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Effect of phenylpyrrole-resistance on fitness parameters and ochratoxin production in *Aspergillus carbonarius*

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ABSTRACT

The risk of resistance development to fludioxonil and the potential implications of resistance mutations to ochratoxin production in *Aspergillus carbonarius* were investigated. Mutants of *A. carbonarius* highly resistant to phenylpyrroles were isolated at a high mutation frequency after N-MNTG-mutagenesis and selection on media containing fludioxonil. A highly reduced sensitivity to fungicides belonging to the same cross-resistance group (AHDs and phenylpyrroles) such as the aromatic hydrocarbon tolclofos-methyl and the dicarboximide fungicides iprodione and vinclozolin was also observed. No cross-resistance relationships were found between fludioxonil and the triazole epoxiconazole, the anilinopyrimidine cyprodinil and the chloronitrile chlorothalonil. Interestingly, fludioxonil-resistant isolates were highly sensitive to the QoI fungicide pyraclostrobin compared to the wild-type parental strain. Fitness studies revealed that resistance mutation(s) had a negative effect on mycelial growth, resistance to osmotic stress and pathogenicity of the fludioxonil-resistant strains. Mycotoxin analysis showed that most fludioxonil-resistant strains produce less quantities of ochratoxin A (OTA) than the wild-type strain both when grown on artificial medium and on grapes. Increased osmotic sensitivity and reduced pathogenicity of the mutant strains were significantly correlated with reduced ochratoxin production *in vivo* but not *in vitro*. The above-mentioned data indicate that fludioxonil is an excellent fungicide for the control of *A. carbonarius* in grapes and a valuable asset for farmers in terms of resistance management and ochratoxin contamination of grapes, vine products and wines.

1. Introduction

Aspergillus carbonarius infection of grapes initiates as early as the veraison period (Battilani et al., 2003) leading to the development of grape sour rot. The growing concern over this fungus originates not from the disease severity and the subsequent yield losses but, mainly, from its ability to produce toxic secondary metabolites (mycotoxins) and specifically ochratoxin A (OTA). Of all the *Aspergillus* OTA-producing species, those belonging to the section *Nigri* – especially *A. carbonarius* – are the ones mainly responsible for OTA contamination in grapes and their products including grape juice, dried vine fruit and wines (Magnoli et al., 2004; Tjamos et al., 2004; Perrone et al., 2006; Guzev et al., 2006; Leong et al., 2006; Battilani et al., 2006; Chulze et al., 2006; El Khoury and Atoui, 2010). A detailed monograph on the toxicological profile of Ochratoxin A, reporting the nephrotoxic, neurotoxic, immunotoxic, teratogenic and carcinogenic properties of this substance, is available by the International Agency for Research on Cancer (IARC, 1993). The European Union, in view of the danger posed by the OTA-contaminated products, has established regulatory levels of this mycotoxin in cereals, grapes and coffee (Commission Regulation (EC), 1881/2006). Specifically for dried vine fruit the limit was set to 10 μg/kg, while for grape-juice and wines the limit is as low as 2 μg/kg.

Various control measures have been utilized to reduce disease incidence and OTA production in vineyards and post-harvest. Reduction of OTA contamination of grapes could result from the application of good cultural practices and control of biotic and abiotic agents (Covarelli et al., 2012). Additionally, the use of biocontrol agents can contribute towards this end, via fungal antagonism or by degrading the ochratoxin produced (Amézqueta et al., 2012), but their effectiveness in the field is yet to be confirmed. The most efficient and cost-effective measure to reduce the incidence of molds in most crops, remains the application of fungicides (Munimbazi et al., 1997). Among a number of fungicides tested for their ability to control *A. carbonarius* and the prevention of OTA accumulation in grapes, the phenylpyrrole fludioxonil (commercially formulated as a mixture with cyprodinil) has been found to be very effective in reducing the incidence and levels of *Aspergillus* populations (Tjamos et al., 2004; Bellí et al., 2006; García-Cela et al., 2012). Fludioxonil is one of the more recently introduced fungicides, a synthetic analog deriving from the natural antibiotic pyrrolnitrin produced by *Pseudomonas pyrociniae* (Nishida et al., 1965), with a broad spectrum of activity against fungal species from the three major

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classes of fungi (Ascomycetes, Basidiomycetes and Deuteromycetes) (Koch and Leadbeater, 1992). Phenylpyrrole as well as dicarboxamide fungicides disrupt the signaling system that is responsible for fungal osmoregulation by stimulating the histidine kinase (HK) – MAPK stress response pathway (Oshima et al., 2002; Motoyama et al., 2005; Kim et al., 2010; Fujimura, 2010; Furukawa et al., 2012).

The development of resistance, typical for site-specific fungicides like fludioxonil is a serious problem compromising the longevity of fungicide effectiveness and has been addressed in a number of studies involving laboratory and field strains of various plant pathogens (Brent and Hollomon, 2007). An additional concern in the case of mycotoxigenic fungi is the effect of fungicide resistance on mycotoxin production and though phenylpyrrole fungicide resistance risk has been excessively studied (Faretra and Pollastro, 1993; Leroux et al., 1999; Ziogas and Kalamarakis, 2001; Ziogas et al., 2005; Kanetis et al., 2007; Li and Xiao, 2008; Gachango et al., 2011; Kuang et al., 2011), very little information is available concerning the impact of resistance mutation(s) on mycotoxin production (Markoglou et al., 2011) while no information is available in the case of *A. carbonarius*.

Thus, the main objectives of the present study were to evaluate: (a) fludioxonil effectiveness *in vitro*, (b) *A. carbonarius* inherent resistance risk to phenylpyrroles by determining the mutation frequency, resistance levels, cross-resistance relationships and fitness parameters of laboratory mutant strains and (c) the impact of resistance mutations on the ochratoxigenic ability of *A. carbonarius*.

2. Materials and methods

2.1. Fungal strains and culture conditions

The wild-type strain NRRL 67 of *A. carbonarius* Speare (obtained from the United States Department of Agriculture, Washington, D.C., USA) was selected for its certified ochratoxicogenic ability to be the parental strain from which all fludioxonil-resistant isolates (Ac/Fld) derived. Isolates were grown on Czapek Dox agar (CZA) medium (0.2% sodium nitrate, 0.05% potassium chloride, 0.05% magnesium sulfate, 0.001% ferric sulfate, 0.1% dipotassium phosphate, 0.001% zinc sulfate, 0.0005% copper sulfate, 3% sucrose and 2% agar) and Malt Extract Agar (MEA) medium (20 g malt extract, 20 g glucose, 1 g peptone and 20 g agar) at 30 °C with 16 h day^{-1} light and 90% relative humidity. Ochratoxin production was evaluated by growing isolates on Yeast Extract Sucrose agar medium (YES) containing 2% yeast extract, 15% sucrose and 2% agar, at the same conditions.

2.2. Fungicides, ochratoxins and solvents

All fungicides used in *in vitro* tests were pure technical grade. Fludioxonil and cyprodinil were kindly supplied by Syngenta Crop Protection AG (Switzerland), tebuconazole by Bayer CropScience (Germany), pyraclostrobin, epoxiconazole and vinclozolin by BASF (Germany), iprodione by Rhone Poulenc Agrochimie S.A. (France), tolclofos-methyl by Sumitomo Chemical Co. (Japan) and chlorothalonil by ISK Biosciences Ltd (United Kingdom). The ochratoxin A (OTA) and ochratoxin B (OTB) standards were purchased from Sigma-Aldrich (USA). Analytical standard stock solutions of the fungicides and ochratoxins were made in appropriate organic solvents at various concentrations. These solutions were stored at −20 °C. Three standard solutions containing mixtures of ochratoxins A and B in methanol at concentrations 10, 25 and 50 μg ml^{-1} were prepared as calibration standards. HPLC grade solvents, methanol and formic acid, 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.

2.3. Mutation induction

Fludioxonil resistant isolates were obtained by chemical mutagenesis using N-methyl-nitrosoguanidine (N-MNTG) purchased from Sigma-Aldrich (USA). A suspension of approximately 10^7 conidia ml^{-1}, harvested from 15 day-old cultures of the *A. carbonarius* wild-type strain, was amended with N-MNTG at a concentration of 40 μg ml^{-1} and incubated at 25 °C for 4 h under continuous agitation. Following mutagenesis, conidia were plated on MEA medium containing 10 μg ml^{-1} fludioxonil and incubated at 30 °C for 10 days. Resistant colonies were transferred on MEA and single spore isolates (Ac/Fld-) were maintained on agar slants containing 0.25 μg ml^{-1} fludioxonil, a concentration that fully inhibited growth of the wild type strain.

2.4. In vitro fungitoxicity tests

Fungicide sensitivity of *A. carbonarius* wild type and fludioxonil-resistant isolates was determined based on the calculation of EC_{50} values (effective concentration causing 50% inhibition of mycelial growth) for each fungicide. MEA amended with 0.01, 0.025, 0.05, 0.1, 0.5, 1, 5 and 10 μg/ml fludioxonil, vinclozolin, toclofos-methyl iprodione, epoxiconazole, pyraclostrobin and chlorothalonil, was used for the calculation of fungitoxicity curves of all isolates. The same concentrations on CZA medium were used to obtain the respective curves in the case of cyprodinil. Each concentration was applied in triplicate. Inoculum, consisting of 5-mm mycelial plugs cut from the edge of 4-day old *A. carbonarius* colonies grown on MEA, was transferred on the fungicide-amended growth media. Cultures were incubated at 30 °C in the dark for 5 days. 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 (Rf) expressed as the ratio of EC_{50} for each resistant isolate to the EC_{50} value of the wild-type strain. Tests for each isolate were replicated twice for each concentration and each fungicide.

2.5. Study of ecological fitness parameters

Fludioxonil-resistant mutants of *A. carbonarius* were tested for mycelial growth rate, sporulation, pathogenicity and sensitivity to osmotic pressure and compared with the wild-type parent strain. Three 5-mm mycelial plugs for each strain were transferred to the center of MEA containing plates for radial growth measurements. After incubation at 30 °C in the dark, the colony diameter of each isolate was measured at 24 h intervals. To determine conidial production, 200 μl from a conidial suspension (10^7 conidia/ml) were plated on fungicide-free MEA and incubated for 10 days at 30 °C with 14 h day^{-1} light. The total mycelial mass produced in each dish was scraped and transferred to a 100-ml Erlenmeyer flask with 20-ml deionized water. The flasks were agitated vigorously and the concentration of conidia in the resulting spore suspension was determined with a Neubauer hemocytometer and expressed as number of conidia/cm^2 of culture. Pathogenicity experiments were conducted on vine-grapes (*Vitis vinifera* cv *kalmeri*). Grapes selected on the basis of size and shape uniformity and absence of any wound were detached from the bunches and surface-disinfected by drenching for 5 min in a 2% sodium hypoclorite solution. Following disinfection, the fruit were rinsed three times with sterile-deionized water and air-dried. The fruit were inoculated at the wound resulting from the detachment of the grape from the bunch with 10 μl of a conidial suspension containing 1×10^4 conidia per ml using a pipette. Twenty grapes for each isolate were inoculated and the experiment was replicated twice. Non-inoculated grapes treated with sterile water were used as control. The fruit were placed on wire mesh platforms (10 grapes per box) in plastic boxes (25 × 15 × 10 cm [length × width × height]).