Chemical Fungicides and *Bacillus siamensis* H30-3 against Fungal and Oomycete Pathogens Causing Soil-Borne Strawberry Diseases

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Chemical and biological agents were evaluated to inhibit *Colletotrichum fructicola*, *Phytophthora cactorum*, and *Lasiodiplodia theobromae* causing strawberry diseases. Mycelial growths of *C. fructicola* were gradually arrested by increasing concentrations of fungicides pyraclostrobin and iminoctadine tris (albesilate). *P. cactorum* and *L. theobromae* were more sensitive to pyraclostrobin compared to *C. fructicola*, but iminoctadine tris (albesilate) was not or less effective to limit *P. cactorum* or *L. theobromae*, respectively. *Bacillus siamensis* H30-3 was antagonistic against the three pathogens by diffusible as well as volatile molecules, and evidently reduced aerial mycelial formation of *P. cactorum*. *B. siamensis* H30-3 growth was declined by at least 0.025 mg/ml of pyraclostrobin. The two fungicides additively inhibited mycelial growths of *C. fructicola*, but not of *P. cactorum* and *L. theobromae*. *B. siamensis* H30-3 volatiles led to less growth of *C. fructicola* than one reduced by the fungicides. Taken together, *in vitro* antimicrobial activities of the two fungicides together with or without

B. siamensis H30-3 volatiles may be cautiously incorporated into integrated management of strawberry diseases dependent on causal pathogens.

Keywords : antimicrobial, *Bacillus siamensis*, fungicides, strawberry diseases, volatiles

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Strawberry production worldwide is strained by *Colletotrichum* and *Phytophthora* species. Strawberry anthracnose by *Colletotrichum* spp. has been serious threat and various controls including chemical fungicides were suggested. *C. fructicola* is a part of *C. gloeosporioides* species complex, and DNA markers were developed to differentiate the fungal species from other *Colletotrichum* spp. causing strawberry anthracnose (Gan et al., 2017). Many Korean *C. gloeosporioides* isolates causing strawberry anthracnose was re-classified as *C. fructicola* (Nam et al., 2013). Fungicides azoxystrobin and prochloraz-Mn protected strawberry crown anthracnose by dipping the seedlings in fungicides before planting in fruit production fields (Kim et al., 2002; Nam et al., 2014). Control efficacies of azoxystrobin, pyraclostrobin and thiophanate-methyl as protectants were found in strawberry plant fields over 3 years against *C. gloeosporioides*—causing crown rot (MacKenzie et al., 2009). *Phytophthora cactorum* infection led to leather rot and crown rot of strawberry (Grove et al., 1985; Lim et al., 1998; Madden et al., 1991). Azoxystrobin, mefenoxam, and pyraclostrobin showed protectively controlled strawberry leather rot, whilst post-infection treatment with mefenoxam only decreased the

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leather rot (Rebollar-Alviter et al., 2007). Metalaxyl and fosetyl-Al reduced the leather rot by foliar-spraying and soil-drenching in strawberry fruit production fields (Ellis et al., 1998). In South Korea, two and six fungicides were registered to control Phytophthora rot in strawberry nursery and fruit production fields, respectively, in 2020. However, pyraclostrobin and iminoctadine tris (albesilate) were not investigated for the strawberry Phytophthora rot yet.

Lasiodiplodia theobromae infects many plants such as grapevine, mango, and peach trees (Burruano et al., 2008; Li et al., 2014; Saeed et al., 2017; Úrbez-Torres et al., 2008). Strawberry dieback by *L. theobromae* was reported in nursery of South Korea and fruit production fields in Turkey (Nam et al., 2016; Yildiz et al., 2014). Strawberry cv. ‘Seolhyang’, planted over 80% of production fields in South Korea, was highly susceptible to the dieback, which can be a great concern (Nam et al., 2016). Unfortunately, appropriate controls have not been demonstrated for the strawberry dieback.

Pyraclostrobin controlled anthracnose on strawberry, bean, grape, and pepper plants (Conner et al., 2004; Gao et al., 2017; Samuelian et al., 2014; Turechek et al., 2006). However, recent occurrences of pyraclostrobin-resistant *C. acutatum* isolates in pepper and strawberry fields warned of frequently using pyraclostrobin (Forcelini et al., 2016; Kim et al., 2019). Iminoctadine tris (albesilate) has shown *in vitro* antifungal activity against *Alternaria dauci* causing carrot Alternaria leaf spot and *Aspergillus tubingensis* causing Shine Muscat bunch rot (Do et al., 2020; Kim et al., 2020), and protectively and curatively reduced cucumber leaf spots by *Corynespora cassiicola* (Zhu et al., 2019). Iminoctadine tris (albesilate) was registered for strawberry anthracnose and grey mould in South Korea, but hardly demonstrated for other strawberry diseases.

Antagonistic microbes have controlled strawberry diseases. Endophytic bacterium *Azospirillum brasilense* REC3 in strawberry roots promoted plant growth as well as controlled anthracnose via enhanced plant immunity including augmented phenolics and pathogenesis-related gene expressions in plants (Tortora et al., 2012). Decreased anthracnose by *Bacillus amyloliquefaciens* S13-3 derived from soil was closely related with the bacterium-produced antimicrobial lipopeptides (Mochizuki et al., 2012; Yamamoto et al., 2015). Dipping strawberry roots in *Trichoderma harzianum* and *T. viride* conidial suspensions decreased leather rot (Porras et al., 2007). *Pseudomonas fluorescence* F113 and *Serratia plymuthica* HRO-C48 from rhizosphere of sugar beet and oilseed rape plants, respectively, reduced the strawberry root rot (Barahona et al., 2011; Kurze et al., 2001).

Bacillus siamensis H30-3 promoted Chinese cabbage plant growth under normal ambient and adverse environments like high temperature- and high temperature-drought stresses (Lee et al., 2018; Shin et al., 2019). *B. siamensis* H30-3 was antagonistic to *Alternaria brassicicola* and *Colletotrichum higginsianum* causing black spot and anthracnose diseases in Chinese cabbage, respectively, but diseases by *A. brassicicola* and *C. higginsianum* were reduced in cv. Ryeokgwang, not cv. Buram-3-ho (Lee et al., 2018). Chinese cabbage soft rot by *Pectobacterium carotovorum* subsp. *carotovorum* was alleviated by *B. siamensis* H30-3 (Shin et al., 2019). These results indicate that *B. siamensis* H30-3 has broad antimicrobial activities against fungi and bacteria.

In this study, *in vitro* antifungal activities of two fungicides pyraclostrobin and iminoctadine tris (albesilate) were demonstrated against *C. fructicola*, *P. cactorum*, and *L. theobromae* by singly or simultaneously. *B. siamensis* H30-3 was applied to the three pathogens to investigate whether the bacterial strain arrests the pathogen growths through diffusible and volatile antifungal machineries. Tolerance of *B. siamensis* H30-3 to the two fungicides was evaluated for simultaneous usage potentials with chemical controls for integrated strawberry disease management. Simultaneous treatment with two fungicides with or without *B. siamensis* H30-3 volatiles on the three pathogen growths was also investigated.

In vitro antimicrobial activities of pyraclostrobin and iminoctadine tris (albesilate) were evaluated against *C. fructicola*, *P. cactorum*, and *L. theobromae* (Fig. 1). Colony formation (Fig. 1A) and mycelial growths (Fig. 1B) of *C. fructicola* were gradually decreased by increasing pyraclostrobin (0, 0.00625, 0.0125, 0.025, 0.05, and 0.1 mg/ml) and iminoctadine tris (albesilate) (0, 0.00625, 0.0125, 0.025, 0.05, and 1 µg/ml). Linear regression analyses demonstrated strong correlation of fungicides concentrations with reduced growths of *C. fructicola*, shown by increasing concentrations of pyraclostrobin ($Y = -14.79X + 100.59$, $R^2 = 0.8943$) and iminoctadine tris (albesilate) ($Y = -17.308X + 123.71$, $R^2 = 0.9689$).

Increasing pyraclostrobin concentrations at 0.00625, 0.0125, 0.025, 0.05, and 0.1 mg/ml resulted in significant decreases in mycelial growth of *C. fructicola* to ca. 56.9%, 50.2%, 42.5%, 28.3%, and 15.2% compared to mock-treated control. Same dosage range of pyraclostrobin showed more drastic suppressions in mycelial growths of *P. cactorum* and *L. theobromae*. Minimal concentration of 0.00625 mg/ml of pyraclostrobin reduced growth of *P. cactorum* and *L. theobromae* by ca. 18.9% and 17.5%, respectively. More doses of pyraclostrobin to 0.1 mg/ml did not change *P.*

Fig. 1. *In vitro* inhibitory efficacies of two chemical fungicides pyraclostrobin and iminocadine tris (albesilate) on mycelial growths of *Colletotrichum fructicola* isolate SAn-3, *Phytophthora cactorum* isolate P-9815 (KACC 40183) and *Lasiodiplodia theobromae* isolate LT120902. (A) Colony formations of *C. fructicola*, *P. cactorum*, and *L. theobromae* on 1/2-potato dextrose agar media containing different concentrations of pyraclostrobin and iminocadine tris (albesilate), and cultured at 25°C for 5, 10, and 2 days for *C. fructicola*, *P. cactorum*, and *L. theobromae*, respectively. (B) Relative mycelial growths (%) of *C. fructicola*, *P. cactorum*, and *L. theobromae*. Bars represent the standard errors of the means of the five independent experimental replications. Each experiment has four replications. Means followed by the same letters are not significantly different at 5% level by least significant difference test.

cactorum suppression efficacy, whilst increasing doses to more than 0.025 to 0.1 mg/ml gradually more suppressed the growths of *L. theobromae*. Increasing iminocadine tris (albesilate) concentrations at 0.00625, 0.0125, 0.025, 0.05, and 0.1 µg/ml led to significant decreases in growth of *C. fructicola* to ca. 89.2%, 78.0%, 62.0%, 34.9%, and 14.6% compared to mock-treated control. Same dosage ranges of iminocadine tris (albesilate) has no antimicrobial activity against *P. cactorum*, and only slightly reduced growth in-

Fig. 2. *In vitro* inhibition of mycelial growths of *Colletotrichum fructicola*, *Phytophthora cactorum*, and *Lasiodiplodia theobromae* by dual cultures with *Bacillus siamensis* H30-3. (A) Mycelial cultures on 1/2-potato dextrose agar media in the absence or presence of *B. siamensis* H30-3 co-culture at 25°C. Photos were taken 8, 16, and 4 days after inoculation of *C. fructicola*, *P. cactorum*, and *L. theobromae*, respectively. (B) Inhibited mycelial growths shown by half of the colony diameter after co-culture. Bars represent the standard errors of the means of the five independent experimental replications. Each experiment has four replications. Asterisks indicate significant differences as determined by Student's *t*-test ($P < 0.05$).

hibition was found against *L. theobromae* by 0.05-0.1 µg/ml. These results suggest that pyraclostrobin can be applied to control both Phytophthora rot and dieback caused by *P. cactorum* and *L. theobromae*, respectively. Antimicrobial activities of iminocadine tris (albesilate) by more than 0.1 µg/ml remains investigated.

Bacterial suspension (10^8 cfu/ml) of *B. siamensis* H30-3 and the three pathogens were co-inoculated on 1/2-potato dextrose agar (PDA) media as described previously (Lee et al., 2018). *B. siamensis* H30-3 showed diffusible antimicrobial activities against *C. fructicola*, *P. cactorum*, and *L. theobromae* during dual cultures (Fig. 2). Compared to each mock-treated control at 8, 16, and 4 days after the cultures, mycelial growths of *C. fructicola*, *P. cactorum*, and *L. theobromae* were markedly limited as cultured with *B. siamensis* H30-3 simultaneously (Fig. 2A). Significantly reduced colony radius of the three phytopathogens were shown in Fig. 2B. Volatiles from *B. siamensis* H30-3 cultured on different media exhibited *in vitro* antimicrobial

Fig. 3. *In vitro* inhibition of mycelial growths of *Colletotrichum fructicola*, *Phytophthora cactorum*, and *Lasiodiplodia theobromae* by *Bacillus siamensis* H30-3 volatiles. (A) Schematic diagram showing antimicrobial volatiles from *B. siamensis* H30-3 against phytopathogens. Nine drops (5 μ l each) of *B. siamensis* H30-3 suspension (10^8 cfu/ml) were inoculated on tryptic soy agar (TSA), nutrient agar (NA), and Luria-Bertani agar (LBA). Arrows indicate releasing volatiles onto mycelia of the phytopathogens. (B) Pathogen cultures on 1/2-potato dextrose agar (PDA) media in the absence and presence of *B. siamensis* H30-3 volatiles on the three different growth media at 25°C. Photos were taken 5, 10, and 2 days after inoculation of *C. fructicola*, *P. cactorum*, and *L. theobromae*, respectively. (C) Colony diameters (mm) of *C. fructicola*, *P. cactorum*, and *L. theobromae* treated with *B. siamensis* H30-3 volatiles. Bars represent the standard errors of the means of the six independent experimental replications. Each experiment has four replications. Asterisks indicate significant differences as determined by Student's *t*-test ($P < 0.05$).

activities against *C. fructicola*, *P. cactorum* and *L. theobromae* (Fig. 3). Volatiles from *B. amyloliquefaciens*, *B. pumilus* and *B. velezensis* strains showed antifungal activities against diverse fungal species (Asari et al. 2016; Lim et al., 2017; Morita et al., 2019). Reduced colonies of the three pathogens were found on 1/2-PDA media covered different culture media tryptic soy agar, nutrient agar, and Luria-Bertani agar media drop-inoculated by *B. siamensis* H30-

Fig. 4. Effects of pyraclostrobin and iminoctadine tris (albesilate) on *in vitro* growth of *Bacillus siamensis* H30-3. Bacterial suspensions in nutrient broth liquid media with or without different concentrations of the two fungicides were cultured and relative bacterial growths in response to the two fungicides were demonstrated as percentage (%) compared to those in untreated culture. Values presented are means and error bars indicated the standard errors of the means of five independent experimental replications. Each experiment has four replications. Means followed by the same letters are not significantly different at 5% level by least significant difference test.

3 (Fig. 3A). Particularly, aerial mycelia of *P. cactorum* was drastically reduced by *B. siamensis* H30-3 volatiles. Distinct difference in growth inhibition by the different bacterial media was not found (Fig. 3B). *B. siamensis* H30-3 has several antimicrobial lipopeptide-encoding genes such as *bacD*, *bmyA*, *ituA*, and *srfA* (Lee et al., 2018), which may be involved in the suppressed *C. fructicola*, *P. cactorum*, and *L. theobromae*.

In vitro bactericidal activities of the two fungicides against *B. siamensis* H30-3 were investigated (Fig. 4). *B. siamensis* H30-3 (10^5 cfu/ml) were initially cultured in 4-ml of nutrient broth with or without increasing concentrations of the two fungicides at 30°C for 24 h and the bacterial growths were evaluated spectrophotometrically at OD₆₀₀ (Hong et al., 2016). Bacterial growth was declined by more than 0.025 mg/ml of pyraclostrobin and no change was found by increased doses (0.05-0.1 mg/ml). Iminoctadine tris (albesilate) doses from 0.00625 to 0.1 μ g/ml has no antibacterial effect on *B. siamensis* H30-3. *Bacillus* spp. as biological control agents were suggested to be integrated into disease management with chemical fungicides (Jacobsen et al., 2004; Korsten et al., 1997; Lee et al., 2012). *B. siamensis* H30-3 can be considered for strawberry disease control with the two fungicides. However, use of less than 0.0125 mg/ml pyraclostrobin will be recommended to avoid its negative effect on the growth of *B. siamensis* H30-3 during their simultaneous application.

Antimicrobial effects of the two chemical fungicides with *B. siamensis* H30-3 volatiles were investigated (Fig.

Fig. 5. Antimicrobial activities of chemical fungicides pyraclostrobin and iminoctadine tris (albesilate) with or without volatiles from *Bacillus siamensis* H30-3. Mycelial growths of *Colletotrichum fructicola*, *Phytophthora cactorum* and *Lasiodiplodia theobromae* on 1/2-potato dextrose agar (PDA) media supplemented with pyraclostrobin (P) (0.00625 mg/ml) and/or iminoctadine tris (albesilate) (I) (0.00625 µg/ml). The PDA media were covered by tryptic soy agar media inoculated by *B. siamensis* H30-3. Colony diameters were measured after cultures at 25°C for 5, 10, and 2 days for *C. fructicola*, *P. cactorum* and *L. theobromae*, respectively. Values presented are means and error bars indicated the standard errors of the means of five independent experimental replications. Each experiment has four replications. Means followed by the same letters are not significantly different at 5% level by least significant difference test.

5). Co-treatment with pyraclostrobin (0.00625 mg/ml) and iminoctadine tris (albesilate) (0.00625 µg/ml) without *B. siamensis* H30-3 volatiles resulted in more significantly suppressed growths of *C. fructicola* compared to the one treated with single fungicide. However, *P. cactorum* and *L. theobromae* was not synergistically suppressed by the co-treatment with the two fungicides. Together with *B. siamensis* H30-3 volatiles, mycelial growths of *C. fructicola* were more efficiently decreased by pyraclostrobin and/or iminoctadine tris (albesilate). The fungicide-suppressed *P. cactorum* was rather relieved by *B. siamensis* H30-3 vola-

tiles. No significant difference in growths of *L. theobromae* treated with the fungicides by *B. siamensis* H30-3 volatiles. It should be solved how *B. siamensis* H30-3 volatiles will apply to the strawberry growing fields. Identification of *B. siamensis* H30-3 volatile compounds and improved production of the antimicrobial volatile compounds may pave a way for more efficient and reliable biological control of strawberry diseases.

Taken together, fungicides pyraclostrobin and iminoctadine tris (albesilate) and *B. siamensis* H30-3 showed their antimicrobial activities against the *C. fructicola*, *P. cactorum*, and *L. theobromae* singly or in combination. Use of *B. siamensis* H30-3 volatiles with pyraclostrobin and/or iminoctadine tris (albesilate) can be a more useful for controlling strawberry anthracnose by *C. fructicola*. *In planta* disease control efficacies of the chemical and biological agents will provide more sustainable production of strawberry fruits.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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