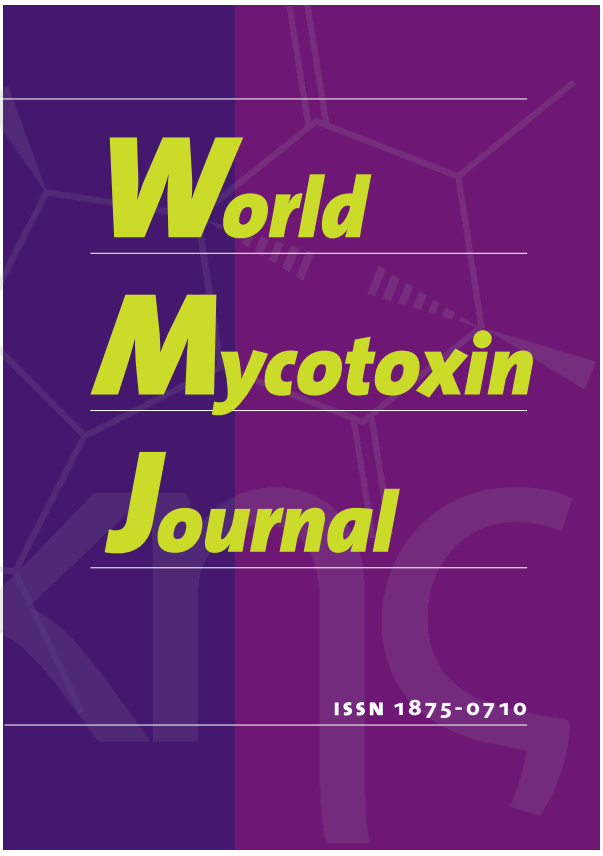

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Effect of *Kluyveromyces thermotolerans* on *polyketide synthase* gene expression and ochratoxin accumulation by *Penicillium* and *Aspergillus*

M.L. Ponsone[1,2], Y.G Kuhn[1], M Schmidt-Heydt[3], R. Geisen[3] and S.N. Chulze[1,2]

[1]Orientación Micología, Departamento de Microbiología e Inmunología, Universidad Nacional de Río Cuarto, Ruta Nacional N° 36 Km 601, 5800 Río Cuarto, Córdoba, Argentina; [2]Members of the Research Career of CONICET; [3]Max Rubner-Institut, Haid-und-Neu-Straße 9, 76131 Karlsruhe, Germany; lponsone@exa.unrc.edu.ar

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RESEARCH PAPER

Abstract

In a previous study, it was demonstrated that *Kluyveromyces thermotolerans* strains can reduce both growth and ochratoxin A (OTA) accumulation by *Aspergillus* section *Nigri* strains. There is no information about the mechanisms related to this reduction. A viable hypothesis can be that the presence of biocontrol agents can affect OTA biosynthesis by influencing the transcriptional activity of the polyketide synthase (pks) gene, one of the key enzymes in the OTA biosynthetic pathway. The aims of this work were to determine the effect of two selected strains of *K. thermotolerans* as potential biocontrol agents and to evaluate if their presence can affect the *otapks* gene expression of ochratoxigenic *Aspergillus* and *Penicillium* species. Growth, OTA and ochratoxin B (OTB) biosynthesis by the fungal strains at the phenotypic and molecular levels were monitored. The results obtained showed that both *K. thermotolerans* strains evaluated had a strong influence on growth, OTA and OTB biosynthesis, and expression of the mycotoxin biosynthesis genes. However, no direct correlation between the influence of the biocontrol yeasts on *pks* gene expression, OTA and OTB production could be found. These results could indicate an inhibitory mechanism by the yeasts, which apparently involve a post-transcriptional mechanism. The data obtained could imply that the production of mycotoxins can be regarded as a kind of adaptation mechanism to environmental stress conditions by these mycotoxigenic species.

Keywords: biocontrol, *Kluyveromyces thermotolerans,* ochratoxigenic species, ochratoxins, polyketide synthase

1. Introduction

Ochratoxins are a group of mycotoxins produced by some *Aspergillus* and *Penicillium* species, which can colonise a range of food products. *Aspergillus* species like *Aspergillus carbonarius, Aspergillus westerdijkiae, Aspergillus steynii, Aspergillus niger* or *Aspergillus ochraceus* are mainly responsible for the occurrence of ochratoxin A (OTA) in food products like grapes, coffee, cocoa or spices (Abarca *et al.,* 2003; Thirumala-Devi *et al.*, 2001), whereas the *Penicillium* species occur either in cereals, like *Penicillium verrucosum,* or in NaCl and protein rich foods, like *Penicillium nordicum*. In the latter products, *P. verrucosum* can also be found occasionally. *P. verrucosum* and *P. nordicum* are more common in the cool damp conditions of northern Europe, whereas *Aspergillus* species are more common in the warmer climatic regions of the world. OTA has been shown to have nephrotoxic, immunotoxic, genotoxic, neurotoxic, and teratogenic properties. The International Agency for Research on Cancer has classified OTA as a possible human carcinogen, group 2B (IARC, 1993). Based on both the available scientific toxicological and exposure data, the European Commission has established 2 µg/kg as the maximum permitted level for OTA in wines, musts and grape juice, 10 µg/kg for raisins, 5 µg/kg for cereals, 5 and 10 µg/kg for coffee and instant coffee, respectively, and 15 µg/kg for spices (EC, 2006).

Structurally, OTA comprises a dihydrocumarin moiety linked to a L-β-phenylalanine molecule derived from the shikimic acid pathway by an amide bond. Several related

compounds were also reported to occur in cultures of OTA-producing organisms, such as the dechlorinated analogue ochratoxin B (OTB), the isocoumarin nucleus of OTA, ochratoxin α (OTα) and its dechlorinated analogue ochratoxin β that are not linked to phenylalanine, methyl and ethyl esters including ochratoxin C, which is an ethyl ester derivative of OTA and several amino acid analogues (Moss, 1996, 1998; Xiao *et al*., 1995). The OTA biosynthetic pathway has not yet been elucidated in any fungal species, although is it clear that the pathway involves some crucial steps such as: (1) the biosynthesis of the isocoumarin group through the catalysing action of a polyketide synthase (PKS); (2) its ligation with the amino acid phenylalanine through the carboxyl group in a reaction catalysed by a peptide synthetase; and (3) the chlorination step. However, the order of the reactions has not yet been well defined (Gallo *et al.,* 2012).

Most of the molecular aspects of OTA biosynthesis have been elucidated in *Penicillium* species. In *P. nordicum*, a putative OTA biosynthetic cluster has been identified containing biosynthetic genes encoding a PKS (*otapks*PN), a non-ribosomal peptide synthetase (*otanps*PN), putatively responsible for the formation of the peptide bond between the polyketide and the phenylalanine, a gene with some homology to chlorinating enzymes (*otachl*PN), thought to be involved in the chlorination step, and a gene with some homology to a transporter protein hypothesised to be involved in OTA export (*otatra*PN) (Geisen *et al.* 2006; Karolewiez *et al.* 2005). The orthologues for these genes have also been identified for *P. verrucosum*. So far, in *Aspergillus* spp., only the pks genes involved in the initial steps of the pathway have been characterised (Bacha *et al.,* 2009; O'Callaghan *et al.,* 2003,). In *A. carbonarius,* Gallo *et al.* (2009) identified a *pks* gene whose expression pattern showed correlation to OTA production, and recently a non-ribosomal peptide synthetase gene was characterised (Gallo *et al.,* 2012).

Growth prevention of OTA-producing fungi is the most effective strategy for controlling the entry of this mycotoxin into the food and feed chains. The resistance to fungicides of major plant pathogens and the public concern over pesticide residues in food and the environment have increased the interest in alternative methods for disease control (Zhang *et al*., 2007). Biological control has been proposed as a strategy to reduce the impact of ochratoxigenic species. Among the microorganisms considered as potential biological control agents, yeasts are particularly promising, due to their capacity of colonising plant surfaces or wounds for long periods under dry conditions (Bleve *et al.,* 2006; Dimakopolou *et al.,* 2008), their simple nutritional requirements, the capacity to grow in fermenters on inexpensive media, and also the ability to survive in a wide range of environmental conditions without production of anthropotoxic compounds (Wilson *et al.*, 1989).

In a previous study, we have demonstrated that *Kluyveromyces thermotolerans* can control growth and OTA accumulation by *Aspergillus* section *Nigri* strains (Ponsone *et al.,* 2011). At present, there is no information about the possible mechanisms implied in this control. A viable hypothesis could be that the presence of biocontrol agents can affect OTA biosynthesis by affecting the transcription patterns of the pks enzyme, one of the key enzymes in the OTA biosynthetic pathway. This study was focussed to evaluate the effect of *K. thermotolerans* on growth, OTA *pks* gene expression, and OTA and OTB accumulation by ochratoxigenic *Penicillium* and *Aspergillus* species..

2. Materials and methods

Strains and culture conditions

The following strains have been used throughout the study: *P. verrucosum* BFE808, *P. nordicum* BFE487, *A. niger* BFE631 (Max Rubner Institute culture collection, Karlsruhe, Germany), *A. carbonarius* RC13I and two strains of *K. thermotolerans*, strains RCKT4 and RCKT5 (Department of Microbiology and Immunology, Universidad Nacional de Río Cuarto culture collection, Río Cuarto, Argentina). Two ml of 10^6 cells/ml of each yeast strain evaluated was added to Petri dishes containing 20 ml of YES media (20 g/l yeast extract, 150 g/l sucrose, 15 g/l agar) before solidification. Prior to yeast addition, the medium was maintained at 45°C on a water bath. After medium solidification, a 10 µl central drop of a conidial suspension (10^4 ml) of each *Aspergillus* and *Penicillium* strain was inoculated onto the Petri dish. Control plates without the yeast strains were included. The conditions for growth and ochratoxin accumulation were: incubation on YES agar for 6 and 10 days at 25 °C under dark conditions. The experiment was performed in triplicate and repeated twice.

Assessment of colony growth rate

For measuring the radial mycelial growth rate, two perpendicular diameters of the growing colonies were measured daily until the colony reached the edge of the plate. The radii of the colonies were plotted against time and linear regression was applied to obtain the growth rate (mm/day) as the slope of the line. The lag phase for growth was defined as the time (days) for the colony to reach a diameter of 10 mm.

Quantitative determination of ochratoxin A and B

For OTA and OTB determination, two agar plugs (diameter 1 cm) of the respective colony was taken from the region between the centre and the edge of the colony with the aid of a sterile corer. The 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. Thereafter, the mycelia were discarded and the chloroform extract was evaporated to dryness in a vacuum concentrator (Speed Vac; Savant Instruments, Farmingdale, NY, USA). The extracts of the triplicates were merged for quantitative determination of OTA and OTB, performed as described by 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 consisted of 250 mM ortho-phosphoric 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: at 0 min – 60% solvent A + 40% solvent B; at 7 min – 40% solvent A, 60% solvent B; at 12 min – 35% solvent A, 65% solvent B; at 16 min – 5% solvent A, 95% solvent B; at 27 min – 60% solvent A, 40% solvent B. The limit of quantification was 25 pg on column. Data collection and handling was done with EZ-Chrome Elite 3.2 (Agilent, Santa Clara, CA, USA). All standards used were obtained from Sigma (Taufkirchen, Munich, Germany) with a purity of ≥98%.

Isolation of RNA from fungal cultures

To perform real-time PCR experiments, RNA was isolated using the RNAeasy Plant Mini kit (Qiagen, Hilden, Germany). 1 g of mycelium was taken from one of the replicated plates of the growth experiment and grounded using a mortar and pestle in liquid nitrogen. 250 mg of the resulting powder was then resuspended in 750 µl lysis buffer, mixed with 7.5 µl beta-mercaptoethanol and 100 glass beads with a diameter of 1 mm (B. Braun Biotech International GmbH, Melsungen, Germany) in a 2 ml RNAse-free micro reaction tube. The extracts were mixed thoroughly and incubated for 15 min at 55 °C and 42 kHz in an S10H ultrasonic bath (Elma, Singen, Germany). All further procedures were essentially the same as suggested by the manufacturer of the kit. For cDNA synthesis, 8 µl of the DNAse I-treated total RNA was used along with the Omniscript Reverse Transcription kit (Qiagen). The reaction mixture was essentially as described by the manufacturer and incubated at 37 °C for 1 h. The cDNA was either directly used for real-time PCR or stored at -20 °C.

Reverse transcriptase real-time PCR

The reverse transcriptase real-time PCR reactions to measure the expression of the *otapks*AC, *otapks*AN, *otapks*PN, and *otapks*PV genes in *A. carbonarius, A. niger* aggregate, *P. nordicum* and *P. verrucosum,* respectively, were performed essentially as described by Schmidt-Heydt *et al.* (2007). Briefly, the qPCR core kit for SYBR Green (Eurogentec, Liege, Belgium) was used according to the recommendations of the manufacturer. For each reaction, 1 µl DNA sample solution was mixed with 2.5 µl reaction buffer, 1.5 µl $MgCI_2$ (50 mM), 1.0 µl dNTP-Mix (2.5 mM), 1 µl of each primer (20 µM), 0.75 µl SYBR Green, 0.25 U AmpErase UNG, 1.0 µ1 AmpliTag Gold, and 15.88 µl aqua bidest. The following temperature profile was used: l cycle of 50 °C for 2 min); l cycle of 95 °C for 10 min; 40 cycles of 95°C for 20 s, 55 °C for 20 s and 72 °C for 30 s; l cycle of 55 °C for 20 s); l cycle of 72 °C for 30 s. The same primer pair was used for both *Aspergillus* section *Nigri* strains, since homology between both *pks* was previously tested and good homology was observed. The primer pairs to generate *otapks*AC and *otapks*AN specific internal gene fragments for SYBR Green fluorescence measurement had the following sequences: *otapks*_SYBR_ for (5'GCCGCCAGCAACCTGATCTTT3') and *otapks*_ SYBR_rev for (5'GCTATACCCTCACCCCGTGCATAG3'). For *otapks*PV specific internal gene fragments, the following sequences were used: *otapks*PV_SYBR_ for (5'TTGCGAATCAGGGTCCAAGTA3') and *otapks*PV_ SYBR_rev for (5'CGAGCATCGAAAGCAAAAACA3'). The *otapks*PN gene was amplified with the primer pair *otapks*PN_SYBR_ for (5'TACGGCCATCTTGAG CAACGGCACTGC3') and *otapks*PN_SYBR_rev for (5'ATGCCTTTCTGGGTCCAGTA3').

Statistical analysis

Statistical analysis was performed using SigmaStat for Windows Version 2.03v (SPSS Inc., Armonk, NY, USA). To evaluate the effect of the yeast strains on growth rate and lag phase, *pks* expression, and OTA and OTB production by the four fungal species analysis of variance (ANOVA) was done. When the analysis was statistically significant, the Fisher (LSD) multiple-comparison test was used for separation of the means. Statistical significance was judged at the level $P<0.05$. To evaluate the correlation between *otapks* expression and production of ochratoxins the Pearson test was applied ($P<0.05$)

3. Results and discussion

Growth and OTA and OTB accumulation by the fungal strains at the phenotypic and molecular levels were monitored in relation to *A. carbonarius* RC13I, *A. niger* BFE631, *P. nordicum* BFE487 and *P. verrucosum* BFE808. Under the conditions evaluated, both *K. thermotolerans* (RCKT4 and RCKT5) strains were able to reduce growth of the *Penicillium* and *Aspergillus* strains assayed. The *Penicillium* strains were more sensitive to the presence of the yeasts compared to the *Aspergillus* species, mainly due to their growth kinetics. Exponential growth of the *Penicillium* strains started one day later than that of the *Aspergillus* strains, and at 10 days of growth the *Penicillium* colonies did not reach the edge of the Petri dish, in contrast

to the *Aspergillus* strains. The disadvantage in growth kinetics increased in the presence of the yeast strains, as these strains fully colonised the Petri dishes within 24 h of incubation. Under conditions of competition for space and nutrients, both yeast strains were able to control fungal growth by decreasing growth rate and increasing the lag phase (P<0.05) (Figure 1).

The effect of *K. thermotolerans* strains on *pks* expression showed a correlation between the expression of the mycotoxin biosynthetic genes and the phenotypic production of OTA an OTB (Figure 2). External growth parameters could moderate OTA and OTB biosynthesis via their influence on gene transcription. It is important to remark that the correlation between *pks* gene expression and OTA production was not directly proportional. An increase in *otapks* transcription does not imply a proportional increase in OTA and OTB (Figure 2). One explanation for these results could be a post-transcriptional regulation mechanism or different half-life of the respective mRNA populations. In addition, it should be kept in mind that the production of OTA and OTB is an accumulation process, whereas the transcriptional status is non-static, e.g. the time point of measurement also plays a role in the correlation between transcription and OTA and OTB concentrations.

On the other hand, another viable explanation could be OTA degradation by the toxigenic strains. This is supported by previous studies demonstrating that some *Aspergillus* section *Nigri,* along with some *Penicillium* species, were able to degrade OTA to other metabolites, such as OTα. Varga *et al.* (2000) tested the kinetics of degradation at 30 °C during 10 days and found that OTA was fully converted to the much less toxic OTα within 5 days. Abrunhosa *et al.* (2002) achieved similar results; at 25 °C and 6 days of incubation, 95% of *Aspergillus* section *Nigri* isolated were able to degrade 80% of OTA present in the culture media. A similar percentage of OTA degradation was also found for *Penicillium* strains. More recently, Gallo *et al.* (2012) studied the kinetics of OTA degradation during 25 days at 25 °C by an *A. carbonarius* strain, and found more than 99% of OTA to be degraded to OTα during a two-day incubation period. It is important to remark that the present study was carried out under the same incubation conditions as the studies mentioned, which support our hypothesis of OTA degradation.

Another mechanism could also be toxin adsorption to the yeast cell wall, as previously reported in studies carried out by Bejaoui *et al.* (2004), Caridi *et al.* (2006), Cecchini *et al.* (2006) and Garcia Moruno *et al.* (2005).

In relation to the data on *otapks*AC and OTA accumulation by *A. carbonarius* RC13I, the results showed an overexpression of *otapksAC* in the presence of *K. thermotolerans* strains during both incubation periods (6 and 10 days), however, OTA production was reduced after 10 days of incubation (Figure 2A). The data on *A. niger* BFE631 showed that at 6 days of incubation in the presence of *K. thermotolerans* strains *otapks*AN expression was similar to or lower than the control; OTA accumulation was lower in all cases, except for strain RCKT5. At 10 days of incubation, *otapks*AN expression was higher in

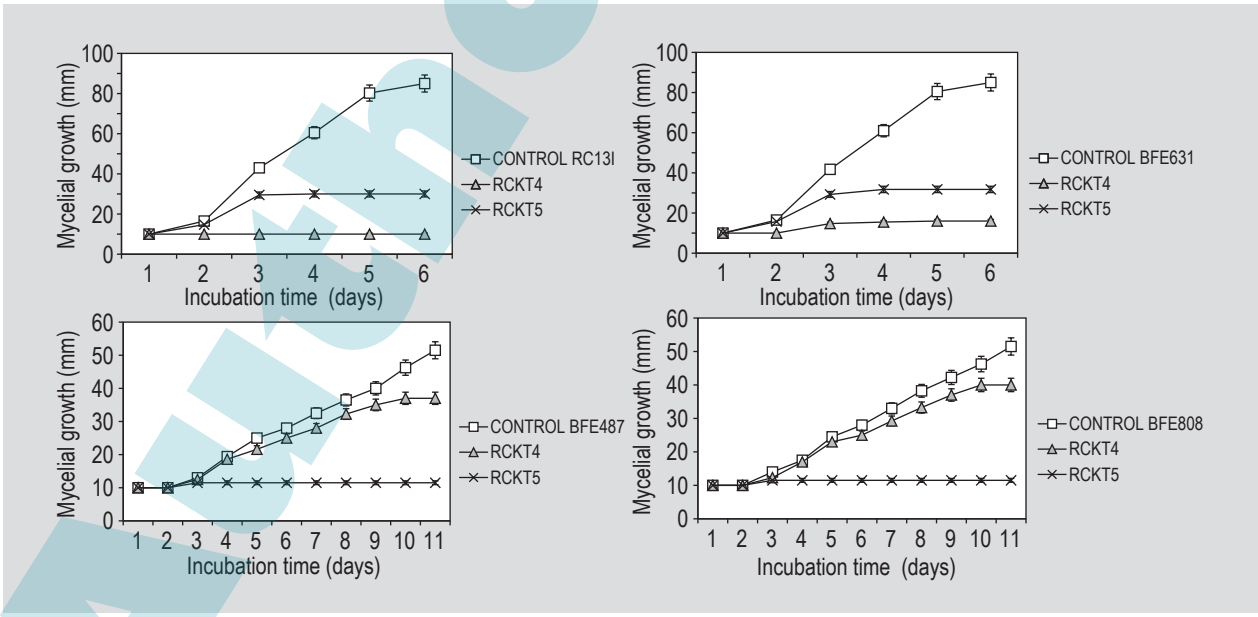

Figure 1. Growth of (A) *Aspergillus carbonarius* RC13I, (B) *Aspergillus niger* BFE631, (C) *Penicillium nordicum* BFE487 and (D) *Penicillium verrucosum* BFE808 (D) in the absence (control) and presence of *Kluyveromyces thermotolerans* strains RCKT4 and RCKT5, respectively, on yeast extract sucrose agar at 25 °C.

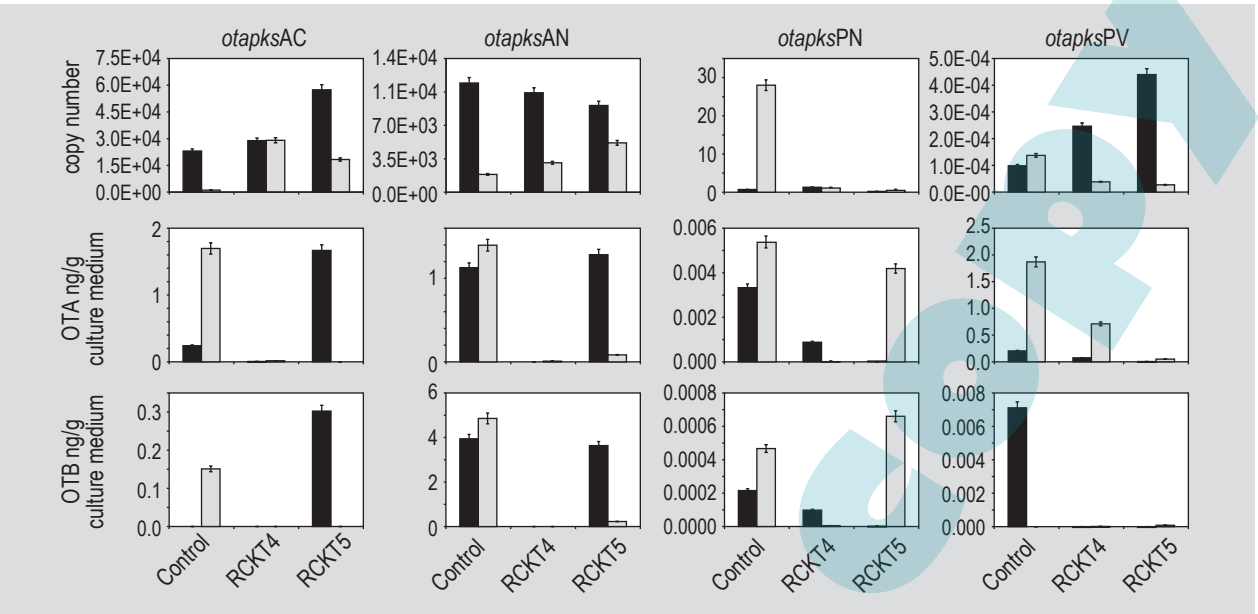

Figure 2. *Otapks*PV, *otapks*PN, *otapks*AC and *otapks*AN gene expression per mass of mycelium, ochratoxin A (OTA) and ochratoxin B (OTB) accumulation by (A) *Aspergillus carbonarius* RC13I, (B) *Aspergillus niger* BFE631, (C) *Penicillium nordicum* BFE487 and (D) *Penicillium verrucosum* BFE808, in the absence (control) or presence of *Kluyveromyces thermotolerans* strains RCKT4 and RCKT5), respectively.

the presence of the *K. thermotolerans* strains, however, OTA accumulation showed an opposite behaviour. In the control, higher OTA levels were found than in the presence of the *K. thermotolerans* strains (Figure 2B). This could be explained by some kind of activation by the inhibiting yeast. Apparently, *A. niger* tried to counteract the OTA inhibiting activity of the yeast by an increased expression of the OTA biosynthesis genes.

According to Gallo *et al.* (2012), OTB could be an intermediate compound in the OTA biosynthetic pathway. Our results agree with that hypothesis, as the kinetic behaviour of OTA and OTB production, under the conditions evaluated, was similar (Figure 2A-B).

The results obtained with *P. nordicun* BFE 487 showed that OTA and OTB accumulation was strongly dependent on the presence or absence of the yeast strains. Activation of the *otapks*PN gene in the control cultures was directly correlated with accumulation of OTA and OTB, whereas in the presence of the yeast strains no direct correlation was observed ($P<0.05$). There was a significant reduction ($P<0.05$) of OTA and OTB accumulation in the presence of the yeast strains compared to the control. These results partially agree with those obtained by Schmidt-Heydt and Geisen (2007), who tested the influence of different environmental conditions, such as pH, temperature and medium composition, on OTA accumulation and *pks* expression. It was observed that growth conditions favouring the expression of the OTA biosynthesis genes also resulted in biosynthesis of OTA. This indicates that the influence of abiotic parameters was mediated via the regulation of the transcription of the OTA biosynthetic genes. Our study showed that the data obtained in the control were similar to those observed by Schmidt-Heydt and Geisen (2007). However, the influence of biotic parameters on OTA and OTB accumulation and the kinetics of toxin accumulation was quite different, indicating that another regulation mechanisms in the OTA biosynthetic pathway could also be involved (Figure 2C).

In the case of *P. verrucosum* BFE808, the activation of the *otapks*PV gene was measured some time before reasonable amounts of OTA were detected. Similar results were previously observed by Geisen and Schmidt-Heydt (2009). In the control cultures, the kinetics of *otapks* expression and OTA accumulation were positively correlated, whereas in the presence of the yeast strains the correlation was negative ($P<0.05$). This suggests once again some other kind of regulation mechanism in the OTA biosynthetic pathway (Figure 2D). The overall analysis of the results showed some kind of post-transcriptional regulation of OTA and OTB accumulation in the presence of antagonistic *K. thermotolerans* strains.

The production of OTA and OTB can be regarded as an adaptation to biotic, e.g. the presence of *K. thermotolerans*, and other stress conditions by these mycotoxigenic species. Whether the activation of mycotoxin biosynthesis is the cause or the consequence of a stress reaction cannot yet be concluded. Similar results have been observed by O'Callaghan *et al.* (2003), who showed a higher level

of expression and OTA biosynthesis by *A. ochraceus* in permissive medium (pH values ≤7.3) compared to restrictive medium (pH ≤5.6). Also, food preservatives such as sorbate and propionate showed both gene expression stimulation and toxin production by *P. verrucosum* (Schmidt-Heydt *et al.*, 2007). Therefore, care is needed to ensure that control strategies are able to inhibit growth, limiting the potential for activation of the mycotoxin biosynthesis genes and thus mycotoxin accumulation.

According to the results presented here, we can conclude that the presence of the *K. thermotolerans* strains can reduce OTA and OTB accumulation by *Penicillium* and *Aspergillus* strains. *Penicillium* strains seemed to be more sensitive to the presence of the biocontrol yeasts strains and the inhibitory effect apparently could involve a post-transcriptional mechanism. Finally, the efficacy of *K. thermotolerans* RCKT4 and RCKT5 to reduce OTA and OTB accumulation by *Aspergillus* and *Penicillium* strains in culture media is promising. Further studies with these potential biocontrol strains under greenhouse and field conditions, in grapes and cereals, and during storage of dry cured meat will be necessary to validate these biocontrol agents.

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