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First report: *Penicillium adametzioides*, a potential biocontrol agent for ochratoxin-producing fungus in grapes, resulting from natural product pre-harvest treatment

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ABSTRACT

Ochratoxin A (OTA) is a secondary metabolite produced mainly by *Aspergillus* section *Nigri* (*Aspergillus carbonarius* is the most relevant strain in the Mediterranean region with a group 2B carcinogenic effect in humans. *In vivo* experiments were conducted in southern France involving applying pre-harvest Stifénia® (elicitor), Scala® (chemical fungicide) and two other control treatments [not contaminated by *A. carbonarius* (OTA-PF) and not treated and artificially contaminated by OTA-PF but not treated]. The Stifénia® and Scala® treatments significantly reduced the OTA juice contamination so that it was under the authorised uptake OTA limit. Stifénia® highly affected the grape fungal ecosystem with new non-*Aspergillus* strains isolated from grape stems and juices. *In vitro* antagonistic tests were performed with Stifénia® non-*Aspergillus* isolates ($n = 10$). Three antagonistic tests were applied using different distances (3 and 5 cm in between the two microorganisms) with two different inoculation times (at the same time and with three day intervals in between). Certain strains had a positive mycelial growth effect on *A. carbonarius* colonies, such as *Penicillium* spp. and *Fusarium* sp. Other strains displayed a reduction effect on OTA production of OTA-PF, such as *Penicillium* spp. (J2, J3). *Penicillium adametzioides* (S3) and *Penicillium expansum* (J1) (at certain stages) reduced the OTA production and mycelial growth. *P. expansum* was excluded as a bio-control agent because of its mycotoxin production ability. The higher challenge distance between certain strains of *P. adametzioides* (S3) and other *Penicillium* strains (as J1, J2, J3 and J4; at three and seven days) reduced the secretion of OTA by OTA-PF. This OTA production reduction could possibly prevent OTA contamination prevention in the case of epidemic favourable conditions by reducing the OTA produced in grape post-harvest products (i.e., juice). This could be accomplished by applying as the elicitor one of the tested fungi with an antagonistic effect on OTA production, such as *P. adametzioides* (at 10 days). Certain strains, such as *P. adametzioides* (S3) and J2 (*P.* spp.) should be further investigated to determine the details of the underlying mechanism of their OTA reduction and their ecosystem effects in cases of *in vivo* application.

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1. Introduction

Ochratoxin A (OTA) is a mycotoxin with nephrotoxic, teratogenic, and immunosuppressive properties (Pitt et al., 2001). It has been classified as a possible human carcinogen (group 2 B) by the International Agency for Research on Cancer (IARC) (Reddy & Bhoola, 2010). OTA is suspected to be involved in Balkan Endemic Nephropathy (BEN) (a fatal kidney disease occurring in some areas of south-eastern Europe) and in the high frequency of urinary tract tumours observed in some Balkan areas (Pfohl-Leszkowicz, Petkova-Bocharova, Chernozemsky, & Castegnaro, 2000). A relationship between kidney disease in association with focal segmental glomerulosclerosis (FSGS) in humans and Ochratoxin A has been demonstrated by Hope and Hope (2012). Human OTA exposure mainly occurs via the food chain. Thus, EU legislation has

Abbreviations: OTA-PF, Ochratoxin A producing fungus.

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established OTA maximum uptake limits for certain plant products, such as grape juice and wine (maximum of 2 μg/L) because the tolerable weekly intake (TWI) of OTA is 120 ng/kg body weight (European Commission Regulation (EC) No. 1881/2005, 2006; EC No. 105/2010, 2010). OTA contamination in grape juice is of great concern, as children are its main consumers, and the consumption of juice is greater than that of wine (Varga & Kozakiewicz, 2006).

OTA is related to grape contamination in the vineyard by several OTA-producing species of fungi, especially the black aspergilli, mainly *Aspergillus carbonarius* and the members of the *Aspergillus niger* aggregates. *A. carbonarius* is considered the major OTA-producing species in vine grapes (Cabañes et al., 2002). Numerous studies have demonstrated that moulds can be found on grapes from the veraison stage, and mould development increases rapidly between veraison and maturation (Bejaoui, Mathieu, Taillandier, & Lebrihi, 2006).

Several strategies have been proposed to prevent the toxic effects of mycotoxins in general, and of ochratoxins in particular, in food and feed: (i) prevention of mycotoxin contamination; (ii) decontamination or detoxification of foods contaminated with mycotoxins; and (iii) inhibition of the absorption of consumed mycotoxin in the gastrointestinal tract (Kabak, Dobson, & Var, 2006). The prevention of mycotoxin contamination in the field is a main goal of the agricultural and food industries. As mycotoxin-producing moulds can usually colonize damaged parts of plants, crops must be protected against damage caused by either mechanical processes or insects. Field treatment with fungicides is the traditional prevention technique (Varga, Kocsubé, Péteri, Vágvölgyi, & Tóth, 2010). The public's demand for reduced pesticides in food and the environment have resulted in a passionate debate over the safety of the present control practices for postharvest diseases. Natural plant extracts and antagonism may provide alternatives to chemical preservatives (Sukorini, Sangchote, & Khewkho, 2013). Biological control for postharvest diseases could be realized by the improvement of the beneficial microflora that already exist on fruit and vegetable surfaces instead of the artificial introduction of antagonists against postharvest pathogens. Our knowledge of methods to manipulate the naturally occurring populations of mixed species of microorganisms in a beneficial manner, however, is meager, and the greatest use of biological control (pre- and postharvest) has been achieved through the artificial introduction of large numbers of known antagonists (Wisniewski & Wilson, 1992). Although it is not possible to entirely prevent the formation of OTA in food products, OTA accumulation can be minimised (Varga et al., 2010). Over the years, much effort has been devoted to the search for new antifungal materials from natural sources for food preservation (Boyraz & Özcan, 2005; Galvano, Piva, Ritieni, & Galvano, 2001; Irkin & Korukluoglu, 2007; Soliman & Badeaa, 2002; Yin & Tsao, 1999). Certain natural compounds have antifungal activity that inhibits ochratoxigenic black aspergillii growth, especially *A. carbonarius*, such as fusapyrone produced by *Fusarium semitectum* (Favilla et al., 2008) and natamycin produced by *Streptomyces natalensis* (Medina, Jiménez, Mateo, & Magan, 2007).

The excessive use of chemical fungicides and the negative effects of their residuals on the microflora natural balance in the environment also motivated this study. This work aimed to find a safe control method for reducing the OTA production by *A. carbonarius* and its accumulation in the human food, particularly in grape juice by applying an alternative to chemical fungicides, including a natural plant extract (elicitor, Stifénia®) at an experimental station. The study examined the microbial interactions in the most effective treatment for reducing the OTA production as a possible explanation for its mode of action by studying the antagonistic activity of its non-*Aspergillus* isolated strains against *A. carbonarius*.

2. Material and methods

2.1. In vivo experiment and fungal strains

Four pre-harvest treatments were applied to the cultivar Mourvèdre, at the IFV (French Institute of Vine and Wine), Pech Rouge, Narbonne, France, during two years (2010 and 2011). The vines were not treated nor contaminated with *A. carbonarius* [previously isolated from French vineyards; OTA producing fungus (OTA-PF)] for the first modality (Control 1). The other three modalities were contaminated with OTA-PF by inoculation at the veraison stage. The first modality involved no treatment application (Control 2), whereas the other two modalities consists of treatment with Stifénia® (homogenised fenugreek seed powder, SOFT, France) from the green-tip stage to harvest with 15-day interval (as recommended by producers to promote the eliciting effect), and the other treatment involved two treatments with Scala® (chemical fungicide with pyrimethanil 400 g/L as active ingredients, Bayer, France) at green-tip stage and leaves-output stage. The samples were harvested in late September, during the grape harvest period. Grape bunches (5 kg/treatments) were randomly taken and placed in two previously sterilised bags, which were kept at 4 °C until analysis. Mycological analysis was immediately performed and the remaining samples were kept frozen at −20 °C.

2.2. Media cultures

PDA (Potato-Dextrose agar; Biokar Diagnostics, Beauvais, France) medium with chloramphenicol was used for fungal isolation at pH 3.5 from grape juices (GJ) and grape stems (GS). Normal PDA was used for strain purification, conservation and antagonistic tests. CYA and MEA culture media were used for morphological and microscopic identification.

2.3. Isolates

The *A. carbonarius* strain that was used for the *in vivo* grape contamination was previously isolated from French vineyards and displays high OTA production (Dachoupakan et al., 2009). All of the other non-*Aspergillus* tested strains were naturally isolated from grape juices (GJ) and stems [empty clusters; (GS)] that were treated with Stifénia®.

2.4. Enumeration

2.4.1. From grape juice

Decimal dilutions of grape juice (approximate 150 g of randomly grape clusters parts were homogenized in a stomacher (Seward, France) without external water and filtered under pressure). Then 100 μL of each dilution were spread on PDA (3.5 pH) in Petri plates and incubated at 25 °C for five to seven days in the dark. After incubation, the number of colony forming units (CFU) of the filamentous fungi per millilitre of juice homogenate was evaluated (ISO 7218:1996/Amd. 1:2001 (F)) (AFNOR, 2002).

2.4.2. From grape stems (GS)

To estimate the microbial population in the grape stems (GS), the same protocol described above was performed using 25–35 g of GS mixed with 100 mL of sterile physiological water and homogenised by stomacher for 2 min and decimal dilutions with sterile physiological water (NaCl; 8.5 g/L). The same spreading dilution quantities that were used for grape juice enumeration were applied on PDA (3.5 pH) with two replicates at the same incubation conditions. The CFU of filamentous fungi number per millilitre of

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