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Characterization of boscalid-resistance conferring mutations in the *Sdh*B subunit of respiratory complex II and impact on fitness and mycotoxin production in *Penicillium expansum* laboratory strains

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ABSTRACT

Laboratory mutants of *Penicillium expansum* highly resistant (Rfs: 90 to >500, based on EC_{50s}) to Succinate Dehydrogenase Inhibitors (SDHIs) were isolated after UV-mutagenesis and selection on media containing boscalid. A positive correlation was found between sensitivity of isolates to boscalid and other SDHIs such as isopyrazam and carboxin but not to fungicides affecting other cellular pathways or processes, such as the triazole flusilazole, the phenylpyrrole fludioxonil, the anilinopyrimidine cyprodinil and the benzimidazole benomyl. Most of the boscalid-resistant strains were more sensitive to the SDHI fluopyram and the QoI pyraclostrobin. In order to investigate the mechanism responsible for the observed resistance profiles, part of the *Sdh*B subunit isolated the wild type and boscalid-resistant isolates, was genetically characterized. Comparison of the deduced amino-acid sequence between resistant and wild-type isolates revealed two point mutations at a position corresponding to codon 272 of the respective *Sdh*B protein in *Botrytis cinerea*. The substitution of histidine by arginine was found in boscalid-resistant isolates which were equally sensitive to fluopyram compared with the wild-type whereas the replacement of histidine by tyrosine was found in strains with increased sensitivity to fluopyram. No adverse effects of resistance mutations were observed on fitness determining parameters such as osmotic sensitivity, sporulation and pathogenicity, while mycelial growth rate and spore germination was negatively affected in some of the mutants studied. *P. expansum* mutant strains displayed significantly perturbed patulin and citrinin levels as compared to the wild-type parent strain both *in vitro* and *in vivo* as revealed by thin layer (TLC) and high performance liquid chromatography (HPLC).

1. Introduction

Blue mold caused by the fungus *Penicillium expansum*, is one of the most economically important post harvest diseases of pome-fruit [1–3]. Results from a recent Washington State survey on stored apples report 32% yield losses due to fruit rot caused by blue mold [4]. Food safety concerns highlight the importance of this pathogen due to its ability to produce a variety of secondary metabolites (mycotoxins) toxic to humans and livestocks, the most important being patulin and citrinin [5,6].

The cyclic tetraketide patulin has been shown to exert highly toxic effects on plant and animal cells due to its interaction with essential cellular sulfhydryl groups of proteins and glutathione [7]. A number of toxic effects have been attributed to patulin contamination including mutagenic, genotoxic, immunotoxic, teratogenic and neurotoxic effects on rodents and/or chickens [8–10]. European commission has established the maximum permitted levels of patulin at 10 μg kg^{-1} for fruit-based baby food, 50 μg kg^{-1} for fruit juice and 25 μg kg^{-1} for solid apple products [11]. Similar toxic effects have been attributed to citrinin including nephrotoxic, immunotoxic and potential teratogenic actions rendering this mycotoxin a potential human health hazard, although no legal requirements about its levels in food and feed have been established yet [12]. Mycotoxin poisoning symptoms caused by contaminated apple fruit and by-products include suppression of the immune system, nausea, vomiting and other gastrointestinal traumas resulting from severe toxicosis [8,13,14].

Measures to prevent mycotoxin contamination of agricultural products by utilizing physical, chemical and biological detoxification methods as well as controlled temperature/atmospheres storage have been implemented, although the most effective measure to reduce the incidence of blue mold remains the use of fungicide applications [15–17]. Postharvest applications against *P. expansum* world-wide traditionally rely on the use of benzimidazoles (TBZ) or the imidazole imazalil but both chemistries have heavily suffered from resistance development [18–23]. Alternative fungicides have been successfully tested

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against the pathogen including cyprodinil, pyrimethanil, fludioxonil and the mixture pyraclostrobin + boscalid (Pristine) [4,24–26]. However, resistance to some of these fungicides such as the anilinoprymidines (cyprodinil and pyrimethanil) and the phenylpyrrole fludioxonil has been recently reported in Greece [27].

Succinate Dehydrogenase Inhibitors (SDHIs) are one of the fastest growing groups of fungicides listing 17 different compounds belonging to a number of chemical classes. Their successful adaptation by farmers as witnessed by rapidly growing sales globally is due to the broad activity spectrum of the newer group members and the need for alternative fungicides to combat QoI- and DMI-resistances [28]. The carboxamide boscalid, the first new generation broad spectrum representative of SDHIs, acts by blocking complex II of the respiratory chain and displays both protective and curative properties [29]. In Greece it is registered for the control of powdery mildews, scabs, gray mold, downy mildews, rusts and other pathogens belonging to the *Alternaria* spp., *Monilinia* spp., *Botrytis* spp. and *Sclerotinia* spp. genera. Although boscalid is not yet registered for use against *Penicillium expansum* in Greece, its specific mode of action and the potential indirect selection pressure exerted to *P. expansum* by boscalid-containing products used to control other apple diseases such as apple scab and powdery mildew indicate an elevated risk for resistance development. Since boscalid is a candidate compound for post-harvest use against *P. expansum* in Greece, the question of resistance risk development and the possible mycotoxin contamination of apple fruit due to occurrence of resistant mutants arises.

Thus, the objectives of this study were to: a) evaluate boscalid's effectiveness against blue mold and its inherent risk of developing resistance utilizing laboratory mutants of *P. expansum* and b) investigate the biochemical mechanism responsible for resistance to SDHIs at the molecular level and possible implications of resistance mutations to mycotoxin production.

2. Materials and methods

2.1. Fungal strains and culture conditions

Boscalid-resistant isolates (RD_1/Bc-) were originated from the RD_1 wild-type strain of *P. expansum* isolated from an infected Red Delicious apple fruit collected from a packinghouse in Northern Greece. Wild-type and boscalid-resistant isolates were grown on PDA medium for inoculum-preparation purposes and kept in a growth chamber at 22 °C under 70% humidity. *In vitro* mycotoxin production was evaluated by growing strains on Yeast Extract Sucrose agar (YES) medium (150 g sucrose, 20 g yeast extract and 20 g agar in 1 L water) at the same conditions. For long-term storage of strains, five 5 mm mycelial plugs from the margin of a rapidly growing *P. expansum* colony on PDA for each strain were placed in 1.5 mL tubes containing 20% v/v of sterilized glycerol:water and stored at −20 °C.

2.1.1. Fungicides, mycotoxins and solvents

All fungicides used in *in vitro* experiments were technical grade. Benomyl and flusilazole were kindly supplied by Du Pont de Nemours and Co. (Wilmington, DE, USA), boscalid, carboxin and pyraclostrobin by BASF AG (Limburgerhof, Germany), isopyrazam, cyprodinil and fludioxonil by Syngenta Crop Protection AG (Basle, Switzerland), fluopyram by Bayer CropScience (Greece). Thifluzamide was purchased from Sigma-Aldrich. Ethanol was used as solvent for all fungicide stock solutions with the exceptions of benomyl, fluopyram, isopyrazam and pyraclostrobin which were dissolved in acetone. Patulin and citrinin standards were purchased from Sigma-Aldrich (St. Louis, USA). Analytical standard stock solutions of patulin and citrinin were made in methanol at various concentrations and stored at −20 °C. Ten standard solutions of each mycotoxin at concentrations from 0.01 to 5 μg mL^{-1} were prepared as calibration standards. HPLC grade solvents, methanol and acetonitrile, were purchased from Lab Scan (Dublin, Ireland). Ultrapure-grade HPLC water was obtained by purification of distilled water through a Mili-Q Gradient system (Millipore, Bedford, USA). All fungicides were added aseptically from stock solutions to sterilized growth medium prior to inoculation and the final amount of solvent never exceeded 1% (v:v) in treated and control samples.

2.2. Mutagenesis procedure

Isolates resistant to boscalid were obtained after mutagenesis using Ultra-Violet (UV) irradiation. A conidial suspension (approximately 10^8 conidia mL^{-1}) from the *P. expansum* wild type strain was prepared after harvesting the biomass from 7 day-old cultures and filtration with a sterilized mesh. Conidia were exposed to UV light (TUV Philips, 15 W, 254 nm) for 5 min under continuous agitation and left in the dark for 30 min to avoid photo-repair. A 95–96% lethality percentage caused by UV-irradiation was calculated by comparing the number of germinating conidia/mL on PDA sampled before and after UV treatment. Following irradiation, conidia were plated on Minimal Medium (containing 10 g a-D-glucose, 1.5 g K_2HPO_4, 2 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0,5 g $MgSO_4 \times 7\ H_2O$, 2 g yeast extract and 15 g agar dissolved in 1 L distilled water) amended with 10 μg mL^{-1} boscalid and incubated at 22 °C for 20 days in the dark. A total of 15 resistant isolates (RD_1/Bc-) were selected and maintained on MM slants containing 0.75 μg mL^{-1} boscalid, the MIC concentration of the wild type strain of *P. expansum*.

2.3. In vitro fungitoxicity bioassays

Sensitivity of wild-type and boscalid-resistant *isolates* of *P. expansum* to inhibitors was evaluated by measuring radial growth of strains on MM for SDHIs and PDA medium for the rest of the fungicides. Inoculum, consisting of 5-mm mycelial plugs cut from the edge of 4-day old *P. expansum* colonies grown on PDA, was transferred onto the fungicide-amended growth media. To obtain fungitoxicity curves, growth medium was amended with 0, 0.1, 0.5, 1, 2.5 and 5 μg/mL benomyl, cyprodinil, carboxin, flusilazole, thifluzamide and isopyrazam while fungicide concentrations of 0, 0.01, 0.025, 0.075, 0.1, 0.25/0.5/1/2.5 and 5 μg/mL where used for pyraclostrobin, fluopyram and boscalid. In the case of boscalid, additional concentrations of 25, 50 and 100 μg/mL were used to determine resistance levels of *P. expansum* mutants using the commercial formulation Cantus (50 WG). The mean colony diameter was measured and expressed as percentage of the mean diameter of the untreated control. Resistance levels were estimated using resistance factors (Rfs) expressed as the ratio of EC_{50} (the concentration causing 50% reduction of mycelial growth) for each resistant isolate to the mean EC_{50} value of the wild-type isolate. Measurements were made following incubation at 20 °C in the dark for 7 days and then fungitoxicity curves were used to determine EC_{50} values for each isoalte/fungicide combination. Each experiment was replicated three times.

2.4. Fitness parameters

Boscalid-resistant and wild-type isolates were tested for mycelial growth, osmotic sensitivity, sporulation, conidial germination and pathogenicity. Four 5-mm mycelial plugs from the colony-margin from each isolate were transferred to the centre of PDA plates for radial growth measurements. After incubation at 20 °C in the dark, the colony diameter of each isolate was measured at 24 h intervals. Osmotic sensitivity of boscalid-resistant and the wild-type isolates was assessed on PDA medium by measuring the inhibitory effect of 5% NaCl on mycelial growth after 5 days incubation at 20 °C in the dark. A quantity of 10^6 conidia/PDA-plate was incubated for 10 days at 20 °C with 14 h/day light in order to determine conidial production. The total mycelial mass produced in each plate was harvested and transferred to a 100-mL Erlenmeyer flask with 20-mL de-ionized water. The flasks were agitated vigorously and the concentration of conidia in the resulting spore suspension was determined with a Neubauer haemocytometer and