Sensitivity of *Phytophthora nicotianae* From Tobacco to Fluopicolide, Mandipropamid, and Oxathiapiprolin

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Abstract

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Black shank incited by *Phytophthora nicotianae* is a devastating disease in the production of tobacco. Fungicides have been commonly used for managing the disease; however, there is only a narrow pool of effective fungicides. A few new fungicides became available in recent years, including fluopicolide, mandipropamid, and oxathiapiprolin, which reduced diseases incited by oomycetes under field conditions. Limited information is available regarding sensitivity of *P. nicotianae* isolates to these new fungicides. Research was conducted to determine effects of the three new fungicides on *P. nicotianae* isolates from tobacco in Georgia. Studies with 106 isolates indicated that they did not grow when agar medium was amended with the fungicides at the rate of 1 µg/ml. Twenty isolates were used for in vitro studies to determine sensitivity to the fungicides. Fluopicolide, mandipropamid, and oxathiapiprolin

inhibited mycelial growth of the isolates with mean EC₅₀ values (effective concentrations that provide 50% growth reduction) of 0.09, 0.04, and 0.001 µg/ml, respectively. EC₅₀ values of fluopicolide, mandipropamid, and oxathiapiprolin for inhibiting sporangial formation were 0.15, 0.03, and 0.0002 µg/ml, respectively. EC₅₀ values for suppressing zoospore germination averaged 0.16, 0.04, and 0.002 µg/ml for fluopicolide, mandipropamid, and oxathiapiprolin, respectively. Results from the study indicated that *P. nicotianae* isolates from tobacco in Georgia were sensitive to the fungicides, with lower EC₅₀ for oxathiapiprolin than for fluopicolide and mandipropamid. The information on effectiveness and baseline sensitivity of fungicides on *P. nicotianae* will facilitate monitoring of resistance development in the pathogen population.

Black shank is a soilborne disease that can kill and significantly reduce yield of tobacco (*Nicotiana tabacum* L.) in major tobacco-producing regions of the world (Lucas 1975; Shew and Lucas 1998). The disease is initiated by *Phytophthora nicotianae* (Breda de Haan) that was also named *Phytophthora parasitica* var. *nicotianae* (Erwin and Ribeiro 1996; Shew and Lucas 1998). In the United States, the disease was first identified in the 1910s in Georgia and is now known to be distributed in all major tobacco-producing areas in the country (Csinos and Bertrand 1994; Lucas 1975; Mila and Radcliff 2008; Shew and Lucas 1998). Typical symptoms of infected tobacco plants include stunting, leaf chlorosis, and black lesions on lower stems (Shew and Lucas 1998). The disease is characterized by the pith becoming plate-like disks that turn black or brown. Infected roots may become blackened and plants may wilt and die eventually.

Phytophthora nicotianae produces asexual spores including sporangia and zoospores that play important roles in infection of plants and disease development (Erwin and Ribeiro 1996; Shew and Lucas 1998). Sporangia may be produced on mycelia in infected plant tissues that can germinate to form germ tubes for infection or produce zoospores. Zoospores are attracted by plant tissues, and they may germinate quickly and initiate infection under favorable environmental conditions (Erwin and Ribeiro 1996; Shew and Lucas 1998). Sporangia and zoospores may be dispersed by wind, splashing water, or surface water and contribute significantly to dispersal, infection of new plants, and epidemics of the disease. Hence, measures can be taken to eliminate or reduce production or germination of the spores of the pathogen to reduce the disease.

Strategies recommended for managing black shank include resistant tobacco cultivars and applications of fungicides (Antonopoulos

et al. 2010; Csinos and Minton 1983). Some commercial cultivars of tobacco are resistant to race 0 of *P. nicotianae*, which occurs most commonly. However, race 1 populations of the pathogen that are aggressive on the tobacco cultivars resistant to race 0 have been reported in some tobacco-producing areas, including Georgia (Csinos and Bertrand 1994; Parkunan et al. 2010). Applications of fungicides are generally used by growers to manage the different races of the pathogen. Metalaxyl and its active ingredient mefenoxam have been used in the past decades for managing black shank. Although *P. nicotianae* isolates from tobacco resistant to mefenoxam have not been reported, it is risky to use a single fungicide continuously, which might enhance development of *P. nicotianae* strains resistant to the fungicide (Shew 1985). In recent years, several new fungicides became available that provided excellent suppression of black shank. Among the new fungicides, fluopicolide and mandipropamid reduced black shank significantly (Johnson 2015; Mila and Radcliff 2013), and application of fluopicolide in conjunction with mandipropamid was more effective than mefenoxam in reduction of the disease (Mila and Radcliff 2013). Repeated field experiments conducted in the southeastern United States in recent years indicated that black shank was reduced significantly by oxathiapiprolin and tobacco yield was increased (Bittner and Mila 2016; Ji et al. 2014; Johnson 2015).

It is evident that the new fungicides are effective in suppression of black shank; however, limited information is available about sensitivity of different life stages of the pathogen to the fungicides. This research was conducted to determine effects of fluopicolide, mandipropamid, and oxathiapiprolin on different life stages of *P. nicotianae* isolated in Georgia from tobacco. Information regarding sensitivity of the pathogen to the fungicides will facilitate monitoring potential fungicide resistance development and guide development of more effective programs for managing black shank by alternated applications of fungicides with different modes of action.

Materials and Methods

***Phytophthora nicotianae* isolates.** Tobacco plants showing symptoms of black shank were sampled from 20 commercial fields in six counties of Georgia in 2013 and 2014 (Fig. 1). Lower stem tissues (2 cm long) were soaked in 0.5% NaOCl for 60 s and were triple-rinsed with

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sterile distilled water (SDW). The tissues were air-dried in an aseptic hood, and a piece of stem tissue from the advancing margin of the lesion was placed on a 9-cm-diameter pimaricin-ampicillin-rifampicin-pentachloronitrobenzene-hymexazol (PARPH) agar plate (Parkunan and Ji 2013). After incubation at 25°C for 5 days, fungal-like colonies growing from the stem tissues were transferred to PARPH agar plates by hyphal-tip subculturing. The isolates were then purified by subculturing hyphal tips on 10% V8 plates.

Sterile soil extract (SSE) was prepared as described previously and was used for inducing zoospore production by the isolates (Hu et al. 2007). Mycelial plugs (1 cm diameter) taken from the margin of 7-day-old cultures on V8 plates were transferred to 10 ml of SSE in 6-cm diameter petri dishes. The dishes were incubated under continuous light at 22 to 23°C for 2 days. SSE in the dishes was then replaced by 10 ml of SDW. After chilling (4°C) for 35 min, the dishes were incubated at 22 to 23°C for 25 min. Zoospore suspensions were transferred to sterile 50-ml capacity plastic tubes and were vortexed for 30 s to encyst zoospores. Single zoospore isolates were prepared by the methods reported previously (Yin et al. 2012).

For further confirmation of the identity of the pathogen, genomic DNA was extracted from mycelia of 10-day-old cultures of the isolates, using DNeasy plant mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was conducted in 25- μ l mixtures with 1.5 μ l, each, of the *P. nicotianae*-specific primers Pn1 and Pn2 (10 μ M), 0.2 μ l of *Taq* polymerase (1 unit), 2.5 μ l of buffer (10 $\times$), 0.5 μ l of dNTP (10 mM), and 2 μ l of template DNA (50 ng/ μ l). PCR reaction was conducted and PCR products detected using the conditions reported previously (Meng and Wang 2010).

Pathogenicity assays. Sand was purchased from a local supplier, was moistened with distilled water, and was autoclaved for 45 min at 121°C on two consecutive days. Sand was mixed with potting mix

(Scotts Miracle-Gro Co., Marysville, OH) at 1:2 ratio (vol/vol). Tobacco seeds (cv. K326) were sown in 10-cm-diameter pots (450 ml capacity) containing the soil mix in a greenhouse. Seedlings were transplanted to seedling trays with cells (3.6 $\times$ 3.6 $\times$ 6.0 cm) containing the soil mix 3 weeks after seeding, one plant per cell. Isolates of *P. nicotianae* mentioned above were grown on V8 plates at 25°C for 10 days in darkness. A mycelial plug (0.7 cm) from the advancing margin of the colonies was transferred to a V8 agar plate. Wheat grains were soaked in distilled water for 6 h and were autoclaved (121°C) for 30 min on 2 consecutive days. Autoclaved grains were placed on the V8 agar plates with the mycelial plugs, 50 grains per plate. After incubating at 25°C for 10 days, wheat grains on the plates were used for inoculation. Tobacco seedlings were inoculated one week after transplanting by placing two wheat grains under the soil surface around each plant. Six plants were inoculated with each isolate, and six plants treated with autoclaved wheat grains were used as a control. The plants were kept in the greenhouse at 20°C (night) to 30°C (day). Disease incidence (% diseased plants) was evaluated weekly until 3 weeks after inoculation. The experiment was conducted twice under the same conditions. At the end of the experiments, three diseased plants were taken from each treatment for isolation and identification of causal agents of the disease, as described above.

Inhibition of mycelial growth by the fungicides. A collection of 106 isolates identified to be *P. nicotianae* was used in the study. Technical grade mandipropamid (Sigma-Aldrich, St. Louis), fluopicolide, and oxathiapiprolin (provided by Valent USA, Walnut Creek, CA, and DuPont USA, Wilmington, DE, respectively) was dissolved in acetone to make stock solutions. V8 agar was autoclaved and cooled to 50°C, and the stock solutions were used to amend the medium at final fungicide concentrations of 1 and 5 μ g/ml. A mycelial plug (0.7 cm diameter) from the advancing margin of the colonies of

Fig. 1. Distribution of sampling sites in south Georgia. Sampling sites are marked using star symbol.

10-day-old *P. nicotianae* cultures was transferred to V8 plates amended with the fungicides, three plates for each treatment (i.e., each concentration of the fungicides for each isolate). Three plates amended with acetone only were used as a control for each isolate. After incubation at 25°C for 7 days in the dark, the plates were observed, to determine growth of the cultures.

Twenty isolates, one from each field sampled, were selected to evaluate sensitivity of *P. nicotianae* to the three fungicides. V8 plates were amended with the fungicides to achieve final concentrations of 0.05, 0.1, 0.25, 0.5, and 1.0 µg/ml for fluopicolide, 0.01, 0.05, 0.1, 0.25, 0.5, and 1.0 µg/ml for mandipropamid, and 0.0001, 0.00025, 0.0005, 0.001, 0.005, and 0.01 µg/ml for oxathiapiprolin. Isolates were grown, transferred, and incubated under conditions as described above. Three fungicide-amended plates were used for each treatment as well as three plates amended with acetone only as controls. Colony diameters were measured and effective concentrations that provide 50% growth reduction (EC₅₀) were calculated by fitting linear regression lines of probit-transformed inhibition data against the log₁₀-transformed fungicide concentration (Koné et al. 2009; Stein and Kirk 2003). Two repeated experiments were conducted, and results of the experiments were pooled with mean EC₅₀ and standard errors calculated.

Inhibition of sporangium formation by fungicides. The 20 isolates were incubated at 25°C for 10 days in darkness. Three mycelial plugs (1 cm diameter) from the advancing edge of the cultures were placed in a petri dish (6 cm) containing 10 ml of SSE amended with the fungicides. The fungicides were prepared as mentioned above and final concentrations of the fungicides used were 0.1, 0.25, 0.5, 1.0, 2.5, and 5.0 µg/ml for fluopicolide, 0.01, 0.025, 0.05, 0.1, 0.5, and 1.0 µg/ml for mandipropamid, and 0.0001, 0.00025, 0.0005, 0.001, 0.005, and 0.01 µg/ml for oxathiapiprolin. Three petri dishes were used for each treatment, and three dishes with SDW amended with acetone only were used as a control. After incubating at 25°C for 48 h under artificial fluorescent light, the mycelial plugs were stained with acid fuchsin (Matheron and Porchas 2000). The agar plugs were observed under a stereo microscope (100×) and numbers of sporangia under three microscopic fields were counted for each plug. Two repeated experiments were conducted, and results from the experiments were combined to calculate EC₅₀ values.

Inhibition of zoospore germination by fungicides. Preparation of zoospore suspensions of the 20 isolates was as described above by soaking agar plugs (1 cm) in SSE and chilling at 4°C to induce zoospore release. Zoospore suspension (100 µl, 2 × 10³ spores per milliliter) was spread plated on a fungicide-amended V8 plate. Final fungicide concentrations were 0.1, 0.25, 0.5, 1.0, and 5.0 µg/ml for fluopicolide, 0.01, 0.025, 0.05, 0.1, and 0.5 µg/ml for mandipropamid, and 0.0001, 0.00025, 0.0005, 0.001, 0.005, and 0.01 µg/ml for oxathiapiprolin. Three plates were used for each treatment and three plates amended with acetone only were used as a control. The number of zoospores that germinated per 100 spores examined on each plate was recorded after incubation for 5 to 6 h at 25°C. Two repeated experiments were conducted and results of the experiments were pooled to calculate mean EC₅₀ and standard errors.

Results

Phytophthora nicotianae isolates. The 106 isolates were identified to be *P. nicotianae*. PCR amplification using primers Pn1 and Pn2 yielded a 389-bp product from all the isolates. When the isolates were used for inoculation of tobacco seedlings in the greenhouse, all the isolates caused disease with 83.3 to 100% of the infected plants 3 weeks after inoculation.

Inhibition of mycelial growth by fungicides. All 106 isolates of *P. nicotianae* were sensitive to fluopicolide, mandipropamid, and oxathiapiprolin, since none of them could grow on V8 agar amended with the compounds at 1 or 5 µg/ml. When tested at lower concentrations of the fungicides with 20 selected isolates, they were more sensitive to oxathiapiprolin than to fluopicolide or mandipropamid. Fluopicolide completely inhibited mycelial growth at 0.5 to 1.0 µg/ml, with EC₅₀ values ranging from 0.06 to 0.15 µg/ml (Fig. 2). Mandipropamid inhibited mycelial growth completely at 0.25 to 0.5 µg/ml, with

EC₅₀ values ranging from 0.008 to 0.07 µg/ml (Fig. 3). Oxathiapiprolin inhibited mycelial growth completely at 0.005 to 0.01 µg/ml. EC₅₀ values of oxathiapiprolin for suppressing mycelial growth ranged from 0.0002 to 0.0018 µg/ml (Fig. 4).

Inhibition of sporangium formation. Fluopicolide completely inhibited sporangial formation of the isolates at 1.0 to 2.5 µg/ml, with EC₅₀ values ranging from 0.07 to 0.4 µg/ml (Fig. 2). Mandipropamid inhibited mycelial growth completely at 0.1 to 0.5 µg/ml, with EC₅₀ values ranging from 0.005 to 0.06 µg/ml (Fig. 3). Oxathiapiprolin completely inhibited sporangial production at 0.001 to 0.01 µg/ml. EC₅₀ values for oxathiapiprolin to inhibit sporangial production ranged from 0.0001 to 0.0005 µg/ml (Fig. 4).

Inhibition of zoospore germination. EC₅₀ values of fluopicolide and mandipropamid for inhibition of germination of zoospores ranged from 0.09 to 0.2 and 0.01 to 0.08 µg/ml, respectively (Fig. 2 and 3). EC₅₀ values for oxathiapiprolin to inhibit germination of zoospores ranged from 0.0005 to 0.004 µg/ml (Fig. 4).

Discussion

Black shank of tobacco remains a serious disease causing significant plant death and yield reduction. Selected use of effective fungicides is important for developing integrated programs for managing the disease. In addition to mefenoxam and metalaxyl, which have been used for black shank management in the past decades, several new fungicides have become available in recent years for black shank and other diseases caused by oomycetes, including fluopicolide, mandipropamid, and oxathiapiprolin. Little information is available regarding sensitivity of *P. nicotianae* from tobacco to these newer fungicides. In this study, effects of fluopicolide, mandipropamid, and oxathiapiprolin on growth and sporulation of *P. nicotianae* isolates from tobacco in Georgia were determined, which indicated that these compounds effectively inhibited mycelial growth, sporangial production, and germination of zoospores of the isolates. This study provides useful information for understanding mechanisms of the fungicides in reduction of black shank on tobacco.

The Fungicide Resistance Action Committee (FRAC) classifies fungicides into different groups according to their mode of action. Fluopicolide (pyridinylmethyl-benzamide, FRAC group 43) acts by delocalization of spectrin-like proteins (Krämer and Schirmer 2008). Studies conducted in recent years indicated that applications of fluopicolide reduced black shank on tobacco significantly under field conditions in the United States (Johnson 2015; Mila and Radcliff 2013). There are no refereed publications regarding effects of fluopicolide on different life stages of *P. nicotianae* from tobacco. Results in the present study showed that fluopicolide was effective in suppressing various asexual life stages of the pathogen, with mean EC₅₀ values for mycelial growth, sporangial production, and zoospore germination at 0.09, 0.15, and 0.16 µg/ml, respectively.

Mandipropamid (mandelic acid amide, FRAC group 40) affects cellulose synthase (Krämer and Schirmer 2008). Application of mandipropamid in combination with mefenoxam was more effective in reducing incidence of black shank on tobacco compared with mefenoxam used alone (Mila and Radcliff 2013). Effects of mandipropamid on growth and sporulation of *P. nicotianae* isolates from tobacco in the United States are unknown. A study with *P. nicotianae* isolates from tobacco in China indicated that mandipropamid was active in inhibiting sporangium production, mycelium growth, and zoospore germination of the isolates, with EC₅₀ values of 0.009, 0.011, and 0.014 µg/ml, respectively (Wang et al. 2013b). In the present study, mandipropamid suppressed sporangium production, mycelium growth, and zoospore germination of the isolates with average EC₅₀ values of 0.03, 0.04, and 0.04 µg/ml, respectively, which were three to four times higher than those for the isolates from China. Average EC₅₀ values of mandipropamid for inhibiting mycelium growth, sporangial production, and zoospore germination of *P. nicotianae* isolates from Georgia were 2.6 to 4.3 times lower compared with fluopicolide.

Oxathiapiprolin is a piperidinyl-thiazole isoxazoline (FRAC group U15) and its mode of action is inhibiting oxysterol-binding proteins (FRAC 2016; Tally et al. 2015). Application of oxathiapiprolin

reduced black shank on tobacco significantly under field conditions (Bittner and Mila 2016; Ji et al. 2014; Johnson 2015). The effect of oxathiapiprolin on growth and sporulation of four *P. nicotianae* isolates in North Carolina was studied (Bittner and Mila 2016). EC_{50} values for sporangium production, mycelium growth, and germination of zoospores averaged 0.0007, 0.004, and 0.004 $\mu\text{g/ml}$,

respectively. These are similar to ranges of the compound on isolates of *P. nicotianae* in Georgia, with average EC_{50} values of 0.0002, 0.001, and 0.0015 $\mu\text{g/ml}$ for sporangium production, mycelium growth, and germination of zoospores, respectively. Oxathiapiprolin inhibited the isolates in Georgia at lower concentrations than fluopicolide and mandipropamid, i.e., 96 to 610 times lower than

Fig. 2. Inhibition of mycelial growth, sporangium production, and zoospore germination of *Phytophthora nicotianae* isolates by fluopicolide. EC_{50} = concentrations of fluopicolide that inhibited mycelial growth, sporangial formation, or zoospore germination by 50%. Error bars are standard errors of the means of two experiments.

fluopicolide and 24 to 143 times lower than mandipropamid in suppressing mycelial growth, sporangial production, and zoospore germination. One of the *P. nicotianae* isolates from tobacco tested in North Carolina grew on agar medium with 1 $\mu\text{g}/\text{ml}$ of oxathiapiprolin (Bittner and Mila 2016); however, none of the isolates in Georgia could do so at 1 or 5 $\mu\text{g}/\text{ml}$.

Mefenoxam has been the commonly used fungicide for managing black shank on tobacco (Antonopoulos et al. 2010; Csinos and Minton 1983; Ji et al. 2014). Studies indicated that *P. nicotianae* isolates from tobacco were sensitive to mefenoxam (Csinos and Bertrand 1994; Parkunan et al. 2010; Shew 1985; van Jaarsveld et al. 2002; Wang et al. 2013a). Although *P. nicotianae* isolates from tobacco with

Fig. 3. Inhibition of mycelial growth, sporangium production, and zoospore germination of *Phytophthora nicotianae* isolates by mandipropamid. EC₅₀ = concentrations of mandipropamid that inhibited mycelial growth, sporangial formation, or zoospore germination by 50%. Error bars are standard errors of the means of two experiments.

resistance to mefenoxam have not been documented, applications of different fungicides in rotation are recommended to reduce the risk for the pathogen to potentially develop fungicide resistance. So far studies on sensitivity of *P. nicotianae* from tobacco to mefenoxam have focused on suppression of mycelial growth by the compound. Results of the present study showed that oxathiapiprolin,

mandipropamid, and fluopicolide inhibited mycelial growth of *P. nicotianae* isolates at lower concentrations than mefenoxam.

In summary, results from this study indicated that oxathiapiprolin, mandipropamid, and fluopicolide were effective in suppressing mycelium growth, sporangial production, and zoospore germination of *P. nicotianae* from tobacco in Georgia. None of the *P. nicotianae*

Fig. 4. Inhibition of mycelial growth, sporangium production, and zoospore germination of *Phytophthora nicotianae* isolates by oxathiapiprolin. EC₅₀ = concentrations of oxathiapiprolin that inhibited mycelial growth, sporangial formation, or zoospore germination by 50%. Error bars are standard errors of the means of two experiments.

isolates evaluated were resistant to the fungicides, suggesting that they could be incorporated in integrated programs for managing black shank. Information on resistance of *Phytophthora* spp. to fluopicolide, mandipropamid, and oxathiapiprolin is limited. The potential of resistance development to fluopicolide is unknown; however, risk of resistance to oxathiapiprolin is assumed to be medium to high, and the risk is low to medium for cross resistance to mandipropamid (FRAC 2016). Hence, resistance management is needed and future research may be conducted to monitor potential resistance development in the pathogen populations to the fungicides. Results from the present study provide valuable information about sensitivity of *P. nicotianae* to the three fungicides, which can be used as a benchmark for determining fungicide resistance development of the pathogen in future studies.

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