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Differences between the succinate dehydrogenase sequences of isopyrazam sensitive *Zymoseptoria tritici* and insensitive *Fusarium graminearum* strains

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ABSTRACT

Forty-one *Zymoseptoria tritici* strains isolated in Luxembourg between 2009 and 2010 were highly sensitive towards the new succinate dehydrogenase inhibitor (SDHI) isopyrazam, with concentrations inhibiting fungal growth by 50% (EC_{50}) ranging from 0.0281 to 4.53 μM, whereas 41 *Fusarium graminearum* strains isolated in Europe and Northern America between 1969 and 2009 were insensitive with the average rate of inhibition converging towards 28% with increasing isopyrazam concentration. Seven isolates of both species covering the range of isopyrazam sensitivities observed in the present study were selected for the sequencing of the subunits B, C and D of the succinate dehydrogenase (sdh) gene. Predicted sdh amino acid sequences of subunits B, C and D were identical among *F. graminearum* strains. By comparing with fungal strains where resistance towards SDHIs was previously reported, three variations were unique to *F. graminearum*; B-D130N located in the iron–sulfur cluster [2Fe–2S], B-A275T located in the [3Fe–4S] cluster and an additional S at amino acid position 83–84 of sdhC, probably modifying structurally the ubiquinone binding site and therefore the biological activity of the fungicide. No variation was found among the *Z. tritici* strains in subunits B and D. Two variations were observed within the subunit C sequences of *Z. tritici* strains: C-N33T and C-N34T. The difference in EC_{50} values between *Z. tritici* strains with the NN and TT configuration was non-significant at $P = 0.289$. Two outliers in the *Z. tritici* group with significantly higher EC_{50} values that were not related to mutations in the sdhB, sdhC, or sdhD were detected. The role of isopyrazam for the control of *F. graminearum* and *Z. tritici* in Luxembourg is discussed.

1. Introduction

Fusarium graminearum Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch) is the main causal agent of FHB (Fusarium Head Blight). The pathogen infects cereals during anthesis if humidity levels for fungal growth are sufficiently high [1] and alters the filling of the kernels, leading to reductions in yield, but also in quality. *F. graminearum* produces mycotoxins that are harmful to humans once they are ingested; therefore, controlling this disease is essential. *F. graminearum* strains are classified according to chemotypes that are named after the mycotoxin they primarily produce. Three chemotypes are currently distinguished in *F. graminearum*, namely the 3-acetylated deoxynivalenol (3-ADON) type, the 15-acetylated deoxynivalenol (15-ADON) type, and the nivalenol (NIV) type. Cultural techniques such as removing plant residue of the previous crop, avoiding maize as a previous crop and using resistant varieties are useful for controlling FHB [2,3], but when weather conditions are favorable for infections, these actions are not sufficient to prevent epidemic outbreaks. In this case, fungicides are the last resort to prevent critical mycotoxin contamination of the grain [4,5]. While the efficacy of azole fungicides on *F. graminearum* populations seems hardly diminished so far [6], efficacy of strobilurin fungicides was reported to be low [7]. Recently, resistance towards the strobilurin trifloxystrobin has been characterized in *F. graminearum* as being natural, and not acquired [8]. Strobilurins target the cytochrome bc1 (also called complex III) in the mitochondrial respiration chain. Rotating fungicides from different mode of action groups in spray regimes contributes to avoiding fungicide resistance [9]. Since chemical control of FHB pathogens almost solely depends on azoles at present, this option hardly exists. Therefore, it is highly desirable to have fungicides available with efficacy against FHB pathogens, but with another mode of action than azoles.

Zymoseptoria tritici (Desm.) Quaedvlieg and Crous, 2011 (formerly *Septoria tritici* Rob. ex Desm.) is the most important cereal pathogen in Northern Europe [10]. Recently, the new genus *Zymoseptoria* was erected to accommodate *Septoria*-like pathogens of cereal hosts [11]. It is the causal agent of Septoria Leaf Blotch (SLB), a foliar disease that causes chlorotic spots on leaves, leading to drastic reductions in yield [12]. Control of *Z. tritici* in the field is

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mostly achieved by using resistant cultivars and applying fungicides, the efficiency of cultural techniques being low [13]. Breeding for *Z. tritici* resistance did not allow producing a completely resistant cultivar so far, mostly because of the lack of efficient resistance genes [14]. Currently, yields are higher for susceptible cultivars sprayed with fungicides than for cultivars with good resistance, an observation that promotes chemical control as the economically most efficient way to harness SLB [15]. Due to the spread of fungal resistance towards strobilurins in Europe [16], chemical control of *Z. tritici* is largely achieved by using demethylase inhibitors (DMIs). Sensitivity towards DMIs is characterized by a shifting of EC_{50} values over time depending partly on the availability and efficacy of other mode of action groups [17]. In 2009, observed EC_{50} values slightly increased, but this trend slowed down in 2010 [18]. However, it appears critical to expand the range of active ingredients available to control SLB.

Isopyrazam belongs to the group of SDHIs (succinate dehydrogenase inhibitors), which target the complex II (or succinate dehydrogenase) in the mitochondrial respiration chain, interrupting the electron flow, and thus altering cellular respiration. The genes coding for the succinate dehydrogenase proteins are nuclear. The enzyme complex succinate dehydrogenase is composed of 4 subunits. Two hydrophilic parts form a peripheral domain and possess the succinate dehydrogenase activity, the flavoprotein sdhA (643 amino acids in *Z. tritici*) and the iron–sulfur protein shdB (297 amino acids in *Z. tritici*) which contain 3 iron–sulfur complexes, [2Fe–2S], [4Fe–4S] and [3Fe–4S]. The subunits sdhC (size: 187 amino acids in *Z. tritici*) and sdhD (size: 193 amino acids in *Z. tritici*) form a lipophilic domain anchoring the complex in the mitochondrial membrane. Finally, a heme b group is complexed between sdhC and sdhD [19]. The enzyme complex is active in the electron transport by the aerobic respiratory chain as well as in the citric acid cycle, by coupling the oxydation of succinate to fumarate with the reduction of ubiquinone to ubiquinol [20]. Isopyrazam binds in the quinone-binding site of the protein complex, which is composed of residues of sdhB, sdhC and sdhD, close to the [3Fe–4S] cluster and heme b [20,21]. The European commission approved the use of isopyrazam in the United Kingdom in 2010, as a mixture with cyprodinil, on winter and spring barley, and in 2011 as a mixture with epoxiconazole on winter wheat, winter and spring barley, rye and triticale [22].

The list of plant pathogenic organisms resistant to disease control agents [23] currently includes 11 fungal species being reported to be resistant towards SDHIs, namely *Alternaria alternata*, *Aspergillus nidulans, Botrytis cinerea*, *Botrytis elliptica*, *Coprinus cinereus, Corynespora cassiicola*, *Didymella bryoniae*, *Z. tritici, Podosphaera xanthii, Ustilago nuda* and *Ustilago maydis*. In *A. alternata*, *B. cinerea, C. cassiicola* and *P. xanthii*, the mutation in the iron–sulfur cluster (subunit B) of the succinate dehydrogenase, causing the substitution of an histidine residue by a tyrosine or an arginine residue (respectively B-H277Y/R, B-H272Y/R and B-H278Y), confers resistance towards carboxamides [24–27]. This mutation has been identified in several field isolates, but it is not the only cause for resistance, as all resistant isolates do not carry it. For instance, in *B. cinerea*, the substitution B-P225T/L also leads to weak resistance towards carboxamides [28]. In *C. cassiicola*, mutations C-S73P, D-S89P have also been detected in some moderately resistant isolates [29]. In *A. alternata*, mutations C-H134R, D-D123E and D-H133R were also detected in isolates resistant towards boscalid [30]. In *B. cinerea*, the overexpression of ATP binding cassette and major facilitator family transporters, leading to the efflux of the active compound, is described as taking part in the resistance towards carboxamides. In the latter fungus, this phenomenon causes also the emergence of multi-drug resistance [25]. In *B. elliptica*, *D. bryoniae* and *U. nuda*, resistance was observed in the field, but the causes were not yet investigated [31,32].

The current climatic conditions in northwestern Europe allow for regular *Z. tritici* epidemics in wheat [33] as well as for occasional FHB epidemics [34]. It was the objective of this study to characterize the sensitivity of *F. graminearum* and *Z. tritici* towards isopyrazam to allow conclusions on its integration into spray programs in regions where these pathogens pose the major threat to grain quantity and quality and to identify so far unknown sdh structures that could confer resistance.

2. Material and methods

2.1. Strains

Z. tritici strains (Table 1) were isolated from wheat leaves showing symptoms of SLB sampled across Luxembourg. First, leaves with pycnidia were incubated on 1.5% water agar in closed Petri dishes. After one day of incubation, each oozing cirrhus arising from a pycnidium was collected using a sterile needle and spread on MYA (Malt Yeast Agar: yeast extract, 4 g; malt extract, 4 g; glucose, 4 g; agar, 15 g for 1 L of deionized water) medium. Plates were incubated at 22 °C in the dark. A piece of growing mycelium was then transferred into 1.5 mL of sterile deionized water, and 300 μL of a 1:400 and a 1:160,000 dilution of the mixture were spread on MYA medium. Water was allowed to evaporate, and plates were incubated at 22 °C in the dark. Colonies originating from a single spore were then transferred to new MYA plates, and incubated at 22 °C in the dark. Once the cultures started to sporulate, plates were washed off with 3 mL of a sterile 15% glycerol-deionized water-solution. Spore suspensions of each isolate were stored at −20 °C until further use.

The identity of the strains was confirmed by PCR using the procedure described in Giraud et al. (2010) [34]. As a positive control, a strain from the MUCL (Mycothèque de l'Université Catholique de Louvain, Belgium), MUCL18, was used. *F. graminearum* strains were obtained from various origins (Table 2) and previously characterized as described in Pasquali et al. [35,36] and Dubos et al. [8].

2.2. In vitro assays

Isopyrazam (analytical grade, chemical purity >99.6%, obtained from Sigma–Aldrich) was solved in ethanol at a concentration of 2.78 mM. Dilutions having a concentration of 100%, 50%, 10%, 5%, 1%, 0.1%, 0.01% and 0% of the stock solution were poured into a clear, flat bottom, sterile 96-wells microtiter plates (Nunc A/S, Denmark). Ethanol was allowed to evaporate. Then, 100 μL of PDB medium (Potato Dextrose Broth, 24 g L^{-1}) for *F. graminearum* strains, or 100 μL of liquid MYA for *Z. tritici* strains were transferred into each well, as well as 100 μL of spore suspension for the treatments or 100 μL of sterile deionized water for the control. Spore concentrations $>5 \times 10^7$ spores L^{-1} were used [8]. Since *Z. tritici* was much more sensitive than *F. graminearum* in preliminary tests, 0.0556 mM was chosen as the maximum concentration for *Z. tritici*.

Each strain was tested in triplicate. Plates were closed with lids, sealed with Parafilm, and incubated in an orbital shaker at 22 °C and 120 rpm in darkness. After 5 days, 10 μL of resazurin (0.6 mg mL^{-1}) were added to each well for *F. graminearum* and 5 μL for *Z. tritici* to increase the color contrast between wells where respiring tissue was present (yellow staining) and wells where respiring tissue was absent (pink staining [8]). Plates were incubated for 24 h in the dark, at 22 °C and 120 rpm. The absorbance of each well was determined at 405 nm using an absorbance reader (Fluorescence, absorbance and luminescence reader, model Genios, Tecan Group Ltd., Männedorf, Switzerland).