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The effect of substrate on mycotoxin production of selected *Penicillium* strains

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Abstract

Analytical methods are presented for detecting simultaneously 11 fungal metabolites (aflatoxins B1, B2, G1 and G2, citrinin, cyclopiazonic acid, mycophenolic acid, ochratoxin A, penicillic acid, penitrem A and roquefortine C) on different matrices. The methods were applied to determine the mycotoxins produced by different *Penicillium crustosum*, *Penicillium nordicum* and *Penicillium verrucosum* strains on yeast extract sucrose (YES) agar and cheese and bread analogues and are based on high-performance liquid chromatography (HPLC) and photodiode array detection (PDA). The growth substrate had a distinctive effect on the mycotoxin production ability of the fungi examined. The *P. crustosum* strains produced roquefortine C on all the substrates, with the highest amounts being detected on the cheese analogue. Penitrem A was synthesised on the cheese analogue only. The strains of *P. verrucosum* produced exclusively citrinin on YES, but both ochratoxin A and citrinin were detected in considerable amounts on the bread analogue. On the bread, toxin profiles varied significantly between the individual *P. verrucosum* strains. The cheese analogue was not favourable for the mycotoxin production of this species. The growth substrate had the least effect on the toxin production of the *P. nordicum* strains, which synthesised ochratoxin A in moderate amounts on all three media.

Keywords: Fungi; Mycotoxin; *Penicillium*; Substrate

1. Introduction

Fungal growth occurs under favourable environmental conditions and is associated with the production of a wide range of secondary metabolites, many of which can be hazardous to vertebrates. These metabolites are collectively called mycotoxins. The amount of nutrients available, the ambient temperature, water activity and oxygen are the most important factors governing the growth and mycotoxin production of fungi (Filtenborg et al., 2000). Moulds are able to grow on all kinds of food (Filtenborg et al., 1996). However, the reason why a

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particular species will predominate in one product is often not known, although it is thought to be correlated with the species characteristics and the properties of the product (Filtenborg et al., 2000).

The conditions which promote toxin production are usually more restricted than those for growth. The prediction, of which mycotoxins may be produced in which foods, can be based on a knowledge of the specific mycobiota of foods and data from laboratory experiments. However, the actual presence of mycotoxins in a food product can be evaluated only after chemical or biological analysis of the product itself (Filtenborg et al., 2000), as the toxin profiles and the amounts produced depend entirely on the ecological and processing parameters of the foodstuff (Filtenborg et al., 1996).

Penicillium species are frequent contaminants of different food products and are known to produce a variety of secondary metabolites (Frisvad and Filtenborg, 1989). *Penicillium crustosum*, *Penicillium nordicum* and *Penicillium verrucosum* are regarded as being food-borne fungal species of particular importance in temperate regions of the world (Frisvad and Thrane, 2000). *P. crustosum* has been found in various foods, including nuts (Overy et al., 2003), fruits, meats, cereals (El-Banna and Leistner, 1988) and cheeses (Lund et al., 1995). *P. verrucosum* and *P. nordicum* are related species of which *P. verrucosum* is primarily encountered on cereals and *P. nordicum* on meat (Larsen et al., 2001; Castella et al., 2002). Both species have also been isolated from cheese (Lund et al., 1995; Larsen et al., 2001).

The use of the secondary metabolite profiles forms an important part of fungal classification and identification, and a number of methods have been developed for screening large numbers of mycotoxins on pure agar culture (Filtenborg et al., 1983; Frisvad, 1987; Smedsgaard, 1997). However, there are very few published multimycotoxin methods for analysing several toxins simultaneously in complex foodstuffs. Thin layer chromatographic (TLC) methods have been developed to determine mycotoxins in cheese (Siriwardana and Lafont, 1979; Taniwaki and van Dender, 1992). Rundberget and Wilkins (2002) presented a multimycotoxin method based on liquid chromatographic–mass spectrometric (LC–MS) analysis for determining 13 fungal metabolites in a food mixture.

Although it is a recognised fact that the mycotoxin profile of a fungal species is dependent on the growth media and conditions, there have been only a few studies published comparing the toxin profiles of fungi growing on different substrates, including food. Engel (1978) investigated the mycotoxin production capacity of certain *Aspergillus* and *Penicillium* species on yeast extract sucrose (YES) agar and cheese. In this study, we have examined the mycotoxin production of selected *P. crustosum*, *P. nordicum* and *P. verrucosum* strains on three different substrates—YES, a cheese analogue and a bread analogue. Simple methods comprising solvent extraction, high-performance liquid chromatographic (HPLC) determination and photodiode array detection (PDA) are presented for analysing simultaneously 11 mycotoxins in these matrices.

2. Materials and methods

2.1. Reagents

Methanol, acetonitrile, ethyl acetate, dichloromethane, diethyl ether and phosphoric acid were purchased from J.T Baker (Deventer, Holland) and were either of HPLC or analytical grade. Formic acid was from Merck (Darmstadt, Germany) and was of analytical grade. Trifluoroacetic acid and NaCl were purchased from Sigma (St. Louis, USA). The standards for aflatoxin B1 (AF B1), aflatoxin B2 (AF B2), aflatoxin G1 (AF G1), aflatoxin G2 (AF G2), citrinin (CIT), cyclopiazonic acid (CPA), mycophenolic acid (MPA), ochratoxin A (OTA), penicillic acid (PEA), penitrem A (PEN A) and roquefortine C (ROQ) were purchased from Sigma (St. Louis, USA). The stock solutions of the mycotoxin standards were prepared either in methanol or in acetonitrile. Further dilutions were made in 50% acetonitrile.

2.2. Fungal strains and substrates

The strains selected for the study were 16 different *Penicillium* strains, i.e., four *P. crustosum*, four *P. nordicum* and eight *P. verrucosum* strains, obtained from the IBT-collection (BioCentrum-DTU, Denmark).

The media used for examining mycotoxin production of the strains were YES (Singh et al., 1991) and cheese and bread analogues. For the preparation of YES, Difco yeast extract and Bacto agar (Becton, Dickinson and Co., Sparks, USA) were used. The cheese analogues were prepared at BioCentrum-DTU, Denmark. Thereby, 15 g of agar (Bie and Berntsen, Rødovre, Denmark) was added to 500 ml of deionised water and autoclaved at 121 °C for 15 min. Then 400 g of melted cheese was mixed with the warm agar. Portions (20 ml) of the final mixture were transferred to Petri dishes. The analogue consisted on average of 51% of water, 24.5% of fat, 21.6% of protein and 2.9% of ash. Bread analogues were prepared at Cranfield University, UK, as described by Needham and Magan (2003). Bread dough was prepared by mixing wheat flour (500 g), margarine (50 g), salt (2.5 g), baking powder (2.5 g), yeast extract (2.5 g), glycerol (50 g) and water (300 ml). Approximately 3-mm-thick discs of 75 mm in diameter were cut from the dough, wrapped in aluminum foil and autoclaved at 121 °C for 15 min.

To examine the toxin production on pure culture, each of the *Penicillium* strains was first cultivated on Czapek yeast autolysate agar (CYA; Singh et al., 1991) at +25 °C for 7 days and then inoculated on YES. The yeast extract and agar used in the preparation of CYA were the same as for YES (see above). The cheese analogues (20 ml) and slices of bread analogues (20–30 g) were inoculated with 100 μl of 10^6 ml^{-1} spore suspension. All of the inoculations were preformed in duplicate. The YES cultures and bread analogues were incubated at +25 °C for 7 days and the cheese analogues at +20 °C for 11 days.

2.3. Sample preparation

Mycotoxins were extracted from the agar plates with a slight modification of the method of Smedsgaard (1997). Five plugs of 1 cm in diameter were cut from the fungal colonies and 1.8 ml of methanol–dichloromethane–ethyl acetate (1:2:3) containing 1% (v/v) formic acid was added. The sample was vortexed (MS 2 Minishaker, IKA-Werke, Staufen, Germany) for 45 s, sonicated (Branson 2510, Branson, Dunbury, USA) for 15 min and vortexed again for 45 s. Thereafter, the sample was centrifuged (Heraeus Megafuge 2.0 R, Kendro, Langenselbold, Germany; 5 min, 3000 rpm, +5 °C). The supernatant was transferred into a vial and evaporated to dryness under a stream of nitrogen at +50 °C. The dry residue was redissolved in 0.2 ml of 50% acetonitrile, mixed and filtered through a 0.2-μm filter into an autosampler vial.

A 20-g sample of cheese analogue was extracted with 45 ml of dichloromethane and 5 ml of 0.2 M phosphoric acid by homogenising the sample with Ultra Turrax (IKA-Werke, Staufen, Germany) for 5 min. The sample was centrifuged (Beckman J2-21 M, Palo Alto, USA; 15 min, 4000 rpm, +10 °C), and the supernatant was filtered through a filter paper (Whatman 41, Whatman, Maidstone, England). Then 30 ml of the filtrate was transferred into a separating funnel and shaken with 40 ml of 20% methanol. If necessary, NaCl was added to break down any emulsion formed. The dichloromethane layer was transferred into a round bottom flask and evaporated to dryness with a rotary evaporator (Büchi, Flawil, Switzerland). The dry residue was dissolved in 5 ml of methanol and quantitatively transferred into a test tube, and 5 ml of hexane plus a few drops of water was added to the sample. After vortexing the sample and letting the solvent layers separate, the hexane layer was discarded and the partition repeated with 4 ml of hexane and 0.5 ml of diethyl ether. The nonpolar layer was discarded, and the sample (methanol phase) evaporated to dryness under a stream of nitrogen at +50 °C. The sample was redissolved in 0.2 ml of 50% acetonitrile, mixed and filtered through a 0.2-μm filter into an autosampler vial.

A slice of bread analogue (20–30 g) was extracted with 60 ml of dichloromethane and 10 ml of 0.2 M phosphoric acid by homogenising with Ultra Turrax for 10 min. The sample was centrifuged (15 min, 4000 rpm, +10 °C), and the supernatant was filtered through a filter paper (Whatman 41). Then 40 ml of the filtrate was transferred into a round bottom flask and evaporated to dryness with a rotary evaporator. The dry residue was dissolved in 5 ml of methanol and quantitatively transferred into a test tube. Hexane (6

ml) and a few drops of water were added to the sample. After vortexing and letting the solvent layers separate, the hexane layer was discarded and the partition repeated with 5 ml of hexane and 1 ml of diethyl ether. The nonpolar layer was discarded, and the sample evaporated to dryness under a stream of nitrogen at +50 °C. The sample was redissolved in 0.2 ml of 50% acetonitrile, mixed and filtered through a 0.2-μm filter into an autosampler vial.

2.4. HPLC analysis

All the samples were analysed with the same HPLC procedure. Separation of the analytes was performed on a Symmetry C-18 column (3.9×150 mm, 5 μm) with a guard column (Waters, Milford, USA) with gradient elution using an Alliance 2960 Separations Module (Waters, Milford, USA). The injection volume was 50 μl. The solvents used were water and acetonitrile, both containing 0.05% (v/v) trifluoroacetic acid. An isocratic elution with water–acetonitrile (75:25) was maintained at 0.7 ml/min flow for the first 26 min, after which the gradient was raised to 5% water and 95% acetonitrile, and the flow increased to 1 ml/min over 14 min. The detection of the mycotoxins was accomplished with a Waters 996 PDA detector (Waters, Milford, USA) at the maximum wavelength of each toxin. The identification of the mycotoxins was based on the comparison of the UV-spectra and the retention times of the detected peaks with those of the standard substances.

3. Results and discussion

3.1. Method

The methods used were simple, inexpensive and allowed the extraction and separation of the mycotoxins of interest. The sample preparation for the YES samples was simple, but complex matrices, i.e., the cheese and bread analogues, needed a more extensive clean up procedures to remove fat and protein. Partition with 20% methanol was necessary for the cheese analogues to remove excess water and hydrophilic interfering substances. These methods can be used to analyse some of the essential secondary metabolites of both *Aspergillus* and *Penicillium* strains. In this study, the methods were applied for *Penicillium* toxins solely.

A maximum of 10 mycotoxins could be analysed and separated in a single HPLC run with the method used. Roquefortine C could not be determined in the same run with the aflatoxins because it produced a broad peak, which interfered with the detection of aflatoxins. In practice, this was not a problem inasmuch as the strains examined were not known to be able to produce aflatoxins. Aflatoxins B2 and G1 could not be separated to the baseline, but the resolution was regarded as adequate. The peak of citrinin was also broad, but it did not affect the chromatographic separation, as there were no peaks eluting near citrinin. An example of a chromatogram of a standard mixture with aflatoxins is presented in Fig. 1. The maximum UV absorbances for the investigated mycotoxins determined from the standard

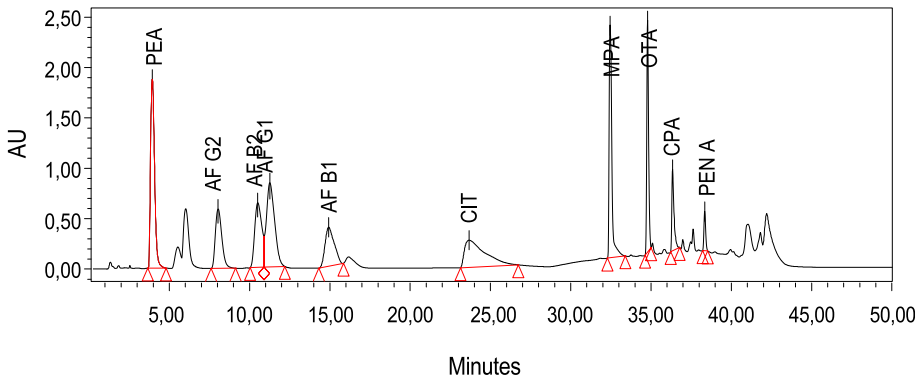

Fig. 1. Chromatogram of a standard mixture showing the separation of 10 mycotoxins possible to analyse with the method developed. Peaks are extracted from the maximum absorbance of each mycotoxin.

Table 1
UV maxima of the mycotoxins investigated

Mycotoxin	UV maxima (nm)[a]
Penicillic acid	227
Roquefortine C	204, 234, 304
Aflatoxin G2	217, 265, 367
Aflatoxin B2	198, 219, 266, 363
Aflatoxin G1	200, 219, 264, 367
Aflatoxin B1	198, 224, 265, 363
Citrinin	214, 328
Mycophenolic acid	214, 250, 304
Ochratoxin A	216, 331
Cyclopiazonic acid	223, 279
Penitrem A	236, 301

[a] At the elution conditions at the retention time of each mycotoxin, the detection wavelength is underlined.

mixture are listed in Table 1. It should be noted that the UV spectrum of a compound is dependent on the composition of the eluent and its pH, and thus, the wavelengths presented here may differ slightly from those published earlier (Frisvad and Thrane, 1987).

3.2. Mycotoxin production

The fungi examined were able to grow on all of the substrates with the incubation periods used. However, the growth of *P. nordicum* strains and five of the *P. verrucosum* strains (IBT 5252, 10039, 13077, 22025, 22123) was only moderate on YES.

The strains of *P. crustosum* were able to produce roquefortine C and penitrem A. These compounds have been reported to be the most important toxins produced by this species (Frisvad and Filtenborg, 1989). Roquefortine C was produced moderately on the bread analogue and YES. Although cheese is in general regarded as a poor substrate for mycotoxin production (Engel, 1978; Scott, 1989), in this study the cheese analogue favoured the toxin production of *P. crustosum*. In particular, roquefortine C was synthesised in high amounts on the cheese analogue. Penitrem A was also produced on this medium but not on YES or on the bread analogue.

P. nordicum was able to produce ochratoxin A. However, none of the substrates favoured the production, as the strains synthesised ochratoxin A only moderately or not at all. The *P. verrucosum* strains synthesised both ochratoxin A and citrinin. In comparison to the situation with *P. nordicum*, the mycotoxin production of *P. verrucosum* strains was more influenced by the media. In particular, the cheese analogue proved to be an unsuitable substrate for the toxin production of *P. verrucosum*, as only one strain synthesised ochratoxin A on this substrate. On YES, citrinin was produced in considerable amounts by almost all the strains of *P. verrucosum*, but ochratoxin A was absent. The bread analogue was a suitable substrate for the toxin production of *P. verrucosum*, and high levels of both ochratoxin A

Table 2
Toxin production of the selected strains on different growth substrates

Fungal strain (IBT number)	Toxin production on YES agar	Toxin production on cheese analogue	Toxin production on bread analogue
P. crustosum (14747)	ROQ+	PEN A+, ROQ+++	ROQ+/–
P. crustosum (16885)	ROQ+	PEN A+, ROQ+++	ROQ+/–
P. crustosum (20233)	ROQ+	PEN A+, ROQ++	ROQ+/–
P. crustosum (22279)	ROQ+	ROQ+++	ROQ+/–
P. nordicum (12803)	OTA+	CPA+/–, OTA+	–
P. nordicum (14745)	OTA+/–	OTA+	OTA+
P. nordicum (19486)	OTA+/–	CPA+/–	–
P. nordicum (22073)	OTA+/–	OTA+	OTA+
P. verrucosum (5252)	CIT++	–	OTA+++, CIT+++
P. verrucosum (10039)	CIT++	OTA+	OTA+++, CIT+
P. verrucosum (13077)	–	–	OTA++
P. verrucosum (21491)	CIT++	–	CIT++
P. verrucosum (22025)	–	–	OTA+++
P. verrucosum (22116)	CIT+	–	CIT++
P. verrucosum (22123)	CIT+	CPA+/–	OTA+++, CIT+
P. verrucosum (22502)	CIT++	–	–

(–) = not detected, plus sign (+) = detected, double plus sign (++) = moderate amount detected, triple plus sign (+++) = large amount detected, plus/minus (+/–) = ambiguous detection due to matrix effect or a small chromatographic peak.

and citrinin were detected. However, there were major variations in the toxin profiles produced by these strains. Small amounts of cyclopiazonic acid were also detected on the cheese analogue inoculated with the strains of *P. nordicum* and *P. verrucosum*. However, this is the first study to report the production of cyclopiazonic acid by these species. As the amounts of cyclopiazonic acid detected were small, and the UV spectrum is not highly specific, the findings should be further confirmed with a mass spectrometric method. The toxin profiles of the strains examined are presented in Table 2. Examples of the chromatograms for the cheese and bread analogues inoculated with *P. verrucosum* IBT 10039 are presented in Fig. 2.

The ochratoxin A and citrinin production of *P. verrucosum* and *P. nordicum* is in accordance with earlier studies. Larsen et al. (2001) and Castella et al. (2002) showed that *P. verrucosum* and *P. nordicum* are closely related species, which are both able to produce ochratoxin A, but only *P. verrucosum* can produce citrinin. In this study, *P. nordicum* strains were only moderate ochratoxin A producers, which is in disagreement with the observations of Castella et al. (2002), who reported that *P. nordicum* strains synthesised high amounts of ochratoxin A both on YES and CYA. On the other hand, the findings concerning ochratoxin A production of *P. verrucosum* are similar in these studies. Castella et al. (2002) found that the ochratoxin A production of *P. verrucosum* strains was more influenced by the media than that of *P. nordicum* strains. Additionally, *P. verrucosum* synthesised ochratoxin A only moderately on YES in their study.

The results demonstrate the distinctive effect of the substrate on the secondary metabolism of the fungi examined. Many fungi have rather simple nutrient requirements, and all foodstuffs for human consumption represent sufficient carbon and nitrogen sources

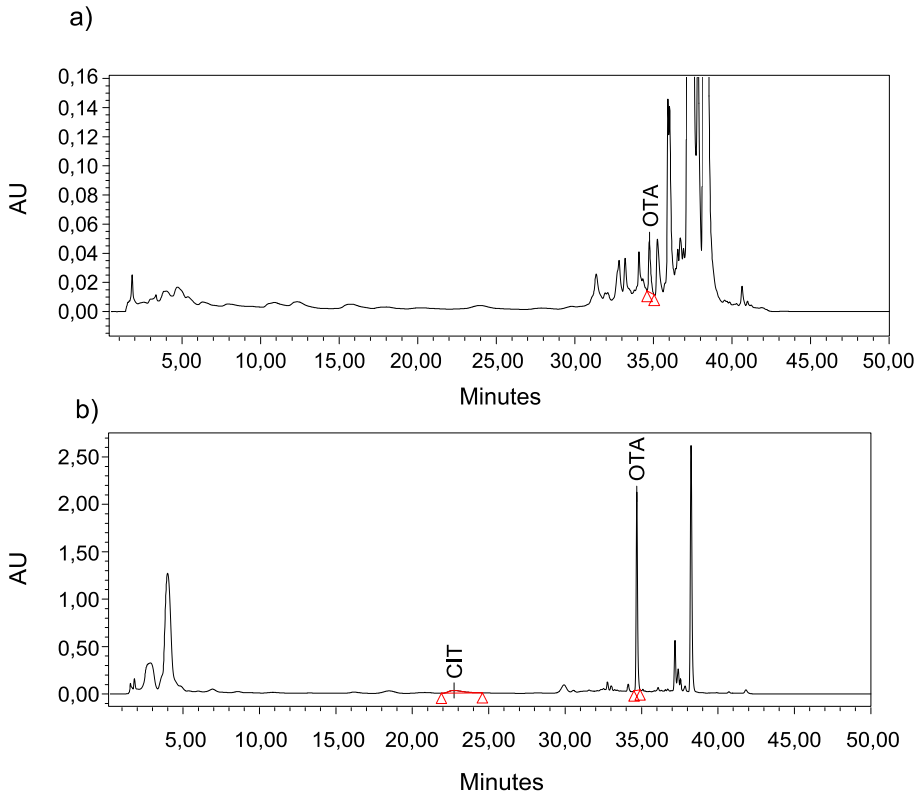

Fig. 2. Chromatograms of the cheese analogue (a) and bread analogue (b) inoculated with *P. verrucosum* IBT 10039. Chromatograms are extracted from the wavelength 330 nm to illustrate the differences in ochratoxin A and citrinin production of the strain between these two substrates.

and also provide all other necessary nutrients for fungal growth (Filtenborg et al., 2000; Carlile et al., 2001). However, foods cannot be automatically regarded as being suitable for maintaining the mycotoxin metabolism. In this study, the composition of the substrates was adequate to support the growth of the strains but not their mycotoxin production in all cases. The specific reasons why a fungal species predominates on a particular food product, which factors govern its mycotoxin production and what are their function, are still mostly unclear (Betina, 1989; Filtenborg et al., 2000). As mycotoxin production can occur in different environments, both under favourable and stress conditions (Magan et al., 2002), the specific factors, which directed the mycotoxin production to the pathways observed in this study, can only be speculated.

The mycotoxin production ability of *P. crustosum* on the cheese analogue and *P. verrucosum* on the bread analogue are likely to be linked with the substrates being similar to the natural habitats of these species. The ability of *P. crustosum* to produce penitrem A on the cheese analogue could be connected to the high protein content of this substrate, as amino acids are required in the synthesis of tremorgenic mycotoxins (Campbell, 1984), such as penitrems. The composition and moisture content of the bread analogue were probably unsuitable for penitrem A production. This toxin is synthesised only at high-water activities, i.e., above 0.92 a_w (ICMSF, 1996). The finding of the penitrem A indicates that the occurrence of *P. crustosum* in cheese should be regarded as a possible health risk. We are unable to find any reason for the considerable mycotoxin production and variable profiles seen with the *P. verrucosum* strains growing on the bread analogue. On the basis of these results, the quantitative toxin production is a strain specific characteristic, especially in the case of *P. verrucosum*.

YES is a highly nutritious medium and recommended for secondary metabolite analysis (Singh et al., 1991). In this study, rather moderate toxin production and simple toxin profiles were observed on YES. This could be explained by primary metabolism prevailing during the incubation period used, with secondary metabolism occurring only later. On the other hand, *P. nordicum* and some *P. verrucosum* strains grew weakly on this substrate, which might also have affected their mycotoxin production efficiency. This study demonstrates that it is necessary to use more than one medium for fungal metabolite screening; otherwise, the mycotoxin profile of a species can remain incomplete.

4. Conclusion

With the analytical methods used, the mycotoxins of interest could be determined semiquantitatively on different substrates. The selected *Penicillium* strains were able to produce various mycotoxins to different extents depending on the strain and the medium. The results of this study confirm the importance of using different substrates when examining the toxin-producing ability of a fungal strain.

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