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Assessing the resistance potential of *Phytophthora nicotianae*, the causal agent of black shank of tobacco, to oxathiopropalin with laboratory mutants

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ABSTRACT

Black shank, caused by *Phytophthora nicotianae* van Breda de Haan, is a devastating disease of tobacco (*Nicotiana tabacum* L.) throughout the world. The newly-discovered fungicide, oxathiapiprolin has shown efficacy against *P. nicotianae*, but the potential for fungicide resistance is unknown. Production of oxathiapiprolin resistant *P. nicotianae* isolates was attempted through mass selection of zoospores, UV light mutagenesis, and mycelial adaptation through repeated culturing on oxathiapiprolin-amended medium. No resistant isolates were detected from mass zoospore selection. UV light mutagenesis of mycelium generated two isolates, EdgeB7-M1 and YadA28-M1, with stable mycelial and sporangial resistance to oxathiapiprolin. Isolate EdgeB7-M1 remained pathogenic to tobacco, while YadA28-M1 was nonpathogenic. For mycelial adaptation, 48 colonies, 12 replicate colonies from four isolates, were transferred 15 times on oxathiapiprolin-amended medium. Twelve of the 48 colonies had a significant increase in mycelial insensitivity to oxathiapiprolin; however, mycelial adaptation was not stable. Mycelial growth and sporangia production of the adapted isolates was significantly less compared to the wild-type whereas only one remained pathogenic to tobacco. In summary, the generation of oxathiapiprolin resistant *P. nicotianae* isolates was feasible. Constant exposure to oxathiapiprolin increased insensitivity, however fitness costs associated with insensitivity reduced the probability of isolate reproduction and survival.

1. Introduction

Black shank, caused by the devastating soil-inhabiting oomycete *Phytophthora nicotianae* van Breda de Hann (=*P. nicotianae* var. *nicotianae*), is a major disease of tobacco (*Nicotiana tabacum* L.) throughout the world and results in millions of dollars in annual loses each year (Lucas, 1975; Mila and Radcliff, 2014; Shoemaker and Shew, 1999). In North Carolina alone, tobacco losses from black shank can total over $10 million annually (Mila and Radcliff, 2014). Black shank was first introduced to the United States in Georgia in 1915. The pathogen eventually spread to North Carolina by 1931 possibly from out-of-state tobacco transplants infected with the pathogen (Lucas, 1975; Shew and Lucas, 1991). Presently, there are three physiological races (0, 1, and 3) of *P. nicotianae* on tobacco in North Carolina, with race 0 and 1 the most frequently found across all tobacco growing areas of the state (Gallup and Shew, 2010). Race 1 of *P. nicotianae* has the ability to infect and cause disease on tobacco cultivars that have the single resistance gene, *Ph* gene, which confirms immunity again race 0 (Mila and Radcliff, 2014).

Management of the black shank disease relies on an integrated approach that includes use of resistant tobacco cultivars, rotation with non-host crops, nematode control, and the use of fungicides (Lucas, 1975; Mila and Radcliff, 2014; Shew and Lucas, 1991). The prevalence of race 1 of *P. nicotianae* increased significantly since the release of cultivars with the *Ph* resistance gene. As a result, fungicides have become an important tool for black shank management in the USA. Currently, there are three systemic fungicides, that is mefenoxam, fluopicolide, and oxathiapiprolin, registered for use on tobacco for black shank control in the USA.

Mefenoxam, released to the market in 1996, is the more active isomer of the fungicide metalaxyl, which was first introduced in

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1977 (Erwin and Ribeiro, 1996; Kannwischer and Mitchell, 1978). Metalaxyl and mefenoxam are phenylamides, with activity against oomycetes, specifically against mycelial growth and sporangia production (Hu et al., 2008; Staub and Young, 1980). The mode of action (MOA) of mefenoxam and metalaxyl is the inhibition of ribosomal RNA synthesis, caused by an interaction of the fungicide with RNA polymerase I (Davidse et al., 1983). Overall, both metalaxyl and mefenoxam have proved to be effective fungicides against the black shank disease since their introduction (Antonopoulos et al., 2010; Csinos and Minton, 1983; Kannwischer and Mitchell, 1978; Reilly, 1980; Staub and Young, 1980). Resistance to these fungicides has been well documented in multiple pathosystems. Metalaxyl resistance was first observed by Davidse et al. (1981) in *P. infestans* strains from a Dutch potato field that was subjected to multiple applications of the fungicide. Shew (1985) demonstrated that a decrease in mycelial sensitivity of *P. nicotianae* to metalaxyl occurred after repeated applications of the fungicide in tobacco fields. Despite the development of phenylamide resistance in multiple oomycetes, including *P. nicotianae* isolates from non-tobacco hosts, metalaxyl and mefenoxam have remained efficacious for use against black shank since their introduction, with no fungicide resistance reported.

Fluopicolide, a systemic fungicide belonging to the benzamides, has shown efficacy against several oomycetes, including *P. nicotianae*. The MOA is different from the phenylamide fungicides, and has been suggested to cause the perturbation of spectrin-like proteins (Toquin et al., 2006). In *P. capsici*, fluopicolide has shown efficacy against all growth stages, including mycelial growth, sporangia production, and zoospore germination and motility (Jackson et al., 2010). The risk of resistance to fluopicolide has been reported in a number of systems. Lu et al. (2011) demonstrated that fluopicolide-resistant mutants of *P. capsici* could be produced with mass spore selection, a method that simulates the resistance naturally occurring in a field. All fluopicolide resistant *P. capsici* isolates had similar fitness levels to the wild type isolates, suggesting that no fitness cost was associated with the resistance mutation (Lu et al., 2011) Fluopicolide was registered for black shank control in the USA in 2015.

Oxathiapiprolin, released for use in the USA in 2016, is an example of the first chemistry of the new piperidinyl thiazole isoxazoline class of fungicides that has shown efficacy against multiple oomycete pathogens, including *P. nicotianae* (Bittner and Mila, 2016; Ji et al., 2014; Pasteris et al., 2016). Bittner and Mila (2016) found that oxathiapiprolin was able to inhibit the mycelial growth, sporangia production, zoospore germination, and motility of zoospores of *P. nicotianae* from tobacco. Similar results were also reported with *P. capsici* from pepper (Ji and Csinos, 2015). In both studies, sporangia production was the most sensitive life-cycle stage (Bittner and Mila, 2016; Ji and Csinos, 2015). Pasteris et al. (2016) found that oxathiapiprolin binds strongly to the oxysterol binding protein (OSBP). This is a novel site of action different from all other previously discovered fungicides. The function of OSBP in oomycetes has yet to be determined. Isolates of *P. capsici* resistant to oxathiapiprolin were produced by irradiating zoospores of *P. capsici* with ultraviolet light and then selecting on oxathiapiprolin-amended media (Pasteris et al., 2016). Oxathiapiprolin-resistant isolates of *P. capsici* then were sequenced to determine mutations within their genome. Pasteris et al. (2016) found that all resistant mutants had a single nucleotide polymorphism in the OSBP gene. Although the possible mechanism of resistance to oxathiapiprolin in *P. capsici* was described, resistant isolates were not further characterized to determine the possible fitness costs of resistance (Pasteris et al., 2016). Miao et al. (2016) also described oxathiapiprolin resistant mutants produced in *P. capsici*. Fitness of the mutants was found to variable when compared to the parent isolates. Some mutants exhibited drastic fitness costs, such as the reduction of sporangia production, while the fitness of other mutants was equal to the parent isolate. Currently, it is unknown if *P. nicotianae* isolates from tobacco are at risk for resistance to oxathiapiprolin.

The objectives of this study were to (i) produce isolates of *P. nicotianae* resistant to oxathiapiprolin through mass zoospore selection, UV light mutagenesis, and mycelial adaptation, and determine (ii) stability, (iii) fitness costs, and (iv) pathogenicity to flue-cured tobacco of produced resistant isolates.

2. Materials and methods

2.1. Isolates

Two race 0 (EdgeB7 and EdgeA12) and two race 1 (YadA3 and YadA28) isolates of *P. nicotianae* from tobacco were selected from the A. L. Mila collection, North Carolina State University, USA. All isolates were sensitive to oxathiapiprolin as previously determined (Bittner and Mila, 2016).

2.2. Fungicide

Oxathiapiprolin (DPX-QGU42, DuPont™ Zorvec™ Enicade™) formulated as DPX-QGU42 (100 g a.i./L, OD), was provided by DuPont, Wilmington, DE. Stock solutions of oxathiapiprolin were prepared in sterilized deionized water and stored at 4 °C in the dark to preserve fungicidal activity. For amended medium, stock solutions of the fungicide were added to the medium when it had cooled to roughly 55 °C. Media amended with oxathiapiprolin was stored in the dark at 4 °C.

2.3. Production of resistant isolates through mass selection from zoospores

P. nicotianae isolates EdgeB7 and YadA3, the two of the four isolates with the most prolific sporangia production, were selected. Zoospores were produced by the method described by McCorkle et al. (2013). The concentration of zoospore suspensions was adjusted by dilution to 1.5×10^5 mL with the aid of a hemacytometer. Zoospores were screened for resistance by plating 0.5 mL of the zoospore suspension onto V8 agar (V8A; V8 juice, 200 mL; $CaCO_3$, 3 g; agar, 17 g; and deionized water, 800 mL) in Petri plates (90 mm × 15 mm) amended with oxathiapiprolin at 0.2 μg a.i.mL^{-1}. This rate was determined to be ten times the minimum inhibition rate for mycelial growth of *P. nicotianae* (data not shown). A 0.5 mL volume of the zoospore suspensions, at 1.5–2 x 10^5 mL^{-1} concentration, was spread evenly over each Petri dish with a sterile L-shaped glass rod. A total of 46.2×10^6 motile zoospores were tested. Non-amended Petri plates of V8A were used as a control. After the addition of zoospore suspensions, Petri plates were incubated in the dark at 28 °C for 14 days. After the 14-day incubation period, Petri plates were examined for *P. nicotianae* colony formation.

2.4. Production of resistant isolates through UV mutagenesis

P. nicotianae isolates EdgeA12, EdgeB7, YadA3, and YadA28 were grown on non-amended V8A at 28 °C until the medium in the Petri plate (90 mm × 15 mm) became completely colonized. Ultraviolet (UV) light mutagenesis was performed under a portable UV lamp (Mineralight Lamp; San Cabriel, CA) at a wavelength of 254 nm. A total of eleven Petri plates of each isolate grown on non-amended V8A were exposed to UV light without lids at a 10 cm distance for 4 (five Petri plates) and 5 (six Petri plates) min. After irradiation, cultures were immediately transferred to an incubator at 28 °C for 1 h in the dark to minimize potential photorepair. Forty agar disks