Botrytis cinerea Botrytis elliptica* and *Botrytis tulipae*. Phytopathology 80: 978.
12. Chastagner GA, Riley K (1987) Occurrence and control of benzimidazole and dicarboximide resistant *Botrytis* spp. on bulb crops in Western Washington and Oregon USA. Phytopathology 77: 1237.
13. Leroux P, Lafon R, Gredt M (1982) Resistance of *Botrytis cinerea* to benzimidazole and dicarboximide fungicides situation in Alsace Bordeaux and Champagne vineyards France. Bulletin OEPP 12: 137-144.
14. Steekelenburg NAM van (1987) Resistance to benzimidazole and dicarboximide fungicides in *Botrytis cinerea* and *Didymella bryoniae* in cucumbers in the Netherlands. Medelingen van de Faculteit Landbouwwetenschappen Universiteit Gent 52: 875-880.
15. Bardas GA, Veloukas T, Koutita O, Karaoglanidis GS (2010) Multiple resistance of *Botrytis cinerea* from kiwifruit to SDHIs, QoIs and fungicides of other chemical groups. Pest Manag Sci 66: 967-973.
16. Chen LS, Chung WC, Chung WH (2009) Sensitivity of *Botrytis cinerea* of strawberry to strobilurins (QoIs) in Taiwan. Plant Pathology Bulletin 18: 89-99.
17. Ishii H (2008) Fungicide research in Japan, an overview. In: Modern Fungicides and Antifungal Compounds IV. Proceedings of the 15th International Reinhardsbrunn Symposium. Eds. Dehne H-W, Gisi U, Kuck K-h, Russell P E, Lyr H.
18. Markoglou AN, Malandrakis AA, Vitoratos AG, Ziogas BN (2006) Characterization of laboratory mutants of *Botrytis cinerea* to QoI fungicides. Eur J Plant Pathol 115: 149-162.
19. Bartlett DW, Clough JM, Godwin JR, Hall AA, Hamer M, et al. (2002) The strobilurin fungicides. Pest Manag Sci 58: 649-662.
20. Taniguchi N (1974) On the basic concept of nanotechnology. In Proceeding of International Conference on Production Engineering (ICPE). Tokyo, Japan pp: 18-23.
21. Murphy CJ (2002) Materials science. Nanocubes and nanoboxes. Science 298: 2139-2141.
22. Nair R, Varghese SH, Nair BG, Maekawa T, Yoshida Y, et al. (2010) Nanoparticulate material delivery to plants. Plant Science 179: 154-163.
23. Tsai SS, Sun GJ (2009) Introduction of nanotechnology: fundamentals and applications. Taipei: New Wun Ching Developmental Publishing Co., Ltd Press, pp: 518.
24. Prasad T, Kambala VSR, Naidu R (2011) A critical review on biogenic silver nanoparticles and their antimicrobial activity. Curr Nanosci 7: 531-544.
25. Baek YW, An YJ (2011) Microbial toxicity of metal oxide nanoparticles (CuO, NiO, ZnO, and Sb2O3) to *Escherichia coli*, *Bacillus subtilis*, and *Streptococcus aureus*. Sci Total Environ 409: 1603-1608.
26. Hsu SH, Tseng HJ, Hung HS, Wang MC, Hung CH, et al. (2009) Antimicrobial activities and cellular responses to natural silicate clays and derivatives modified by cationic alkyl amine salts. ACS Applied Material and Interfaces 1: 2556-2564.
27. Liu SB, Wei L, Hao L, Fang N, Chang MW, et al. (2009) Spharper and faster "nano darts" kill more bacteria: a study of antibacterial activity of individually dispersed pristine single-walled carbon nanotube. ACS Nano 3: 3891-3902.
28. Su HL, Chou CC, Hung DJ, Lin SH, Pao IC, et al. (2009) The disruption of bacterial membrane integrity through ROS generation induced by nanohybrids of silver and clay. Biomaterials 30: 5979-5987.
29. Jo YK, Kim BH, Jung G (2009) Antifungal activity of silver ions and nanoparticles on phytopathogenic fungi. Plant Disease 93: 1037-1043.
30. Kim SW, Kim KS, Lamsal K, Kim YJ, Kim SB, et al. (2009) An in vitro study of the antifungal effect of silver nanoparticles on oak wilt pathogen *Raffaelea* sp. J Microbiol Biotechnol 19: 760-764.
31. Aguilar-Me´ndez MA, Marti´n-Marti´nez ES, Ortega-Arroyo L, Cobia´n-Portillo G, Sa´nchez-Espi´ndola E (2011) Synthesis and characterization of silver nanoparticles: effect on phytopathogen *Colletotrichum gloeosporioides*. J Nanopart Res 13: 2525-2532.
32. Lamsal K, Kim SW, Jung JH, Kim YS, Kim KS, et al. (2011) Application of silver nanoparticles for the control of *colletotrichum* species in vitro and pepper anthracnose disease in field. Mycobiology 39: 194-199.
33. Kim HJ, Park HJ, Choi SH (2011) Antimicrobial action effect and stability of nanosized silica hybrid Ag complex. J Nanosci Nanotechnol 11: 5781-5787.
34. He L, Liu Y, Mustapha A, Lin M (2011) Antifungal activity of zinc oxide nanoparticles against *Botrytis cinerea* and *Penicillium expansum*. Microbiol Res 166: 207-215.
35. Norman DJ, Chen JJ (2011) Effect of foliar application of titanium dioxide on bacterial blight of geranium and Xanthomonas leaf spot of poinsettia. Hortscience 46: 426-428.
36. Benefield LD, Judkins JF, Weand BL (1982) Process chemistry for water and wastewater treatment. New Jerseye: Prentice-Hall Press, pp: 510.
37. Gajbhiye M, Kesharwani J, Ingle A, Gade A, Rai M (2009) Fungus-mediated synthesis of silver nanoparticles and their activity against pathogenic fungi in combination with fluconazole. Nanomedicine 5: 382-386.
38. Liu Y, Yan L, Heiden P, Laks P (2001) Use of nanoparticles for controlled release of biocides in solid wood. Journal of Applied Polymer Science 79: 458-465.
39. Morones JR, Elechiguerra JL, Camacho A, Holt K, Kouri JB, et al. (2005) The bactericidal effect of silver nanoparticles. Nanotechnology 16: 2346-2353.
40. Park HJ, Kim SH, Kim HJ, Choi SH (2006) A new composition of nanosized silica-silver for control of various plant diseases. Plant Pathol J 22: 295-302.