Suppression of Ripe Rot on ‘Zesy002’ Kiwifruit with Commercial Agrochemicals

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Ripe rot caused by *Botryosphaeria dothidea* is one of the serious diseases of postharvest kiwifruit. In order to control ripe rot on *Actinidia chinensis* cultivar ‘Zesy002’, several commercial agrofungicides were selected by an antifungal test on an artificial medium. Furthermore, disease suppression by the selected fungicides was evaluated on the kiwifruit by inoculation with a conidial suspension of *B. dothidea*. On the artificial media containing boscalid + fludioxonil was shown to be the most effective antifungal activity. However, in the bio-test pyraclostrobin + boscalid and iminoctadine-tris were the most effective agrochemicals on the fruit. On the other hand, the infection structures of *B. dothidea* on kiwifruit treated with pyraclostrobin + boscalid were observed with a fluorescent microscope. Most of the fungal conidia had not germinated on the kiwifruit treated with the agrochemicals whereas on the un-

treated fruit the fungal conidia had mostly germinated. Electron microscopy of the fine structures showed morphological changes to the conidia and branch of hyphae on the kiwifruit pre-treated with pyraclostrobin + boscalid, indicating its suppression effect on fungal growth. Based on this observation, it is suggested that ripe rot by *B. dothidea* may be suppressed through the inhibition of conidial germination on the kiwifruit treated with the agrochemicals.

Keywords : dimple rot, fluorescence microscopy, infection structure, plant protection, postharvest disease

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Kiwifruit contains a lot of vitamin C and low calories and gives beneficial healthy effects to human's body. Furthermore, it has been reported that kiwifruit had effects to prevent cardiovascular disease, cancer, and other degenerative disorders (Singletary, 2012). For these reasons kiwifruit is one of the popular fruit in South Korea and extent of kiwifruit cultivation was taken at 1,300 ha in 2015 (Kim et al., 2017a). The consumption of kiwifruit in South Korea is increasing and reached approximately 35,000 tons in 2018 less than 10,000 tons were harvested in Korea (Tridge, 2021). However, the amount of kiwifruit yield could not be increased as much as expected because of outbreak of the diseases.

One of the most serious diseases in kiwifruit vines is bacterial canker caused by *Pseudomonas syringae* pv. *actinidiae* (Psa; Greer and Saunders, 2012). In many South Korean

kiwifruit orchards, the susceptible kiwifruit cultivar ‘Hort16A’ has been replaced with another cultivar, ‘Zesy002’, which is tolerant to *Psa* (Donati et al., 2014). However, over the past decade postharvest diseases have occurred on ‘Zesy002’ kiwifruit which was found in several orchards of Jeju Island.

During the storage of kiwifruit several diseases often occur during low temperature storage. In a study of post-harvest rots of ‘Hort16A’ in New Zealand most of the rots were found to be caused by *Diaporthe* spp., *Botrytis cinerea*, or *Botryosphaeria dothidea* (Manning et al., 2003). A survey of Korean kiwifruit orchards in 1999 showed that as much as 32% of kiwifruit had soft rot caused by these diseases (Koh et al., 2003). Among these, *B. dothidea* was the main fungus causing postharvest diseases and accounted for 83.3% of the fungal pathogens (Koh et al., 2003).

In Korea, 20.5% of kiwifruit cultivar ‘Hayward’ (*Actinidia chinensis* var. *deliciosa*) was lost with ripe rot caused by *B. dothidea* in 2014 (Kim and Koh, 2018). Another cultivar of *A. chinensis* var. *deliciosa* (Gamrok) was known to be more susceptible to *B. dothidea* because of its higher sugar contents than other cultivars (Kim et al., 2017b). To control postharvest rot on kiwifruit, many agrochemicals were tested in kiwifruit orchards, especially cultivating kiwifruit cultivar *A. chinensis* ‘Zesy002’.

In this study, in order to evaluate the relative efficacy of the currently used chemical products, the direct protection rate of the chemicals were evaluated by agar plate assays. To further optimize agrochemicals used on-orchard, agrochemicals which were shown to inhibit antifungal activity in the agar plate test were assayed for efficacy in a bio-test with ‘Zesy002’ fruit. Furthermore, to reveal the disease suppression mechanism on kiwifruit by these chemicals, the infection structures of *B. dothidea* were observed using a fluorescence microscope.

Material and Methods

Fruit material. Kiwifruit (*Actinidia chinensis* var. *chinensis* cultivar ‘Zesy002’) were supplied by Zespri Co. Ltd. in Jeju Korea. The kiwifruit were sterilized with 1% sodium hypochlorite, 70% ethyl alcohol each for 60 s and washed with sterilized water three times. After drying at room temperature the kiwifruit were used for inoculation.

Fungal inoculum. *B. dothidea* isolate C1.1 was isolated from ‘Zesy002’ kiwifruit showing ripe rot symptom, which is visually similar to dimple rot, from an orchard of Jeju Island Korea during the 2018 harvest season (Ledesma et al., 2021). This isolate was held in Jeju University plant pa-

thology general culture collection at -80°C and used in this experiment. For sporulation, *B. dothidea* was grown on an oatmeal agar medium (Becton, Dickinson and Company, Claix, France) under aerobic conditions at 28°C with 7,000 lux in daytime for 7 days. Mycelium of *B. dothidea* was scraped with a loop and incubated another 7 days under the same conditions. In the plate 10 ml of sterilized water was added and conidia were harvested with a brush. The conidial suspension was filtered with double folded Miracloth (Calbiochem, Darmstadt, Germany). The concentration of inoculum was adjusted to 1×10^5 conidia/ml using a haemocytometer (Hausser Scientific Inc., Horsham, PA, USA) and used as inoculum.

Agrochemical evaluation

In vitro test. The effect of several agrochemicals were tested in potato dextrose agar (PDA; Becton, Dickinson and Company) medium. All chemicals were added into the PDA at the recommended concentration for field application, respectively. Some of the agrochemicals were subsequently diluted because the inoculated fungi did not grow. The list of agrochemicals and recommended concentrations are given in Table 1. As the control, PDA without chemicals was used.

Mycelium of *B. dothidea* ($\phi = 5$ mm) was inoculated on the PDA medium added with the different agrochemicals and incubated at 25°C for 7 days. The diameter of the colony was measured with a ruler. This experiment was replicated three times including three PDA plates each.

In vivo test. Agrochemicals which revealed direct anti-

Table 1. Agrochemicals used and their applied concentration in potato dextrose agar media

Ingredients	Concentration (ppm)	Diluted concentration (ppm)
Iminoctadine-tris	1,000	- ^a
Azoxystrobin	1,000	-
Tebuconazole	500	50
Thiophanate methyl	1,000	-
Iprodione	1,000	100
Boscalid	500	-
Trifloxystrobin	250	-
Boscalid-fludioxonil	1,000	10
Fluazinam	500	-
Fluopyram	250	-
Cyprodinil	500	-
Pyraclostrobin + boscalid	500	-

^aNot tested.

fungal effect on agar plates such as iprodione, tebuconazole, boscalid-fludioxonil, fluazinam, iminoctadine-tris, thiophanate methyl, trifloxystrobin, cyprodinil and pyraclostrobin + boscalid were used in this experiment. All chemicals were applied to 'Zesy002' kiwifruit previously wounded with a sterile stick ($\phi = 0.5$ mm) at the recommended concentration for field application. After drying at room temperature the treated fruit were used for fungal inoculation.

A drop of 70 μ l of conidial suspension of *B. dothidea* with 0.01% Tween-20 was inoculated onto the wounded part of each kiwifruit. The inoculated fruit were placed in an incubator maintained at 28°C, 90% relative humidity, for 7 days. As a control, sterilized 0.01% Tween-20 solution was applied instead of the conidial suspension. The symptoms on the fruit were examined and the lesions were measured with a ruler. The experiments were separately replicated 3 times which contained six kiwifruit each treatment.

Observation with a fluorescence microscope. To investigate the infection behavior of *B. dothidea* kiwifruit treated with pyraclostrobin + boscalid was observed with a fluorescence microscope (BX60, Olympus, Tokyo, Japan). The kiwifruit surfaces were inoculated with 60 μ l of *B. dothidea* conidial suspension and the inoculated parts were excised with a razor in size of 5×5 mm² at 3, 6, and 12 h after inoculation. The peel tissues were fixed with 2% glutaraldehyde in sodium phosphate buffer (pH 7.2) at 4°C for 2 h. After fixation, the samples were washed with sodium phosphate buffer three times (10 min each) and dyed with 0.2% diethanol (UVtex-2B, Polysciences, Inc., Muellheim, Germany) for 30 min at room temperature. After washing with H₂O three times the samples were mounted on glass slides with 70% glycerin (glycerin, OCI Company Ltd., Seoul, Korea). Infected sites were observed with a fluorescent filter set (exciter filter, BP 400-440; interference beam splitter, FT 460; barrier filter, LP 470). The germinated conidia were counted and the length of hyphae was measured. Experiments were separately replicated three times using three samples for each treatment.

Scanning electron microscopy of the kiwifruit after fungal inoculation. Kiwifruit surfaces of 'Zesy002' pre-treated with agrochemicals and then inoculated with *B. dothidea* were observed at 1, 2, and 3 days after inoculation using a scanning electron microscope (FE-SEM Mira3, Tescan Ltd., Brno, Czech Republic).

The inoculated kiwifruit were cut to 0.4×0.6 mm² using a sterile blade. Fixation, dehydration and embedding of

the fruit were performed according to Hayat (1989). The leaf samples were fixed in 2% (v/v) glutaraldehyde in 0.05 M phosphate buffer (pH 7.4) for 2 h. After washing with phosphate buffer for 10 min three times each, post fixation was performed in 2% (v/v) osmium tetroxide in phosphate buffer for 2 h at room temperature. After washing three times, the samples were dehydrated through an alcohol series (30%, 50%, 70%, 90%, and 100% two times for 30 min each). The samples were gently dried using a critical point drier (EM CPD 300, Leica, Jena, Germany). Samples were mounted on metallic stubs, gold-coated (~ 100 Å) with a sputter coater (Q150R Plus – Rotary Pumped Coater, Quorum Technologies Ltd., Sussex, UK) and viewed under the FE-SEM at 20 kV.

Statistical analysis. Data of diameters of mycelia on the PDA for determining the efficacy of agrochemicals, disease severity on kiwifruit treated with agrochemicals, rate of conidial germination, and hyphal lengths on surfaces of kiwifruit were analyzed with Duncan's multiple range test using statistical analysis system (SAS) program version 9.0 (SAS Institute Inc., Cary, NC, USA). Statistical significance was considered at $P < 0.05$.

Results

Antifungal effect on PDA medium amended with agrochemicals. Hyphal growth suppression of *B. dothidea* by 12 commercial agrochemicals was evaluated on PDA medium. At 7 days after inoculation, hyphal growth was inhibited on all of the agrochemical amended media compared with the untreated control (Fig. 1). Especially, mycelium of *B. dothidea* did not grown on PDA amended with tebuconazole, iprodione and boscalid + fludioxonil at field application concentration (data not shown). Even when tebuconazole or iprodione were diluted 10 times, the antifungal effect was high compared with other agrochemicals tested (Fig. 2). Above all, PDA treated with boscalid + fludioxonil diluted 100 times was shown to have the best suppression effect compared with other chemicals in the *in vitro* experiment (Fig. 2).

Disease suppression on kiwifruit treated with agrochemicals. To test the disease suppression effect on kiwifruit treated with the agrochemicals, *B. dothidea* was inoculated onto kiwifruit pre-treated with the most effective nine agrochemicals selected from the *in vitro* experiment. On untreated kiwifruit, symptoms of *B. dothidea* were visible at 3 days after inoculation. The lesion sizes were larger on the untreated fruit compared with all of the chemicals-

Fig. 1. Mycelium of *Botryosphaeria dothidea* grown on potato dextrose agar (PDA) medium added with different agrichemicals. The present photos were taken at 7 days after inoculation with the fungus. The recommended concentration for field application were added on the PDA medium, respectively. (A) Control. (B) Iminoctadine-tris. (C) Azoxystrobin. (D) Tebuconazole dilluted 10 times. (E) Flupyrim + thiophanate-methyl. (F) Iprodione dilluted 10 times. (G) Boscalid. (H) Trifloxystrobin. (I) Boscalid + fludioxonil dilluted 100 times. (J) Fluazinam. (K) Flupyrim. (L) Cyprodinil. (M) Pyraclostrobin + boscalid.

Fig. 2. Diameter of mycelium of *Botryosphaeria dothidea* grown on potato dextrose agar (PDA) medium added with different agrichemicals. The present photos were taken at 7 days after inoculation with the fungus. The recommended concentration for field application and were added on the PDA medium, respectively. Different letters on the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple test.

Fig. 3. Wounded ‘Zesy002’ kiwifruit control (A), pre-treated with iminoctadine-tris (B), tebuconazole (C), iprodione (D), boscalid + fludioxonil (E), fluazinam (F), fluopyram + thiophanate-methyl (G), trifloxystrobin (H), cyprodinil (I), and pyraclostrobin + boscalid (J) which were inoculated with conidial suspension of *Botryosphaeria dothidea* and as a negative control both untreated/uninoculated kiwifruit (F). The recommended concentration for field application were pre-treated before fungal inoculation, respectively. The concentration of the conidial suspension of *B. dothidea* were 1×10^5 conidia/ml. The present photos were taken at 7 days after inoculation with the fungus.

treated kiwifruit.

At 7 days after inoculation, the symptom diameter on all of the agrochemical pre-treated kiwifruit were reduced compared with those symptoms on the untreated control

(Fig. 3). Especially, pyraclostrobin+boscalid and iminoctadine-tris were the most effective agrochemicals on kiwifruit, on which the disease suppression rates were 75% and 44% compared with the untreated control, respectively (Fig.

Fig. 4. Diameter of ripe rot lesion on wounded ‘Zesy002’ kiwi fruits treated with agrichemicals after inoculated with conidial suspension of *Botryosphaeria dothidea*. The recommended concentration for field application were pre-treated before fungal inoculation, respectively. The concentration of the conidial suspension of *B. dothidea* were 1×10^5 conidia/ml. The diameter of lesions were measured at 7 days after inoculation with the fungus. Different letters on the columns indicate significant differences ($P < 0.05$) according to Duncan’s multiple test.

Table 2. Germination rate of conidia of *Botryosphaeria dothidea* on the surface of kiwifruit ‘Zesy002’ untreated and treated with agrochemical pyraclostrobin + boscalid

Treatment	Germination rate (%)			DMRT ^a
	Hours after inoculation (h)			
	3	6	12	
Untreated	13.7 ± 19.1 ^b	72.5 ± 15.4	96.9 ± 2.1	a
Pyraclostrobin + boscalid	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0	b

^aThe letter indicates significant differences according to Duncan’s multiple range test (DMRT) ($P < 0.01$).

^bMean ± standard deviation.

4). This indicates that the agrochemicals have a disease suppression effect on kiwifruit.

Fluorescent microscopy. To reveal the mechanism of disease suppression on kiwifruit treated with pyraclostrobin + boscalid, which was shown to be the most effective against *B. dothidea*, the infection structure of *B. dothidea* on kiwifruit were observed with a fluorescence microscope. At 3 h after inoculation some conidia were germinated on the untreated kiwifruit and 6 h later most conidia were germinated (Table 2). Hyphal growth developed rapidly and the surfaces of kiwifruit were covered with hyphae 12 h after inoculation (Fig. 5A-C).

However, most conidia did not germinate on the agrochemical-treated kiwifruit during the whole observation period from 3 to 12 h after inoculation (Table 2, Fig. 5D-F). These observations indicated that the agrochemicals inhibited the conidial germination which might result in the

suppression of disease severity. Even on kiwifruit treated with pyraclostrobin + boscalid 100 times diluted from field rates, most conidia had not germinated at 12 h after inoculation (data not shown), indicating that pyraclostrobin + boscalid was the most effective fungicide to suppress ripe rot on kiwifruit.

Scanning electron microscopy. On the untreated kiwifruit, conidia of *B. dothidea* were morphologically intact and mostly germinated at 1 day after inoculation (Fig. 6A). One day later the hyphae of the fungus had grown on the surface of the kiwifruit and were extended widely at 3 days after inoculation (Fig. 6B and C).

However, on the pyraclostrobin + boscalid pre-treated kiwifruit, some conidia of *B. dothidea* were morphologically changed (Fig. 6D, arrow) and most conidia had not germinated. The tapered conidia were often found at 2 days after inoculation (Fig. 6E, arrow) and most conidia still had not

Fig. 5. Fluorescent microscopic observation of infection structures at 3, 6, 12 hours after inoculation on peels of kiwifruits untreated (A-C) and fungicide pyraclostrobin-boscalid (D-F). The concentration of fungicide and conidia suspension of *Botryosphaeria dothidea* were field application and 1×10^5 conidia/ml. Scale bars = 20 μm. c, conidium; h, hyphae; gt, gum tube.

Fig. 6. Scanning electron microscopic observation of infection structures at 1, 2, and 3 days after inoculation on peels of kiwifruits untreated (A-C) and fungicide pyraclostrobin-boscalid (D-F). The concentration of fungicide and conidia suspension of *Botryosphaeria dothidea* were field application and 1×10^5 conidia/ml. Morphological change was observed on conidium (D and E, arrow) and branched hyphae (F, double arrows). Scale bars = 10 μ m. c, conidium; h, hyphae; gt, gum tube.

germinated. At 3 days after inoculation some hyphae were observed which were frequently branched (Fig. 6F, double arrows), indicating the fungicide may suppress the hyphal growth.

Discussion

Although ripe rot caused by *B. dothidea* has been known in kiwifruit *A. chinensis* cultivars ‘Haegum’ and ‘Hayward’, there was no report announcing ripe rot on the cultivar ‘Zesy002’. Over the last decade the postharvest disease has often occurred on ‘Zesy002’; this cultivar is known to be tolerant to Psa (Zespri International Limited, 2016). In a previous study, *B. dothidea* was isolated from symptomatic kiwifruit and confirmed as the causal fungus of ripe rot by fulfilling Koch's Postulates (Ledesma et al., 2021).

Protection efficacy of agrochemicals against ripe rot in cultivar ‘Hort16A’ and ‘Hayward’ has been previously reported. Among three agrochemicals tested, benomyl WP showed the highest efficacy, followed by thiophanate-methyl WP and carbendazim + diethofencarb WP when they were treated in the orchards Jeju Island, Korea (Kim et al., 2013).

In this study, among the agrochemicals tested boscalid + fludioxonil was shown to have the most effective antifungal activity in the artificial medium (Figs. 1 and 2). The efficacy of this chemical was so high that its concentration had

to dilute as 100 fold in the *in vitro* experiment. However, in the bio-test on fruit, this mixture was not the most effective among the tested fungicides. Unexpectedly, pyraclostrobin + boscalid mixture was the most effective against ripe rot on ‘Zesy002’ fruit (Fig. 4). It is not clearly explained why the efficacy of the agrochemicals was different between the *in vitro* and *in vivo* experiments. One of the reasons for this difference in efficacy could be that different inoculum types were used in each test (mycelium in the *in vitro* test and conidia in the *in vivo* test). It was also suggested that some factors such as fruit exudates or pH may influence the activity of the agrochemicals. (Mihaylova-Kroumova et al., 2020; Zhao et al., 2016).

Boscalid is a pyridinecarboxamide which is active against a broad range of fungal pathogens including *Botrytis cinerea* causing grey mold (Veloukas and Karaglanidis, 2012) or *Sclerotinia sclerotiorum* causing stem rot in canola (Munoz, 2016). Boscalid has been known as an inhibitor of succinate dehydrogenase which has a role in electron transport chain in mitochondria (Hatefi, 1985). Also, pyraclostrobin is a carbamate ester which is widely used as a fungicide for control of many plant pathogens such as *Alternaria* spp. (Survilienė and Dambrauskienė, 2006) or *Ascochyta rabiei* (Chang et al., 2007). This agrochemical inhibits energy transfer in mitochondria in the fungal cell (Sierotzki and Scalliet, 2013). Mixtures with pyraclostrobin and boscalid were effective against brown

spot on the leaves of pear in orchards (Petré et al., 2015).

The infection behavior of *B. dothidea* in kiwifruit is not well known yet. In this study, it was observed that the conidia of *B. dothidea* germinate fast similar to some other fungal pathogens. Also, it seems the fungus can invade the host cells without forming appressoria (Fig. 5). In order to illustrate how the agrochemicals inhibit fungal infection, the infection structures were observed on the surface of kiwifruit 'Zesy002' treated with pyraclostrobin + boscalid. On the agrochemical-treated kiwifruit there was a very low germination rate and restricted hyphal growth of *B. dothidea* (Table 2, Fig. 5). There have been previous reports that germination rates and hyphal development are decreased by this fungicide. Both germination rate and hyphal growth of *Alternaria alternata* and *Penicillium digitatum* were inhibited by boscalid treatment (Vega and Dewdney, 2015; Xu et al., 2020). Also, it was reported that the germination rate of *Puccinia striiformis* causing stripe rust in wheat (Kang et al., 2019) or *Colletotrichum acutatum* causing anthracnose in chili (Gao et al., 2017), was decreased by pyraclostrobin. Conidia and hyphae of *Monilinia fructicola* and *Botrytis cinerea* treated with mixtures of pyraclostrobin and boscalid did not germinate or develop well compared with untreated conidia and hyphae (Amiri et al., 2010; Kim and Xiao, 2010).

Electron microscopy of the fine structure of *B. dothidea* on the untreated kiwifruit showed the intact conidia and hyphae (Fig. 6A-C); this was similar to those observed with the fluorescence microscope (Fig. 5). Strangely, the fungal samples for SEM grew more slowly than those used for fluorescence microscopy; this was likely to be due to environmental differences during the incubation period such as temperature. Through the observation with SEM it was clearly shown that mixtures of pyraclostrobin and boscalid may suppress the growth of *B. dothidea* resulting in the morphological change of conidia and branch of hyphae. Similar observations were found on the kiwifruit pretreated with other fungicides such as iminoctadine-tris or fluazinam (data not shown).

Therefore, it was suggested that the function of the agrochemicals in the suppression of ripe rot on kiwifruit may be the decreasing of germination rate and hyphal development of *B. dothidea*. However, for applying of agrochemicals to the fields, it would be necessary to undertake more experiments in order to prove their efficacy.

Conflicts of Interest

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