Characterization of Fludioxonil-Resistant and Pyrimethanil-Resistant Phenotypes of *Penicillium expansum* from Apple

H. X. Li and C. L. Xiao

Department of Plant Pathology, Washington State University, Tree Fruit Research and Extension Center, 1100 North Western Avenue, Wenatchee, WA 98801.

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ABSTRACT

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Penicillium expansum is the primary cause of blue mold, a major postharvest disease of apple. Fludioxonil and pyrimethanil are two newly registered postharvest fungicides for pome fruit in the United States. To evaluate the potential risk of resistance development in *P. expansum* to the new postharvest fungicides, one isolate of each of thiabendazole-resistant (TBZ-R) and -sensitive (TBZ-S) *P. expansum* was exposed to UV radiation to generate fungicide-resistant mutants. Four fludioxonil highly-resistant mutants ($EC_{50} > 1,000 \mu\text{g/ml}$) and four pyrimethanil-resistant mutants ($EC_{50} > 10 \mu\text{g/ml}$) were tested for sensitivities to thiabendazole, fludioxonil, and pyrimethanil, and fitness parameters including mycelial growth, sporulation on potato dextrose agar (PDA), sensitivity to osmotic stress, and pathogenicity and sporulation on apple fruit. The stability of resistance of the mutants was tested on PDA and apple fruit. Efficacy of the three fungicides to control blue mold incited by the mutants was evaluated on apple fruit. Six fungicide-resistant phenotypes were identified among the parental wild-type isolates and their mutants based upon their resistance levels. All four fludioxonil highly-resistant mutants were sensitive to pyrimethanil and retained the same phenotypes of resistance to TBZ as the parental isolates. All four pyrimethanil-resistant mutants had a low level of resistance to fludioxonil with a resistance factor >15 . The two pyrimethanil-resistant mutants derived from a TBZ-S isolate be-

came resistant to TBZ at $5 \mu\text{g/ml}$. After 20 successive generations on PDA and four generations on apple fruit, the mutants retained the same phenotypes as the original generations. All mutants were pathogenic on apple fruit at both 0 and 20°C , but fludioxonil highly-resistant mutants were less virulent and produced fewer conidia on apple fruit than pyrimethanil-resistant mutants and their parental wild-type isolates. Compared with the parental isolates, all four fludioxonil highly-resistant mutants had an increased sensitivity to osmotic stress on PDA amended with NaCl, while the pyrimethanil-resistant mutants did not. Pyrimethanil was effective against blue mold caused by fludioxonil-resistant mutants at both 0 and 20°C . Pyrimethanil and fludioxonil reduced blue mold incited by pyrimethanil-resistant mutants during 12-week storage at 0°C but were not effective at 20°C . TBZ was not effective against pyrimethanil-resistant mutants derived from TBZ-S wild-type isolates at room temperature but provided some control at 0°C . The results indicate that: (i) a fitness cost was associated with fludioxonil highly resistant mutants of *P. expansum* in both saprophytic and pathogenic phases of the pathogen but not pyrimethanil-resistant mutants; (ii) pyrimethanil possessed a higher risk than fludioxonil in the development of resistance in *P. expansum*; and (iii) triple resistance to the three apple-postharvest fungicides could emerge and become a practical problem if resistance to pyrimethanil develops in *P. expansum* populations.

Additional keywords: anilinoypyrimidine, fungicide resistance, multi-drug resistance, phenylpyrrole.

Penicillium expansum (Link) Thom. is the primary cause of blue mold, a major postharvest fruit rot disease in apple (35). Conidia are the main type of inoculum responsible for fruit infection by *P. expansum*. Conidia of *P. expansum* are present in the orchards, fruit storage rooms, dump-tank and flotation-tank water, and packing facilities (35,37,38,40). *P. expansum* is essentially a wound-invading pathogen. Wounds such as punctures, and bruises on the fruit created at harvest and during postharvest handling are the primary avenue for infection of fruit by the fungus (35).

Chemical control remains the main measure for the control of postharvest diseases in pome fruits (1,8). Benzimidazole fungicides, including benomyl, thiabendazole (TBZ), and thiophanate-methyl, were introduced in the late 1960s for postharvest use on pome fruits (1). Resistance of *P. expansum* to benzimidazole fungicides was first observed in the mid-1970s on pear in the mid-Columbia region of Oregon and Washington (4), and on apple in New York (36). Benomyl and thiophanate-methyl are currently no longer registered for postharvest use in the United

States. TBZ is commonly used as either a drench treatment prior to storage or an online treatment at packing for control of postharvest diseases of apple, but resistance to TBZ in *P. expansum* often results in the failure of blue mold control (10,22).

In 2004, two new fungicides, fludioxonil and pyrimethanil, were registered for postharvest use on pome fruits in the United State. Fludioxonil and pyrimethanil belong to phenylpyrrole and anilinoypyrimidine classes, respectively. Both fungicides are highly effective in inhibiting conidial germination and germ-tube elongation of *P. expansum* (9,26). The baseline sensitivity to fludioxonil and pyrimethanil in *P. expansum* populations from apple in Washington State has been established (26). The *P. expansum* populations from apple in Washington State prior to the commercial use of these two fungicides consisted of a mixture of phenotypes varying in sensitivity to fludioxonil and pyrimethanil (26), but both thiabendazole-sensitive (TBZ-S) and -resistant (TBZ-R) isolates of *P. expansum* were sensitive to fludioxonil and pyrimethanil, and can be effectively controlled by the two new fungicides (11,26,39). Fludioxonil and pyrimethanil can thus be used as alternatives to TBZ for control of blue mold and other postharvest diseases of pome fruits, and particularly as a tool for control of blue mold caused by TBZ-R strains of *P. expansum* (10,26).

Inherent characteristics of both pathogen and fungicide contribute to the development of resistant populations of a fungal

Corresponding author: C. L. Xiao; E-mail address: clxiao@wsu.edu

pathogen to a fungicide (5). Fludioxonil and pyrimethanil are considered medium-risk resistance fungicides, but *P. expansum* is a pathogen, like *B. cinerea*, because of their short life cycles and abundant reproduction, have the propensity to acquire fungicide resistance rapidly and are high-risk pathogens for the development of fungicide resistance (5). Field resistance to fludioxonil in *Alternaria* spp. from crucifers (17) and in *P. digitatum* from lemon has been reported (18,19). There are numerous reports on the occurrence of field isolates of *B. cinerea* resistant to anilino-pyrimidines several years after its registration for commercial use (6,13,14,21,23,31-33), and multiple resistance of *B. cinerea* to anilino-pyrimidines, dicarboximides, and fenhexamid has also been reported (23), indicating a high risk for build-up of resistance to this class of fungicides in fungal pathogens.

Before the fungicide-resistant population of a fungal pathogen becomes detectable in the field, laboratory fungicide-resistant mutants could be used in early studies to assess the risk in the development of resistance to the fungicide. Fitness of fungicide-resistant isolates and the potential cross resistance among the commonly used fungicides are very important parameters that could provide an indication for the risk of build-up of field fungicide resistance in the population of a target pathogen (5). Experience with TBZ resistance in *P. expansum* and the occurrence of resistance/multiple resistance to pyrimethanil and fludioxonil in other fungal pathogens suggest that it is prudent to assess the potential patterns of cross resistance/multiple resistance to the three apple-postharvest fungicides in *P. expansum*. Currently no information is available about the risk of the development of resistance to fludioxonil and pyrimethanil in *P. expansum* populations from pome fruits. In the present study, we used a mutagenesis approach to generate fungicide-resistant mutants of *P. expansum* in the laboratory, and then used the mutants to assess parameters associated with the risk of development of fungicide resistance. A proactive approach to understand the potential risk in the development of resistance to these two new postharvest fungicides in *P. expansum* would help us develop relevant strategies to avoid or delay the build-up of such resistance and thus prolong the effectiveness of fludioxonil and pyrimethanil for controlling blue mold in apple.

The specific objectives of this study were to (i) generate fludioxonil- and pyrimethanil-resistant mutants of *P. expansum* in the laboratory; (ii) assess potential cross resistance or multiple resistance of the fungicide-resistant mutants to the three apple-postharvest fungicides; (iii) estimate saprophytic and pathogenic fitness parameters of the fungicide-resistant mutants; (iv) assess resistance stability of fungicide-resistant mutants; and (v) test the efficacy of the three apple-postharvest fungicides for controlling blue mold incited by the fludioxonil-resistant and pyrimethanil-resistant mutants.

MATERIALS AND METHODS

Fungicides and fruit. Technical grade fludioxonil (a.i., 93%; Syngenta Inc., Switzerland) and pyrimethanil (a.i., 96%; Janssen Pharmaceutica N.V., Belgium) were dissolved in acetone and adjusted to make 50 and 30 mg/ml stock solutions, respectively. Thiabendazole (a.i., 99%; Sigma-Aldrich Co., St. Louis, MO) was dissolved in methanol and adjusted to 0.5 mg/ml in a stock solution. The formulated thiabendazole (Mertect 340F; Syngenta Crop Protection, Greensboro, NC), fludioxonil (Scholar 50WP; Syngenta Crop Protection), and pyrimethanil (Penbotec 400SC; Janssen Pharmaceutica N.V) were suspended in deionized water following the labels.

We used organic 'Red Delicious' apple fruit throughout the study to avoid potential interference by preharvest fungicide residues on the fruit. Fruit were either harvested from a commercial orchard or purchased from a local fruit packinghouse. No known disease-resistance-inducing products were applied to the fruit.

Fruit were stored at 0°C either in air or in controlled atmosphere (CA) until use.

Mutagenesis. Two wild-type isolates of *P. expansum*, CLX 3354 (TBZ-S) and CLX 3294 (TBZ-R) isolated from decayed apples sampled from commercial fruit packinghouses, were used as parental isolates to generate fungicide-resistant mutants. Conidial suspensions were prepared by transferring dry conidia from a 7-day-old potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) culture with a sterile 4-mm-diameter plastic loop to a 20-ml screw-cap tube containing 10 ml of sterile deionized water with 0.01% Tween 20, and the concentrations of conidia of resulting suspensions were estimated with a hemacytometer (37). An aliquot of 0.2 ml of conidial suspension each of isolate W1 with 3.2×10^7 conidia per ml and isolate W2 with 3.7×10^7 conidia per ml was spread with a stainless steel rod onto PDA amended with 10 µg/ml fludioxonil or onto an L-asparagine-based agar (15) (1 g of K_2HPO_4 , 1 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KCl, 0.01 g of $FeSO_4 \cdot 7H_2O$, 2 g of L-asparagine, 22 g of glucose, and 15 g of agar) amended with pyrimethanil at 10 µg/ml. There were five replicate plates for each isolate. Plates were incubated at 20°C for 5 h in the dark and then exposed to ultraviolet light (plates were placed 27 cm from the light at 253.7 nm) for 30 s. This mutagenic treatment resulted in at least 99% lethality. Plates were then sealed with Parafilm, packed in plastic bags and incubated at 20°C in the dark for 7 days. The emergent colonies were transferred twice to the fungicide-amended media of the same composition as used in the selection procedure, and thereafter cycled twice on fungicide-free PDA before testing for fungicide sensitivity (42). A mycelial growth assay for fludioxonil and a germ-tube elongation assay for pyrimethanil (26) were used to determine whether mutants remained resistant to these two fungicides. Resistance to fludioxonil was screened by examining the colony growth on PDA amended with fludioxonil at 0 and 0.5 µg/ml, after incubation for 5 days at 20°C. The mutants that were able to grow on PDA amended with fludioxonil were considered fludioxonil-resistant (FR). For pyrimethanil, at least three parallel lines were marked on the reverse of an agar plate (10 g of glucose, 2 g of K_2HPO_4 , 2 g of KH_2PO_4 , and 12.5 g of agar) (25) amended with pyrimethanil at 0 and 1 µg/ml. A loop of the conidial suspension (approximately 10^5 conidia/ml) was streaked along each line. After incubation for approximately 44 h, plates were examined under a microscope for germ-tube elongation. Mutants with similar length of germ tubes on plates amended with pyrimethanil at 1 µg/ml with those on plates without the fungicide were considered pyrimethanil-resistant (PR). After subculturing for 10 successive generations on PDA without fungicides, fludioxonil- and pyrimethanil-resistant mutants were tested again for resistance to fludioxonil and pyrimethanil, respectively. Two fludioxonil-resistant and two pyrimethanil-resistant mutants derived from each of the isolates W1 (TBZ-S) and W2 (TBZ-R) with stable resistance to fludioxonil and pyrimethanil were selected for further study.

Phenotypes of fungicide-resistant mutants. The in vitro fungicide sensitivities of the two wild-type parental isolates (W1 and W2) and the eight representative fungicide-resistant mutants (FR1 to FR4 for fludioxonil and PR1 to PR4 for pyrimethanil) were determined by a mycelial growth assay for fludioxonil or a germ-tube elongation assay for pyrimethanil (26). Sensitivity to fludioxonil (0, 3.125, 6.25, 12.5, 25, and 50 µg/ml) of the fludioxonil-resistant mutants, sensitivity to fludioxonil (0, 0.015, 0.05, 0.15, 0.5, 1, 1.5, and 5 µg/ml) of the pyrimethanil-resistant mutants, and sensitivity to thiabendazole (0, 0.2, 0.4, 0.6, 1, 2.5, 5, and 10 µg/ml) of W1 isolate and the fungicide-resistant mutants derived from W1 were assessed with mycelial growth assays. In order to obtain the EC_{50} values of thiabendazole for the fungicide-resistant mutants derived from W2 (TBZ-R) and EC_{50} values of fludioxonil for the fludioxonil-resistant mutants, a formulated thiabendazole (Mertect 340F) (0, 100, 250, 500, 750, and 1,000 µg/ml)

and a formulated fludioxonil (Scholar 50WP) (0, 100, 250, 500, 750, and 1,000 µg/ml) were used. To make fresh mycelial inoculum, 1 ml of conidial suspension (approximately 10^6 conidia/ml) was mixed into 16 ml of molten PDA at 50°C, and the medium was then poured immediately into a petri plate. Cultures were incubated at 20°C for 24 h. Agar plugs were cut from the cultures with a 6-mm-diameter cork borer. One such plug was placed upside-down on fungicide-amended PDA in each plate. Three replicate plates were used for each concentration for each isolate. Colony diameters were measured after 7 days at 20°C, and EC₅₀ values for all isolates were estimated.

To determine EC₅₀ values of pyrimethanil, conidial suspensions with approximately 10^5 conidia/ml of the four fludioxonil-resistant mutants (F1 to F4) and the parental isolates were streaked onto the previously described L-asparagine-based agar plates amended with pyrimethanil at 0, 0.15, 0.25, 0.35, and 0.5 µg/ml. Six concentrations of pyrimethanil at 0, 5, 10, 15, 20, and 30 µg/ml were used to determine the EC₅₀ values of pyrimethanil for the four pyrimethanil-resistant mutants. Cultures were incubated at 20°C for approximately 42 h. The lengths of germ tubes (50 germinated conidia for each treatment) were estimated under a microscope using a micrometer. The experiment was performed three times.

Resistance factor (RF, a ratio of EC₅₀ for a fungicide-resistant mutant relative to the EC₅₀ for the parental isolate) (7) and minimum inhibitory concentration (MIC) (31) are commonly used to describe phenotypes of fungicide-resistant isolates or mutants. For resistance to TBZ, an MIC of 5 µg/ml is commonly used (37). In our study, we determined if EC₅₀ values of TBZ for mutants derived from TBZ-S were shifted compared to the wild-type parental isolate. To determine whether these mutants derived from TBZ-S became resistant to TBZ or more sensitive to TBZ, mycelial growth of these mutants was also tested on PDA amended with TBZ at 2.5 and 5 µg/ml. In this study, for TBZ, an isolate was classified as sensitive (S) if the EC₅₀ value was <1 µg/ml and MIC for mycelial growth ≤5 µg/ml; low level of resistance (LR), with the EC₅₀ value between 1 and 10 µg/ml; and highly resistant (HR), with the EC₅₀ value >100 µg/ml. For fludioxonil, an isolate was classified as sensitive (S) with the EC₅₀ value <0.1 µg/ml for mycelial growth; LR with the EC₅₀ value between 0.1 and 1 µg/ml; HR with the EC₅₀ value >50 µg/ml (12).

Saprophytic fitness of fungicide-resistant mutants. The eight fungicide-resistant mutants were tested for mycelial growth, conidial germination, germ-tube elongation, sporulation, sensitivity to osmotic pressure, and stability of resistant phenotypes compared with the parental isolates. One 6-mm-diameter mycelial plug from a 24-h-old culture of each isolate was placed at the center of each of the three PDA plates, and colony diameter in each plate was measured after 7 days at 20°C and 10 weeks at 0°C in the dark. The reverses of PDA plates were marked with four to five parallel lines. A loop of the conidial suspension (approximately 10^5 conidia/ml) made from 7-day-old cultures were streaked along each line. After incubation at 20°C in the dark for 24 h, the percentage of conidial germination (about 300 conidia for each isolate) was determined and the lengths of germ tubes (150 germinated conidia for each isolate) were measured with a micrometer. Only conidia with a germ tube at least twice its length were considered germinated.

To determine conidial production in the absence of the fungicides, an aliquot of 1 ml of each conidial suspension (approximately 1×10^4 conidia/ml) was added to a 125-ml flask containing 20 ml of PDA at 50°C, shaken at 150 rpm for 30 s and poured into a petri plate (95-mm-diameter). Three plates for each isolate were incubated in the dark at 20°C for 7 days and 0°C for 10 weeks. Five agar plugs were cut from the colony in a “+” pattern in each plate with a 6-mm-diameter cork borer, gently crushed with a pestle in a mortar, and then poured into a flask containing either 100 or 200 ml of sterile deionized water with

0.01% Tween 20. The suspension was stirred for 5 min, and the concentration of the conidial suspension was estimated with a hemacytometer. The number of conidia produced on five agar plugs from each plate was calculated.

To test osmotic sensitivity, three 6-mm-diameter mycelial plugs from 24-h-old PDA cultures of each fungicide-resistant mutant and their parental isolates of *P. expansum* were transferred to PDA amended with 0, 1, 2, 4, 6, or 8% (wt/vol) NaCl, respectively (30). The colony diameters were measured after 7 days at 20°C. The percentage of the mycelial radial growth inhibition was calculated. This experiment was performed twice.

Pathogenic fitness of fungicide-resistant mutants. Red Delicious fruit that had been stored in air at 0°C for 1 to 2 months were used in this study. Prior to experiment, fruit were surface-disinfested for 5 min in 0.6% sodium hypochlorite solution, rinsed three times with deionized water, and air dried. Each fruit was wounded with a 4-mm-diameter finish-nail head into 3 mm in depth and subsequently inoculated by placing 20 µl of the conidial suspension (approximately 1×10^4 conidia/ml) with a pipette into the wound. Fruit treated with sterile water were used as a noninoculated control. Nine replicate fruit per isolate were inoculated and then placed on muffin trays in a plastic box covered with its lid. A 50-ml volume of water was added to the bottom of the box to maintain high relative humidity. The plastic boxes were kept at 20°C for 10 days. In addition, 20 replicate apples per isolate were inoculated and placed on fiberboard fruit trays and stored in cardboard boxes at 0°C for 12 weeks. Fruit were considered decayed when a lesion developed around the wound and lesion diameter was measured. Percent fruit with decay symptoms and virulence index (the ratio of the lesion size of the fungicide-resistant mutant relative to the lesion size of its parental wild-type isolate) were calculated. Three decayed fruit from each treatment were selected for re-isolation of the causal agent.

Sporulation of fungicide-resistant mutants on apple fruit was evaluated. Fruit that had been stored at 0°C in CA for 5 months were used in this study. Fruit were surface-disinfested for 5 min in 0.6% sodium hypochlorite solution, rinsed three times with deionized water, and air dried. Five apples were sliced longitudinally with a sterile knife to create a similar size area (approximately 70 × 60 mm) on each fruit. The center of the flat surface of the sliced apple was wounded with a finish-nail head (4-mm-diameter and 3-mm depth). Each wound was inoculated with 20 µl of conidial suspension (approximately 1×10^4 conidia/ml) of each isolate and incubated at 20°C for 7 days and 0°C for 10 weeks. Lesion diameter of each fruit was measured and the decayed area was removed with a sterile scalpel and put into a beaker containing either 100 or 200 ml of sterile deionized water with 0.01% Tween 20. The suspension in each beaker was stirred for 5 min. The concentration of the conidial suspension was estimated with a hemacytometer. The number of conidia produced on each apple was calculated.

Stability of resistance of fungicide-resistant mutants. Stability of fungicide-resistant isolates in vitro was assessed by successive transfers of the fungicide-resistant mutants on fungicide-free PDA for 20 generations. At the end of the 20th generation, the EC₅₀ values of fludioxonil for the fludioxonil-resistant mutants and EC₅₀ values of pyrimethanil, thiabendazole, and fludioxonil for the pyrimethanil-resistant mutants were determined with the assays as previously described. This experiment was performed two times.

Resistance stability of fungicide-resistant mutants on apple fruit was evaluated. Fruit that had been stored at 0°C in CA for 5 months were used in this study. Fruit were surface-sterilized, wounded, and inoculated as previously described with the conidial suspensions (approximately 1×10^4 conidia/ml) of the eight fungicide-resistant mutants and two parental isolates, and incubated at 20°C. Nine replicate apples per isolate were inoculated

and placed on muffin trays in plastic boxes, and 50 ml of water was added to the box to maintain high relative humidity. Lesion diameter was measured 10 days after inoculation. One decayed apple from each inoculum of each generation was selected for re-isolation. The inoculum of the isolate recovered from decayed fruit was used for inoculation of fruit for the following generation. After four successive generations of inoculation using the same method as described above, EC₅₀ values of fludioxonil for the fludioxonil-resistant mutants of the fourth generation on apple fruit and EC₅₀ values of pyrimethanil, thiabendazole, and fludioxonil for the pyrimethanil-resistant mutants, and the virulence index of each generation were determined. This experiment was performed three times.

Efficacy of the fungicides for control of blue mold incited by fludioxonil- and pyrimethanil-resistant mutants of *P. expansum*. Two fludioxonil-resistant (FR2 and FR3) and two pyrimethanil-resistant mutants (PR2 and PR4) and their parental wild-type isolates (W1 and W2) were included in the test. Conidial suspensions (approximately 1×10^4 conidia/ml) made from 7-day-old PDA cultures grown at 20°C were used as inoculum for fruit inoculation. Organic Red Delicious fruit that had been stored in CA at 0°C for about 4 months were used for this study. Fruit were surface-sterilized, wounded, and inoculated as described previously. At approximately 1 h after inoculation, fruit were dipped for 1 min in either deionized water (control) or in one of the three following fungicide solutions: thiabendazole applied as Mertect 340F (1.25 ml/liter), fludioxonil applied as Scholar 50WP (0.6 g/liter), and pyrimethanil applied as Penbotec 400SC (1.25 ml/liter). Twenty apple fruit per replicate and three replicates were used for each isolate. Fruit were air-dried for 15 min and then placed on fruit trays and stored in cardboard boxes at room temperature for 10 days (average 23.8°C, from 21.7 to 24.8°C) and 0°C for 12 weeks, after which decay was evaluated. Fruit was considered decayed when a lesion developed at the inoculation site. Lesion diameter was measured and percent fruit with decay symptoms was calculated. For the experiment conducted at 0°C, after cold storage fruit were also stored at room temperature for an additional week, after which decay was evaluated. In order to confirm the results with the pyrimethanil-resistant mutants, another pyrimethanil-resistant mutant (PR1) also was tested in a separate experiment following the same method and incubated at 20°C for 10 days. This experiment was performed twice.

Statistical analysis. All experiments were conducted at least twice and the data were pooled for analysis because results from repeated experiments did not differ statistically ($P > 0.05$). A liner

regression of the percent inhibition of mycelial growth relative to the control versus the logarithmic transformation for each concentration of fludioxonil and thiabendazole were obtained using SAS PROC REG (version 9.1, SAS Institute, Cary, NC). EC₅₀ values were then calculated based on the models. The EC₅₀ values of pyrimethanil based on the germ-tube elongation assay were calculated based on liner regression models without the logarithmic transformation of fungicide concentrations. A *t* test was performed to compare the stability of fungicide resistance at original generation versus at the 20th generation on PDA or the fourth generation on apple fruit using SAS. The differences between the fungicide-resistant mutants and their parental isolates on saprophytic and pathogenic fitness parameters and the differences among treatments in the fungicide efficacy experiment were analyzed by analysis of variance using PROC GLM of SAS and means were separated by the Waller-Duncan K-ratio *t* test. Sporulation data were transformed to logarithms and all data in percentages were transformed with the arcsine-square-root transformation prior to statistical analysis.

RESULTS

Induction of fludioxonil- and pyrimethanil-resistant mutants. Fludioxonil- and pyrimethanil-resistant mutants of *P. expansum* were readily obtained by exposing conidia on PDA amended with fludioxonil at 10 µg/ml and an L-asparagine-based agar amended with pyrimethanil at 10 µg/ml to UV irradiation, respectively. The frequency of UV-induced mutagenesis was approximately 10^{-7} among the total number of conidia that were exposed. Twenty-four fludioxonil-resistant and 20 pyrimethanil-resistant mutants derived from the TBZ-S isolate (W1), and 16 fludioxonil-resistant and eight pyrimethanil-resistant mutants derived from the TBZ-R isolate (W2) were obtained. After the mutants were subcultured on fungicide-amended media twice and thereafter on fungicide-free PDA twice, 29 fludioxonil-resistant and 10 pyrimethanil-resistant mutants remained resistant to the fungicides according to the initial screening (fludioxonil at 0.5 µg/ml and pyrimethanil at 1 µg/ml as discriminatory concentrations). Four representative fludioxonil-resistant mutants and four pyrimethanil-resistant mutants (two derived from each of W1 and W2 for both fungicides) were chosen for further studies.

Phenotypes of fungicide-resistant mutants. Six phenotypes of *P. expansum* were detected among the fungicide-resistant mutants and their wild-type parental isolates based upon their resistance levels (Table 1). All four fludioxonil-resistant mutants (FR1 to FR4) were highly resistant to fludioxonil (EC₅₀ > 1,000 µg/ml)

TABLE 1. In vitro sensitivity of fludioxonil- and pyrimethanil-resistant mutants and their parental wild-type isolates of *Penicillium expansum* to thiabendazole, fludioxonil, and pyrimethanil

Culture no. ^v	Isolate code ^w	Phenotype ^x	EC ₅₀ (µg/ml) ^y		
			Thiabendazole	Fludioxonil	Pyrimethanil
CLX3354	W1	TBZ ^S Flu ^S Pyr ^S	0.595 ± 0.019 ^z	0.017 ± 0.001	0.191 ± 0.018
CLX3294	W2	TBZ ^{HR} Flu ^S Pyr ^S	>1,000	0.021 ± 0.001	0.189 ± 0.018
CLX4277	FR1	TBZ ^S Flu ^{HR} Pyr ^S	0.220 ± 0.005	>1,000	0.195 ± 0.032
CLX4284	FR2	TBZ ^S Flu ^{HR} Pyr ^S	0.461 ± 0.021	>1,000	0.166 ± 0.020
CLX4262	FR3	TBZ ^{HR} Flu ^{HR} Pyr ^S	>1,000	>1,000	0.158 ± 0.026
CLX4272	FR4	TBZ ^{HR} Flu ^{HR} Pyr ^S	>1,000	>1,000	0.160 ± 0.025
CLX4256	PR1	TBZ ^{LR} Flu ^{LR} Pyr ^R	1.602 ± 0.017	0.421 ± 0.016	13.997 ± 2.135
CLX4258	PR2	TBZ ^{LR} Flu ^{LR} Pyr ^R	1.406 ± 0.064	0.657 ± 0.032	15.133 ± 0.926
CLX4252	PR3	TBZ ^{HR} Flu ^{LR} Pyr ^R	>1,000	0.321 ± 0.009	13.638 ± 1.124
CLX4253	PR4	TBZ ^{HR} Flu ^{LR} Pyr ^R	>1,000	0.365 ± 0.007	13.554 ± 0.936

^v All cultures are maintained at the Tree Fruit Research and Extension Center, Washington State University, Wenatchee, WA.

^w W1 = thiabendazole-sensitive wild-type isolate; W2 = thiabendazole-resistant wild-type isolate; FR1 to FR4 = fludioxonil-resistant mutants; PR1 to PR4 = pyrimethanil-resistant mutants. FR1, FR2, PR1, and PR2 were derived from W1; and FR3, FR4, PR3, and PR4 were derived from W2.

^x TBZ = thiabendazole, Flu = fludioxonil, Pyr = pyrimethanil, S = sensitive, R = resistant, LR = lowly resistant, HR = highly resistant.

^y The EC₅₀ values of thiabendazole and fludioxonil were determined by mycelial growth assays and calculated using liner regression models. The EC₅₀ values of pyrimethanil were determined by the germ-tube elongation assay and calculated using liner regression models.

^z Mean EC₅₀ ± SE.

and sensitive to pyrimethanil and to TBZ as their parental isolates, although the two fludioxonil-resistant mutants derived from the TBZ-S wild-type isolate (W1) became more sensitive to thiabendazole ($MIC \leq 2.5 \mu\text{g/ml}$) than W1 ($MIC = 5 \mu\text{g/ml}$) (data not shown). The resistance factors (RF) of the four pyrimethanil-resistant mutants to pyrimethanil were >73 . All four pyrimethanil-resistant mutants showed a low level of resistance to fludioxonil,

with a resistance factor >15 . The two pyrimethanil-resistant mutants derived from TBZ-S became resistant to TBZ at $5 \mu\text{g/ml}$, with a resistance factor >2 .

Saprophytic fitness of fludioxonil- and pyrimethanil-resistant mutants of *P. expansum*. All four fludioxonil highly-resistant mutants (FR1 to FR4) were sensitive to osmotic pressure and grew slower on PDA amended with NaCl than their parental isolates (W1 and W2) and pyrimethanil-resistant mutants (Fig. 1). The mycelial growth of fludioxonil highly-resistant mutants was inhibited by more than 50% on PDA amended with 4% NaCl. All four pyrimethanil-resistant mutants had the similar levels of sensitivity to NaCl as the parental isolates. The four pyrimethanil-resistant mutants and the parental isolates grew faster on PDA amended with low concentrations of NaCl (1 and 2%) than on the non-NaCl control.

All four fludioxonil highly-resistant mutants grew significantly slower on PDA at 20°C compared with pyrimethanil-resistant mutants and the wild-type parental isolates, but the differences in mycelial growth at 0°C were not consistent with those at 20°C (Table 2). Pyrimethanil-resistant mutants PR1 and PR2 grew slower than the parental isolate W1. PR3 grew faster at 20°C but slower at 0°C than its parental isolate W2. PR4 did not differ significantly in mycelial growth compared with that of W2. There were no significant differences in sporulation among the mutants and wild-type isolates except that FR1 produced fewer spores at 20°C and FR4 mutant produced fewer spores on PDA at both 20 and 0°C compared with pyrimethanil-resistant mutants and the wild-type parental isolates (Table 2). No significant difference in the conidial germination rate was observed among the mutants and wild-type isolates. No consistent trends in the difference in germ-tube elongation was observed or the difference was marginal (Table 2).

Pathogenic fitness of fungicide-resistant mutants. All fungicide-resistant mutants were pathogenic on apple fruit (Table 3). No decay developed on the fruit in the noninoculated control. At 20°C , nearly all inoculated fruit developed blue mold 10 days after inoculation. At 0°C , 8 to 88% of the fruit inoculated with fludioxonil highly-resistant mutants developed blue mold, whereas all fruit inoculated with pyrimethanil-resistant mutants developed blue mold after 12 weeks at 0°C .

Virulence indices of fludioxonil highly-resistant mutants (FR1 to FR4) were significantly ($P < 0.05$) less than one, indicating these mutants were less virulent than their parental wild-type isolates. It also appeared that fludioxonil highly-resistant mutants were less pathogenic and virulent at 0°C than at 20°C . Virulence indices of pyrimethanil-resistant mutants ranged from 0.8 to 1. Except for PR1 at 0°C and PR2 at both 0 and 20°C , pyrimethanil-

Fig. 1. Inhibition of mycelial growth of *Penicillium expansum* on potato dextrose agar amended with different NaCl concentrations. **A**, Parental wild-type isolate W1 (TBZ-S) and the mutants derived from W1; **B**, parental wild-type isolate W2 (TBZ-R) and the mutants derived from W2. FR1 to FR4 are fludioxonil-resistant mutants. PR1 to PR4 are pyrimethanil-resistant mutants. Bars represent standard errors.

TABLE 2. Growth characteristics of fludioxonil- and pyrimethanil-resistant mutants of *Penicillium expansum* and their parental wild-type isolates on potato dextrose agar

Parental wild-type isolate	Mutant	Radial growth ^y		Log ₁₀ unit of sporulation ^w		Conidia germination (%) ^x	Germ-tube elongation ^y
		20°C	0°C	20°C	0°C		
W1		44.8 a ^z	26.3 b	8.2 a	7.6 a	99.6 a	21.7 ab
	FR1	33.3 e	16.9 d	8.1 b	7.3 a	99.9 a	22.1 ab
	FR2	39.4 c	30.1 a	8.2 a	7.6 a	100.0 a	28.8 a
	PR1	43.5 b	19.3 c	8.2 a	7.5 a	100.0 a	27.1 a
	PR2	36.6 d	19.8 c	8.2 a	7.7 a	100.0 a	18.0 b
W2		46.0 b	17.9 a	8.1 a	7.6 a	99.9 a	17.6 ab
	FR3	29.4 d	14.8 b	7.9 ab	7.6 a	100.0 a	14.0 b
	FR4	33.6 c	16.9 a	7.8 b	7.3 b	99.6 a	15.8 ab
	PR3	46.8 a	14.9 b	8.0 a	7.6 a	99.6 a	19.8 ab
	PR4	45.9 b	17.0 a	8.1 a	7.6 a	100.0 a	21.6 a

^y Mean colony diameter (mm) measured after 7 days of incubation at 20°C and 10 weeks at 0°C .

^w Conidia were counted after incubation at 20°C for 7 days and 0°C for 10 weeks. The data were logarithm-transformed before analysis.

^x Percentage of germinated conidia after 24 h incubation. The data were arcsine-transformed before analysis.

^y Mean length (μm) of germ tube after 24 h incubation.

^z Values within the same column for the mutants from the same parental wild-type isolate followed by the same letter are not significantly different at K ratio = 100 ($P = 0.05$) according to the Waller-Duncan K-ratio *t* test.

resistant mutants were as virulent as their parental isolates (Table 3).

Fludioxonil highly-resistant mutants produced significantly fewer spores on apple fruit than pyrimethanil-resistant mutants and the wild-type parental isolates at both 20 and 0°C, regardless of whether the total number of conidia or the number of conidia produced per unit of lesion area was considered (Table 3). FR4 did not produce a detectable number of spores on apple fruit. There was no significant difference in sporulation on apple fruit between pyrimethanil-resistant mutants and the parental isolates.

Stability of fungicide-resistant phenotypes. After having been subcultured on PDA following mycelial propagation for 20 successive generations or successive inoculation with conidia as inoculum on apple fruit for four generations, fungicide-resistant mutants had the same levels of resistance to either fludioxonil (data not shown) or pyrimethanil (Table 4). The pyrimethanil-resistant mutants of the twentieth generation on PDA and the fourth generation on apple fruit also retained the same levels of resistance to fludioxonil and thiabendazole as their original generations (Table 4). There was no significant difference in virulence index on Red Delicious apples among four generations of each mutant (data not shown).

Efficacy of postharvest fungicides for control of blue mold incited by fungicide-resistant mutants. Pyrimethanil was effective in controlling blue mold caused by fludioxonil-resistant mutants at both 0°C and room temperature (Table 5). Fludioxonil was not effective in controlling fludioxonil highly-resistant mutants. Thiabendazole was effective against the fludioxonil-resistant mutant FR2 derived from W1 (TBZ-S), but not FR3 derived from W2 (TBZ-R).

Pyrimethanil reduced decay incidence on fruit inoculated with pyrimethanil-resistant mutants by 17 to 27% and 53 to 59% at room temperature and 0°C, respectively (Table 5). The effectiveness of fludioxonil in controlling pyrimethanil-resistant mutants was temperature dependent. At room temperature, fludioxonil was not effective against pyrimethanil-resistant mutants which exhibited a low level of resistance to fludioxonil, whereas at 0°C decay caused by pyrimethanil-resistant mutants was considerably

reduced by fludioxonil during the 12-week storage. Pyrimethanil-resistant mutants (PR1 and PR2) that were derived from TBZ-S showed a low level of resistance to TBZ. At room temperature, TBZ did not provide effective control of blue mold incited by PR1 and PR2. At 0°C, however, TBZ reduced blue mold incited by PR2 to 6.7% in comparison with 97.5% blue mold in the nontreated control (Table 5).

DISCUSSION

The goal of our study was to assess the potential risk in the development of resistance, including multi-drug resistance, to fludioxonil and pyrimethanil in *P. expansum* populations from apple. Of the six fungicide-resistance phenotypes detected among the fungicide-resistant mutants and their wild-type parental isolates, the four pyrimethanil-resistant mutants of *P. expansum* we examined in this study had multi-drug resistance. Multi-drug resistance to chemically unrelated fungicides has also been reported in *Botrytis cinerea* (23). Three anilinopyrimidine-resistant phenotypes (Ani R1, Ani R2, and Ani R3) were identified in field populations of *B. cinerea* in French vineyards (23,24). Phenotype Ani R1 is moderately to highly resistant to anilinopyrimidines with a resistance factor ranging from 15 to 250, and retains a similar level of sensitivity to phenylpyrroles and other classes of fungicides as the wild type strains (24). Phenotype Ani R2 has a low to moderate level of resistance to anilinopyrimidines (resistance factor 5 to 15) and exhibits a low level of resistance to phenylpyrroles and dicarboximides. Phenotype Ani R3 has a low level of resistance to anilinopyrimidines (resistance factor 3 to 7) and dicarboximides, but remains sensitive to phenylpyrroles. Ani R2 and Ani R3 are multi-drug resistance phenotypes and resistance also extends to some other classes of fungicides (6,23). In our study, in terms of resistance to fludioxonil, pyrimethanil-resistant phenotypes are similar to the resistant phenotype Ani R2 of *B. cinerea* (6,23). However, in our study the TBZ resistance in the phenotype TBZ^{LR}Flu^{LR}Pyr^R appears to be pyrimethanil-mediated resistance, because this phenotype was originally derived from a TBZ-S wild-type isolate. Our findings indicate

TABLE 3. Pathogenic fitness parameters of fludioxonil- and pyrimethanil-resistant mutants of *Penicillium expansum* and their parental isolates on apple fruit

Parental wild-type isolate	Mutant	Incidence (%)		Virulence index ^y		Log ₁₀ unit of conidia harvested from fruit		Log ₁₀ unit of conidia per cm ² lesion size	
		20°C	0°C	20°C	0°C	20°C	0°C	20°C	0°C
W1		100	100 a ^z	1 a	1 a	7.3 a	7.4 a	6.3 a	6.2 a
	FR1	100	55.0 c	0.244 d	0.159 c	1.6 c	2.9 b	1.5 c	2.4 b
	FR2	100	85.0 b	0.583 c	0.208 c	3.9 b	2.9 b	3.5 b	2.5 b
	PR1	100	100 a	0.941 a	0.882 b	7.8 a	7.6 a	6.8 a	6.4 a
	PR2	100	100 a	0.797 b	0.878 b	6.9 a	6.8 a	6.1 a	5.7 a
W2		100	100 a	1 a	1 a	6.9 a	6.2 a	6.1 a	5.2 a
	FR3	100	87.5 b	0.481 b	0.248 b	1.6 b	0.6 b	1.5 b	0.6 b
	FR4	94.4	7.5 c	0.214 c	0.061 c	0 c	0 b	0 c	0 b
	PR3	100	100 a	0.994 a	0.960 a	7.1 a	6.7 a	6.2 a	5.7 a
	PR4	100	100 a	1.032 a	0.963 a	7.1 a	6.3 a	6.2 a	5.2 a

^y Virulence index = the lesion size of a mutant on decayed apple divided by the lesion size of the parental wild-type isolate.
^z The values within the same column for the mutants derived from the same parental wild-type isolate followed by the same letter are not significantly different according to the Waller-Duncan K-ratio *t* test at K ratio = 100 (*P* = 0.05).

TABLE 4. Comparison of EC₅₀ values of pyrimethanil, fludioxonil, and thiabendazole for pyrimethanil-resistant mutants of *Penicillium expansum* after 20 successive generations on potato dextrose agar (PDA) and four successive generations on apple fruit

Origin	Pyrimethanil ^x				Fludioxonil ^y				Thiabendazole ^y			
	PR1	PR2	PR3	PR4	PR1	PR2	PR3	PR4	PR1	PR2	PR3	PR4
Original	13.997	15.133	13.638	13.554	0.421	0.657	0.321	0.365	1.602	1.406	>10	>10
20th on PDA	13.782	17.354	15.818	15.343	0.403	0.639	0.324	0.374	1.399	1.347	>10	>10
4th on apple	14.880	18.747	14.012	15.881	0.330* ^z	0.644	0.298*	0.366	1.565	1.272	>10	>10

^x The EC₅₀ values of pyrimethanil were determined by the germ-tube elongation assay and calculated using liner regression models.
^y The EC₅₀ values of fludioxonil and thiabendazole were determined by the mycelial growth assays and calculated using liner regression models.
^z “*” indicates that the EC₅₀ value was significantly different from that of original generation according to a *t* test at *P* = 0.05.

that once an isolate of *P. expansum* becomes resistant to pyrimethanil, it could also extend resistance to fludioxonil and possess a low level of resistance to TBZ. It is unknown whether pyrimethanil-mediated resistance to TBZ also occurs in *B. cinerea*. In the reports of Chapeland et al. (6) and Leroux et al. (23,24), sensitivities of Ani R1, Ani R2, and Ani R3 in *B. cinerea* to TBZ were not tested. To our knowledge, the present study is the first report of pyrimethanil-mediated resistance to TBZ in a fungal pathogen.

Resistance to TBZ in TBZ-resistant mutants of *P. expansum* generated in the laboratory by UV irradiation was associated with point mutations at codons 167, 198, and 200 of β -tubulin gene (2). A point mutation at codon 198 of β -tubulin gene has also been observed in highly benzimidazole-resistant isolates of *P. expansum* from natural populations (2,20,39). However, A mutation at codon 198 was not detected in the isolates of *P. expansum* which had a low level of resistance to benomyl; those isolates were able to grow on the medium amended with benomyl at 0.5 $\mu\text{g/ml}$ but not at 5 $\mu\text{g/ml}$ (20). Ma et al. (29,30) also reported a point mutation at codon 6 and codon 240 of the β -tubulin gene resulting in a level of resistance to benomyl in *Monilinia fructicola* and *M. laxa*, respectively. In our study, the pyrimethanil-resistant mutants derived from W1 (TBZ-S) exhibited a low level of resistance to TBZ (active growth on media amended with 5 $\mu\text{g/ml}$ but not with 10 $\mu\text{g/ml}$). It is not known whether pyrimethanil-mediated resistance to TBZ also is related to any point mutation of β -tubulin gene of these mutants. Various ATP-binding cassette and major facilitator superfamily transporters have been reported to be involved in multi-drug resistance development in *B. cinerea* (41). Future research is needed to understand mechanisms of multi-drug resistance in *P. expansum*.

In our study, fludioxonil-resistant mutants of *P. expansum* with phenotypes TBZ^SFlu^{HR}Pyr^S and TBZ^{HR}Flu^{HR}Pyr^S were sensitive

to osmotic stress. The four pyrimethanil-resistant mutants exhibited a low level of resistance to fludioxonil but were not sensitive to osmotic stress. Previous reports indicated that laboratory fludioxonil-resistant mutants of *B. cinerea* were sensitive to osmotic stress (16,44,45). Similar findings have also been reported on laboratory induced iprodione-resistant mutants of *Botryosphaeria dothidea* (27) and *Alternaria alternata* (28), and field iprodione-resistant isolates of *P. expansum*, *B. cinerea*, and *Aspergillus nidulans* (3). However, there was no correlation between multiple phenylpyrrole-dicarboximide resistance and osmotic stress regulation in *P. digitatum* from lemon (18). Thus, responses of fludioxonil resistance to osmotic stress regulation may vary by fungal pathogens and phenotypes of fungicide resistant strains. In contrast, in our study pyrimethanil-resistant mutants of *P. expansum* responded to osmotic stress similarly as the wild-type parental isolates and were not sensitive to osmotic stress on PDA amended with 4 to 8% NaCl. It was considered that osmotic sensitivity may be related to the change in cell wall composition, thereby the fitness of the resistant isolates may be decreased in the field (3,27). The results suggest that fludioxonil-resistant strains may not fit well in the environment compared with pyrimethanil-resistant mutants and their wild-type parental isolates.

Pathogenic fitness parameters are important factors in evaluating the potential build-up of fungicide-resistant population of a fungal pathogen (5). Previous reports indicated that pathogenicity or virulence of fludioxonil-resistant isolates or mutants vary by pathogens or phenotypes of the pathogen. For example, osmotic-sensitive phenylpyrrole-resistant mutants of *B. cinerea* are as virulent as the wild-type parental isolate (44) or lose their pathogenicity on apple fruit (16). Kanetis et al. (18) found that fludioxonil-resistant isolates of *P. digitatum* from citrus with EC₅₀ values between 0.21 and 0.45 $\mu\text{g/ml}$ were less pathogenic (low

TABLE 5. Efficacy of three apple-postharvest fungicides for the control of blue mold incited by fludioxonil- and pyrimethanil-resistant mutants and their parental wild-type isolates of *Penicillium expansum* in Red Delicious apple fruit

Isolate	Treatment ^y	Room temperature (RT) for 10 days		Cold storage (0°C) for 12 weeks		Cold storage (0°C) for 12 weeks and RT for another week
		Incidence (%)	Lesion size (mm)	Incidence (%)	Lesion size (mm)	Incidence (%)
W1	Nontreated	100 a ^z	41.8 a	95.8 a	43.8 a	100 a
	Thiabendazole	0 b	0 b	0 b	0 b	0 c
	Fludioxonil	0 b	0 b	0 b	0 b	3.3 b
	Pyrimethanil	0 b	0 b	0 b	0 b	0.8 bc
FR2	Nontreated	100 a	19.1 b	81.7 a	17.2 a	100 a
	Thiabendazole	0 b	0 c	0 b	0 b	2.5 b
	Fludioxonil	100 a	22.5 a	91.7 a	16.9 a	99.2 a
	Pyrimethanil	0 b	0 c	0 b	0 b	0.8 b
PR1	Nontreated	100 a	36.7 a			
	Thiabendazole	57.3 b	15.8 d			
	Fludioxonil	100 a	27.6 b			
	Pyrimethanil	97.5 a	19.7 c			
PR2	Nontreated	100 a	23.8 a	97.5 a	30.6 a	100 a
	Thiabendazole	74.2 b	15.4 bc	6.7 d	4.6 c	10 c
	Fludioxonil	97.5 a	16.7 b	20.8 c	19.9 b	84.2 b
	Pyrimethanil	72.5 b	13.1 c	45.8 b	19.8 b	82.4 bc
W2	Nontreated	100 a	38.8 a	93.3 b	25.6 b	100 a
	Thiabendazole	100 a	39.0 a	99.2 a	30.4 a	100 a
	Fludioxonil	0 b	0 b	0 c	0 c	0 b
	Pyrimethanil	0 b	0 b	0 c	0 c	0 b
FR3	Nontreated	99.2 a	12.4 b	49.2 b	12.6 ab	92.5 b
	Thiabendazole	100 a	12.7 b	74.8 a	14.2 a	98.3 a
	Fludioxonil	100 a	17.4 a	62.5 ab	10.1 b	99.2 a
	Pyrimethanil	0 b	0 c	0 c	0 c	0 c
PR4	Nontreated	100 a	35.2 a	93.3 a	26.1 a	100 a
	Thiabendazole	99.2 a	34.4 a	97.5 a	28.1 a	100 a
	Fludioxonil	99.2 a	24.8 b	10.0 c	9.1 c	60.0 c
	Pyrimethanil	83.3 b	15.8 c	37.5 b	16.0 b	84.2 b

^y Nontreated, thiabendazole, fludioxonil, and pyrimethanil were applied as sterile deionized water, Mertect 340F at 1.25 ml/liter, Scholar 50WP at 0.6 g/liter, and Penbotec 400SC at 1.25 ml/liter, respectively.

^z The data within the same column for each isolate followed by the same letter are not significantly different according to the Waller-Duncan K-ratio *t* test at K ratio = 100 (*P* = 0.05).

disease incidence) but highly virulent (large decay lesion) on citrus fruit, and the isolates with EC₅₀ values >0.79 µg/ml were highly pathogenic but less virulent. In our study, pathogenicity of fludioxonil-resistant mutants with phenotypes TBZ^SFlu^{HR}Pyr^S and TBZ^{HR}Flu^{HR}Pyr^S was temperature dependent. These four fludioxonil highly-resistant mutants were as pathogenic as the wild-type parental isolates at 20°C but less pathogenic at 0°C, and they were all less virulent and produced fewer spores on apple fruit compared with the parental wild-type isolates regardless of temperature. Similar results were found in osmotic-sensitive isolates of *B. cinerea* resistant to phenylpyrrole (45). However, all pyrimethanil-resistant mutants in *P. expansum* were as pathogenic as the wild-type parental isolates and exhibited equivalent or slightly reduced virulence on apple fruit compared with the wild-type parental isolates.

Taken together, our results indicate that a fitness cost was associated with fludioxonil highly resistant phenotypes of *P. expansum* in both saprophytic and pathogenic phases of the pathogen but not pyrimethanil-resistant phenotypes. Furthermore, pyrimethanil likely possesses a higher risk than fludioxonil in the development of resistance in *P. expansum*.

Resistance stability also is an important factor affecting the build-up of a fungicide resistant population in a fungal pathogen (5). It appears that resistance stability of fungicide-resistant mutants or field isolates is dependent upon the pathogen and fungicide involved. For example, fludioxonil-resistant mutants of *B. cinerea* that are sensitive to osmotic stress exhibit a reduced resistance when grown on a fungicide-free medium (45). Iprodione-resistance of *B. dothidea* significantly declined on pistachio leaves in the absence of fungicide (27). Whereas, *B. cinerea* populations resistant to thiophanate-methyl and vinclozolin remain the same level of resistance after being sub-cultured for 20 generations on PDA or 15 generations on geranium seedlings (43). Similarly, persistence of thiabendazole-resistance of *P. expansum* on apple fruit (34) was reported. In our experiments, the sub-cultured mutants of *P. expansum* from the twentieth generations on a fungicide-free PDA and fourth generations on apple fruit exhibited the same phenotypes as their parental generations. The results indicate that resistance of *P. expansum* to fludioxonil and pyrimethanil was stable regardless of whether the tests were done in vitro or in vivo.

The results on the efficacy of the three postharvest fungicides for controlling blue mold in apple fruit incited by the fungicide-resistant mutants were generally in agreement with that of in vitro sensitivity tests. The efficacy of fludioxonil on control of blue mold incited by pyrimethanil-resistant mutants (PR2 and PR4) was reduced during the 12-week storage at 0°C compared with that at 20°C, but the incidence of blue mold increased to 60 to 84% after the fruit were kept for one more week at room temperature. This was likely due to slow growth of the pathogen and stable residues of fludioxonil on the fruit at 0°C. The efficacy of TBZ against blue mold incited by pyrimethanil-resistant mutant PR2 (derived from TBZ-S wild-type isolate) was reduced at 0°C as compared with that at room temperature, but the decay incidence remained at the similar level after the fruit were stored at room temperature for one more week following cold storage. This may suggest that effectiveness of TBZ in controlling multi-drug resistant phenotypes exhibiting a low level of resistance to TBZ was temperature dependent. Although pyrimethanil did not provide effective control of blue mold incited by pyrimethanil-resistant mutants at room temperature, it reduced blue mold incidence by 53 to 60% at 0°C compared with the nontreated control. The results indicate that if fludioxonil and pyrimethanil are used as a postharvest drench treatment prior to cold storage or as a dip treatment in the packing line, they still have some effects on blue mold caused by triple-resistant isolates of *P. expansum* during cold storage, but after the fruit have been shipped to the market, blue mold incidence will likely increase because fludioxonil and

pyrimethanil lose their efficacy at room temperature for control of blue mold incited by the triple-resistant phenotypes of *P. expansum*.

In conclusion, using a mutagenesis approach we found that pyrimethanil-resistant phenotypes of *P. expansum* also extended resistance to fludioxonil, that pyrimethanil-resistant mutants derived from TBZ-S became resistant to TBZ, and that a fitness cost was associated with fludioxonil highly-resistant phenotypes of *P. expansum* in both saprophytic and pathogenic phases of the pathogen but not pyrimethanil-resistant phenotypes. Our results indicate that pyrimethanil possesses a higher risk than fludioxonil in the development of resistance in *P. expansum*, and that triple resistance to TBZ, fludioxonil, and pyrimethanil could develop and become a practical problem if the pathogen develops resistance to pyrimethanil in the pathogen populations. However, it also is worthwhile to mention that fungicide-resistance phenotypes of laboratory mutants may or may not become established under commercial conditions, depending on the presence of selection pressure, fitness, and competition. Nonetheless, these findings will be instrumental in the development and implementation of relevant strategies for management of fungicide resistance in *P. expansum* and control of blue mold in apple.

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