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Metabolic effects of azoxystrobin and kresoxim-methyl against *Fusarium kyushuense* examined using the Biolog FF MicroPlate

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ABSTRACT

Azoxystrobin and kresoxim-methyl are strobilurin fungicides, and are effective in controlling many plant diseases, including Fusarium wilt. The mode of action of this kind of chemical is inhibition of respiration. This research investigated the sensitivities of *Fusarium kyushuense* to azoxystrobin and kresoxim-methyl, and to the alternative oxidase inhibitor salicylhydroxamic acid (SHAM). The Biolog FF MicroPlate is designed to examine substrate utilization and metabolic profiling of micro-organisms, and was used here to study the activity of azoxystrobin, kresoxim-methyl and SHAM against *F. kyushuense*. Results presented that azoxystrobin and kresoxim-methyl strongly inhibited conidial germination and mycelial growth of *F. kyushuense*, with EC_{50} values of 1.60 and 1.79 μg ml^{-1}, and 6.25 and 11.43 μg ml^{-1}, respectively; while not for SHAM. In the absence of fungicide, *F. kyushuense* was able to metabolize 91.6% of the tested carbon substrates, including 69 effectively and 18 moderately. SHAM did not inhibit carbon substrate utilization. Under the selective pressure of azoxystrobin and kresoxim-methyl during mycelial growth (up to 100 μg ml^{-1}) and conidial germination (up to 10 μg ml^{-1}), *F. kyushuense* was unable to metabolize many substrates in the Biolog FF MicroPlate; while especially for carbon substrates in glycolysis and tricarboxylic acid cycle, with notable exceptions such as β-hydroxybutyric acid, γ-hydroxybutyric acid, α-ketoglutaric acid, α-D-glucose-1-phosphate, D-saccharic acid and succinic acid in the mycelial growth stage, and β-hydroxybutyric acid, γ-hydroxybutyric acid, α-ketoglutaric acid, tween-80, arbutin, dextrin, glycerol and glycogen in the conidial germination stage. This is a new finding for some effect of azoxystrobin and kresoxim-methyl on carbon substrate utilization related to glycolysis and tricarboxylic acid cycle and other carbons, and may lead to future applications of Biolog FF MicroPlate for metabolic effects of other fungicides and other fungi, as well as providing a carbon metabolic fingerprint of *F. kyushuense* that could be useful for identification.

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1. Introduction

Tobacco (*Nicotiana tabacum* L.) is a leafy, annual, solanaceous plant grown commercially for its leaves. China is the biggest single tobacco market and accounts for more than 40% of the global tobacco consumption [1]. During tobacco seedling development period, various fungal pathogens attack seedlings in the greenhouse. In the last five years, Fusarium wilt of tobacco, caused by *Fusarium kyushuense* O'Donnell & T. Aoki, was frequently observed in some regions of Guizhou province in southwest China [2,3]. The pathogen attacks seedlings leading to symptoms of severe wilting, chlorosis and stunting with poorly developed root systems, and eventual death. Control of the disease is based on an integration of several cultural methods with the use of fungicides. For *Fusarium* diseases caused by *Fusarium oxysporum* f. sp. *nicotianae* in China, multisite inhibitors, such as chlorothalonil and mancozeb, and the target site-specific fungicides such as carbendazim have been used to control this disease [4–6]. Multisite inhibitors may be effective when applied in protective fashion [7]. Carbendazim was a major fungicide for Fusarium disease management twenty years ago, but carbendazim-resistant strains developed causing all benzimidazoles to lose their efficacy [8–10].

Recently, the strobilurin fungicide azoxystrobin and kresoxim-methyl have been registered in China and other countries to control Fusarium diseases, including wheat scab, potato Fusarium dry rot, and tomato Fusarium wilt [11–13]. They have also been used to manage many other disease management on tobacco in the USA and Germany [14–16]. Few studies have been published to describe the sensitivity to azoxystrobin and kresoxim-methyl by *F. kyushuense*, and because of the relatively high cost of these chemicals locally, they have not been used for tobacco disease management in China. Azoxystrobin, kresoxim-methyl, pyraclostrobin, trifloxystrobin and famoxadone [17–20] are Quinone outside inhibitor (QoI) fungicides (Fungicide Resistance Action Committee

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[FRAC] group 11) also known as strobilurins. Preliminary studies revealed that azoxystrobin and kresoxim-methyl inhibit mitochondrial respiration by blocking electron transfer at the cytochrome bc1 complex [21]. They have high activity against spore germination and less activity against mycelial growth because hyphae may make use of the terminal alternative oxidase (AOX) that bypasses the blockage site [22,23]. Salicylhydroxamic acid is the special inhibitor for the terminal alternative oxidase. The metabolic effects of azoxystrobin and kresoxim-methyl have not yet been fully elucidated, and more research is needed to understand the diverse secondary effects of strobilurins on fungal plant pathogens.

Recently, the Biolog FF MicroPlate was introduced by Biolog Company for characterizing filamentous fungi (FF MicroPlate™ Instruction, Biolog, Hayward, CA, USA). The MicroPlate uses 95 biochemical tests to profile substrate utilization and phenotypic profiling of many microorganisms with each well containing substrates that change color with metabolic activity (Fig. 1) [24,25]. There have been few previous reports of its use for investigating mode of action of fungicides [26]. Therefore, the objectives of this study were: (i) to document the sensitivities of *F. kyushuense* to azoxystrobin, kresoxim-methyl and SHAM and (ii) to evaluate the phenotypic profiling of *F. kyushuense* under pressures of azoxystrobin, kresoxim-methyl and SHAM. The outcome of this study will provide useful information on the effects and mode of action of azoxystrobin and kresoxim-methyl.

2. Materials and methods

2.1. Pathogen, media and chemical preparation

A strain of *F. kyushuense* with wild-type strobilurin sensitivity and pathogenicity to tobacco [2] was collected in 2013 from an infected tobacco seedling in a commercial field in Guizhou province, China. A monoconidial isolate was obtained and used for tests. The isolate was grown and maintained on a lima bean agar medium (LBA, 60.0 g l^{-1} lima beans boiled for 1 h and strained, 16.0 g l^{-1} agar), in a controlled climate cabinet at 25 °C in the dark. For conidial production, agar plugs were removed from the edge of an actively growing culture and placed on LBA plates. After 7 days, conidia were washed off with distilled water, and filtered through a double-layer of sterile cheesecloth (Grade #40: 24 × 20 threads per inch) to remove mycelial fragments. The resulting conidial suspension was quantified with a hemacytometer and adjusted to 1×10^5 spores/ml for subsequent use. For long-term storage, 5-mm agar plugs from the leading edge of individual colonies were transferred into several sterile 1.5-ml microcentrifuge tubes containing 1 ml of 30% sterile glycerol, and tubes were stored at −20 °C in darkness.

Stock fungicide solutions were prepared by dissolving technical grade azoxystrobin (a.i. 93%; Syngenta China Co., Ltd, Shanghai, China), kresoxim-methyl (a.i. 96%; BASF China Co., Ltd, Shanghai, China) and SHAM (a.i. 99.9%; Sigma-Aldrich China Co., Ltd, Shanghai, China) in methanol. Solutions were diluted as required and stored at 4 °C in the dark. The methanol concentration never exceeded 1% of the testing solution. This concentration of methanol was not observed to affect different life stages of *F. kyushuense* (data not shown). Controls always contained the same methanol concentration as the test samples in the experiments. Fungicides were added to LBA after autoclaving when the agar had cooled to approximately 50 °C. Filamentous Fungi inoculating fluid (FF-IF, catalog # 72106) (containing 2.5 g l^{-1} Phytagel and 0.3 g l^{-1} Tween 40) and FF MicroPlate test panels (catalog # 1006) containing 95 different carbon sources were purchased from Biolog Inc. (Hayward, CA, USA) and stored at 4 °C until needed.

A1 Water	A2 Tween 80	A3 N-Acetyl-D-Galactosamine	A4 N-Acetyl-D-Glucosamine	A5 N-Acetyl-β-D-Mannosamine	A6 Adonitol	A7 Amygdalin	A8 D-Arabinose	A9 L-Arabinose	A10 D-Arabitol	A11 Arbutin	A12 D-Cellobiose
B1 α-Cyclodextrin	B2 β-Cyclodextrin	B3 Dextrin	B4 i-Erythritol	B5 D-Fructose	B6 L-Fucose	B7 D-Galactose	B8 D-Galacturonic Acid	B9 Gentiobiose	B10 D-Gluconic Acid	B11 D-Glucosamine	B12 α-D-Glucose
C1 α-D-Glucose-1-Phosphate	C2 Glucuronamide	C3 D-Glucuronic Acid	C4 Glycerol	C5 Glycogen	C6 m-Inositol	C7 2-Keto-D-Gluconic Acid	C8 α-D-Lactose	C9 Lactulose	C10 Maltitol	C11 Maltose	C12 Maltotriose
D1 D-Mannitol	D2 D-Mannose	D3 D-Melezitose	D4 D-Melibiose	D5 α-Methyl-D-Galactoside	D6 β-Methyl-D-Galactoside	D7 α-Methyl-D-Glucoside	D8 β-Methyl-D-Glucoside	D9 Palatinose	D10 D-Psicose	D11 D-Raffinose	D12 L-Rhamnose
E1 D-Ribose	E2 Salicin	E3 Sedoheptulosan	E4 D-Sorbitol	E5 L-Sorbose	E6 Stachyose	E7 Sucrose	E8 D-Tagatose	E9 D-Trehalose	E10 Turanose	E11 Xylitol	E12 D-Xylose
F1 γ-Aminobutyric Acid	F2 Bromosuccinic Acid	F3 Fumaric Acid	F4 β-Hydroxybutyric Acid	F5 γ-Hydroxybutyric Acid	F6 p-Hydroxy-phenylacetic Acid	F7 α-Ketoglutaric Acid	F8 D-Lactic Acid Methyl Ester	F9 L-Lactic Acid	F10 D-Malic Acid	F11 L-Malic Acid	F12 Quinic Acid
G1 D-Saccharic Acid	G2 Sebacic Acid	G3 Succinamic Acid	G4 Succinic Acid	G5 Succinic Acid Mono-Methyl Ester	G6 N-Acetyl-L-Glutamic Acid	G7 L-Alaninamide	G8 L-Alanine	G9 L-Alanyl-Glycine	G10 L-Asparagine	G11 L-Aspartic Acid	G12 L-Glutamic Acid
H1 Glycyl-L-Glutamic Acid	H2 L-Ornithine	H3 L-Phenylalanine	H4 L-Proline	H5 L-Pyroglutamic Acid	H6 L-Serine	H7 L-Threonine	H8 2-Aminoethanol	H9 Putrescine	H10 Adenosine	H11 Uridine	H12 Adenosine-5'-Monophosphate

Fig. 1. Layout of assays in the Biolog FF MicroPlate.

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