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Short communication

Fungicides effectively used for growth inhibition of several fungi could induce mycotoxin biosynthesis in toxigenic species

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ABSTRACT

Seven different commercial fungicides (Aliette, Rovral, Cantus, Ortiva, Luna Experience, Fenomenal and Mancozeb) were tested for their ability to inhibit the growth of the fungal species *Penicillium nordicum, Penicillium verrucosum, Verticillium dahliae* and *Cladosporium* sp. In case of the mycotoxigenic strains *P. nordicum* and *P. verrucosum*, the biosynthesis of the mycotoxins ochratoxin and citrinin was determined. Interestingly individual fungicides were only able to inhibit the growth of the analyzed fungi to some extent. In case of *P. verrucosum* the fungicide "Rovral", an iprodion belonging to the substance class of imidazoles, led to a decrease in the growth rate but to a strong induction of mycotoxin biosynthesis as has been described earlier for the strobilurins. Consequently before using a given fungicide to protect crops and enhance storage life, the applicability of this chemical compound should be tested not only for its ability to inhibit fungal growth but also for its effect on level of secondary metabolite biosynthesis.

1. Introduction

Fungicide resistance has greatly affected control of pre- and postharvest fungal diseases. Among plant pathogens, filamentous fungi in general have a particularly high risk of becoming resistant to fungicides, mainly due to their extensive asexual reproduction potential (Kendall and Hollomon, 1998). Individual spores of these highly variable spore conglomerates may be favored under chemical selection pressure. In pre- and postharvest management of plant derived food, the risk of development of fungicide resistance in species of *Cladosporium, Penicillium* and *Verticillium* populations are exacerbated by the year-round availability of susceptible host tissues because food commodities are stored almost continuously after harvest. *Cladosporium* is a fungal genus which is commonly found on diverse plant materials. Some species are classified as plant pathogens, for example *Cladosporium fulvum* which causes tomato leaf rot (Thomma et al., 2005). The same species has also been an important genetic research model for host resistance (De Wit et al., 1994). Despite the fact that *Cladosporium* species produce no major mycotoxins, they may be pathogenic to humans, trigger allergies if the air borne spores are ingested and have been reported to cause infections of the skin and nails, sinusitis, pulmonary infections and irritation of the lung by the volatile organic compounds (VOCs) that may be produced. Another common fungal genus, *Verticillium*, induces the so called *Verticillium* wilt or maple wilt (Pegg and Brady, 2002). *Verticillium* was first identified on potatoes in Germany in 1870 and can infect over 300 different cultivated plants including apricots, almonds, peaches, plums and avocados and also vegetables such as mint, tomatoes and eggplants (Knoll, 1972). *Verticillium* can persist as a saprotrophic soil organism for several years. During infection the fungal mycelium moves up the xylem vessels. *Verticillium* produces no major mycotoxins. Representative toxigenic fungal species which may affect plants before and after harvest are *P. nordicum* and *P. verrucosum*. These Penicillia are able to produce the mycotoxins ochratoxin and citrinin. Both toxins have polyketide backbones and are structurally highly related. Ochratoxin A as well as citrinin are mainly nephrotoxic and hepatotoxic and may act synergistically (Braunberg et al., 1994). For ochratoxin A, which is rated as a class B carcinogen, regulatory limits have been set in several countries. The level of citrinin is not currently regulated. Both *Penicillium* species are phylogenetically high related (Larsen et al., 2001). *P. nordicum* can mainly be isolated from NaCl rich fermented foods, such as cheeses or meats (Larsen et al., 2001; Lund and Frisvad, 2003), whereas the main habitats of *P. verrucosum* are cereals, olives and meat products. It was recently shown that *P. verrucosum* adapts its secondary metabolite profile depending on the environmental conditions (Schmidt-Heydt et al., 2012). For example oxidative stress usually induces defensive reactions such as radical scavenging mechanisms for reactive oxygen species (ROS). Due to the fact that citrinin is described to have antioxidative properties (Heider et al., 2012) it could act as radical scavenger. In this respect it was shown that *P. verrucosum* changes its secondary metabolite profile from ochratoxin A to citrinin under oxidative stress conditions, for example, those induced by irradiation with light of short wavelength (Schmidt-Heydt et al., 2011).

Different fungicides are available to control fungal infections on pre- or postharvest plants. In this study seven different fungicides were used representing different substance classes in order to investigate whether

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the respective fungicide is effective in inhibiting fungal growth. In case of the mycotoxigenic *Penicillium* strains which were used as representatives for possibly coincidentally occurring toxigenic fungi, the biosynthesis of the mycotoxins ochratoxin A/B and citrinin were also determined.

2. Materials and methods

2.1. Fungal strains and growth conditions

Penicillium nordicum BFE487, *Penicillium verrucosum* BFE808, *Verticillium dahliae* MRI178 and *Cladosporium* sp. MRI031 from the culture collection of the Max Rubner-Institut were used for the experiments. The strains were grown in triplicate on MEA agar plates (17 g/l malt extract, 5 g/l glucose, 20 g/l agar) either supplemented with 100 μl of the respective fungicide at the concentration suggested by the manufacturer or without supplement as control. Aliette WG 803 kg/ha = 1.70 g/100 ml (Bayer Cropscience, Monheim, Germany); Rovral WG 75 0.7 kg/ha = 0.397 g/100 ml (COMPO, Münster, Germany); Cantus WG 1 kg/ha = 0.567 g/100 ml (BASF, Ludwigshafen, Germany); Ortiva L 1 l/ha = 0.567 ml/100 ml (Syngenta, Ketsch, Germany); Luna Experience L 0.125 l/ha = 0.0709 ml/100 ml (Bayer Cropscience, Monheim, Germany); Fenomenal WG 3 kg/ha = 1.70 g/100 ml (Bayer Cropscience, Monheim, Germany); Mancozeb WG 2 kg/ha = 1.134 g/100 ml (Isagro S.p.A. Italia). A spore suspension containing 10^5 spores per ml was used for inoculation of triplicates. The cultures were incubated at 25 °C for 7 days.

In addition to growth analyses on laboratory medium (MEA), ochratoxin A biosynthesis in *P. verrucosum* was determined after growth on wheat kernels mimicking the natural habitat of this species, supplemented with different amounts of the fungicide Rovral WG 75. For this purpose, 20 g sterilized wheat kernels where weighed into Petri dishes and hydrated in 10 ml of sterile H_2O. A 100 μl aliquot of a spore suspension containing 10^5 spores per ml was mixed in triplicate with 0–200 μl Rovral (0.397 g/100 ml) in 25 μl steps and evenly distributed over the prepared wheat kernels. The wheat samples were incubated at 25 °C for 7 days.

2.2. Growth assessment

The growth experiments were carried out using three independent replicates. The morphology of the colonies was documented photographically. The growth rate on MEA agar plates was ascertained by measurement of the colony diameters at the beginning of the experiments and after seven days, the mean values and standard deviations of the growth within seven days were calculated.

2.3. Quantification of ochratoxin A/B and citrinin by HPLC

For determination of ochratoxin A/B and citrinin biosynthesis, an agar plug (Ø 1 cm) of the respective colony was taken from the region between center and edge of the colony with the aid of a sterile corer. This agar plug with the adhering mycelium was transferred into 2 ml micro-reaction tubes and 1 ml of chloroform was added. The fungal mycelia were extracted for 30 min at room temperature on a rotary shaker; the mycelia were discarded and the chloroform extract was evaporated to dryness in a vacuum concentrator (Speed-Vac, Savant Instruments, Farmingdale, USA). Because preliminary tests showed that the variability between the replicates was acceptably low, the extracts of the triplicates were combined for quantitative determination of ochratoxin A and citrinin using the method of Sato et al. (2010) on a Hitachi D-7000 HPLC system (Merck, Tokio, Japan) equipped with an auto-injector, column oven and fluorescence detector. The column oven was set to 40 °C; the fluorescence detector was set to an excitation of 331 nm and an emission of 500 nm. The flow rate was 0.7 ml/min and the injection volume 10 μl. Solvent A consist of 250 mM orthophosphoric acid and solvent B of methanol. Separation was carried out on a LiChrospher 100, C18 (250 mm, Ø 4 mm i.d., particle size 5 μm) reversed phase column (VWR International GmbH, Darmstadt, Germany) using the following gradient: 0 min – solvent A 60%, solvent B 40%; 7 min – 40%, 60%; 12 min – 35%, 65%; 16 min – 5%, 95%; 27 min – 60%, 40%. The limit of quantification was 25 pg on column. Data collection and handling was done with EZ-Chrome Elite 3.2. All standards were obtained from Sigma (Taufkirchen, Munich, Germany) with a purity of ≥ 98%. The mean of three independent replicates was used for further calculation.

In the case of inoculated wheat samples, 10 kernels were ground in 1 ml ethylacetate using a cell disruptor (TissueLyser, Qiagen, Hilden, Germany) and then extracted for 30 min at room temperature on a rotary shaker. After centrifugation at 13,000 RPM for 5 min the supernatant was evaporated to dryness in a vacuum concentrator (Speed-Vac, Savant Instruments, Farmingdale, USA) and ochratoxin A was quantified as mentioned above. The mean and standard deviation of three independent replicates were calculated.

3. Results

For the present study the following fungicides representing different substance classes were used: Aliette, a fosetyl-aluminum (ethylhydrogenphosphat-aluminum); Rovral, an iprodione (3- (3,5-dichlorophenyl) -N-isopropyl-2,4-dioxoimidazolidine-1-carboxamide); Cantus, a boscalid (2-Chlor-N- (4'-chlorobiphenyl-2-yl) nicotinamid); Ortiva, an azoxystrobin (methyl (2E) -2- (2-{[6- (2-cyanophenoxy) pyrimidin-4-yl]oxy}phenyl) -3-methoxyacrylate) belonging to the substance class of the strobilurins; and Fenomenal, a femanidone ((5S)-5-methyl-2-(methylsulfanyl)-5-phenyl-3-(phenylamino)-3,5-dihydro-4H-imidazol-4-one). The fungicides Luna Experience, a fungicidal combination of tebuconazol and fluopyram, and Mancozeb, a dithiocarbamate, were also used.

The fungal strains were grown in triplicate on MEA medium after one point inoculation with a spore suspension containing 10^5 spores per ml. The phenotypical growth of the different fungal colonies is shown in Fig. 1; the mean of the measured growth rate is shown in Fig. 2. In case of the two mycotoxigenic strains *P. nordicum* and *P. verrucosum* the most effective growth inhibiting fungicide inhibiting growth was Cantus, a boscalid, which caused total inhibition of spore germination and growth. The measured trace amount of toxin in the non-growing strains originated from the spores which may contain them self ochratoxin, depending on the physiological status of the spores. This fungicide had little effect on the other fungi, *Verticillium dahliae* and *Cladosporium* sp. Less effective but also leading to a substantial growth decrease in all four fungal strains was the fungicide Rovral, an iprodione. Rovral almost completely inhibited the growth of *P. nordicum*, but putatively resistant single spores were able to germinate. *P. verrucosum* was able to grow only very weakly and without apparent sporulation. *Verticillium dahliae* and *Cladosporium* sp. showed a sharp decrease in the growth rate, which was in case of *Cladosporium* sp. associated with mycelial segmentation. Such response is often a result of chemically induced genetic instability or an attempt to adapt to a given set of environmental conditions. The fungicide Aliette, a fosetyl-aluminum, showed no effect at all on growth of any of the studied fungal strains. The azoxystrobin Ortiva caused growth inhibition in *P. nordicum, P. verrucosum* and *V. dahliae* but not *Cladosporium*, which showed the same growth rate as in the control without fungicide supplementation. The fungicide Fenomenal, a femanidone, did not cause any substantial decrease in the growth rate of *P. nordium* but resulted in a phenotypical thin and colorless mycelial mat without visible spores and pigmentation. Pigmentation was also absent in *P. verrucosum. Verticillium dahliae* was nearly not affected, *Cladosporium* showed a slightly increased growth rate in comparison to the control under this condition. Luna Experience, a fungicidal combination of tebuconazol and fluopyram had little effect