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The influence of different nitrogen and carbon sources on mycotoxin production in *Alternaria alternata*

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ABSTRACT

The aim of this study was to determine the influence of different carbon and nitrogen sources on the production of the mycotoxins alternariol (AOH), alternariol monomethyl ether (AME) and tenuazonic acid (TA) by *Alternaria alternata* at 28 °C using a semi-synthetic medium (modified Czapek-Dox broth) supplemented with nitrogen and carbon sources. Additionally the effect of shaken and static cultivation on mycotoxin production was tested. Initial experiments showed a clear dependency between nitrogen depletion and mycotoxin production. To assess whether nitrogen limitation in general or the type of nitrogen source triggers the production, various nitrogen sources including several ammonium/nitrate salts and amino acids were tested. In static culture the production of AOH/AME can be enhanced greatly with phenylalanine whereas some nitrogen sources seem to inhibit the AOH/AME production completely. TA was not significantly affected by the choice of nitrogen source. In shaken culture the overall production of all mycotoxins was lower compared to static cultivation. Furthermore tests with a wide variety of carbon sources including monosaccharides, disaccharides, complex saccharides such as starch as well as glycerol and acetate were performed. In shaken culture AOH was produced when glucose, fructose, sucrose, acetate or mixtures of glucose/sucrose and glucose/acetate were used as carbon sources. AME production was not detected. The use of sodium acetate resulted in the highest AOH production. In static culture AOH production was also stimulated by acetate and the amount is comparable to shaken conditions. Under static conditions production of AOH was lower except when cultivated with acetate. In static cultivation 9 of 14 tested carbon sources induced mycotoxin production compared to 4 in shaken culture. This is the first study which analyses the influence of carbon and nitrogen sources in a semi-synthetic medium and assesses the effects of culture conditions on mycotoxin production by *A. alternata*.

1. Introduction

Mycotoxins are secondary metabolites of low molecular weight formed by a wide diversity of different moulds. Well-known mycotoxin producers are *Aspergillus*, *Fusarium, Alternaria* and *Penicillium* species. Fungi of the genus *Alternaria* contaminate fruits, vegetables and cereals both as plant-pathogens and saprophytes. Besides the economic loss due to pre-harvest and post harvest decay, *Alternaria* mycotoxin contaminated food can be ingested by both livestock and humans (Andersen et al., 2006; Logrieco et al., 2009; Patriarca et al., 2007). *Alternaria alternata* produces several different mycotoxins of which alternariol (AOH), alternariol monomethyl ether (AME) and tenuazonic acid (TA) are best studied (Coombe et al., 1970; Montemurro and Visconti, 1992; Pero et al., 1973; Rosett et al., 1957; Visconti et al., 1986). *Alternaria* is known to produce mycotoxins when growing on wheat (Li and Yoshizawa, 2000), sunflower seeds (Nawaz et al., 1997; Pozzi et al., 2005), oilseed rape (Nawaz et al., 1997), pecans (Schroeder and Cole, 1976), fruits and fruit juices (Delgado et al., 1996; Lau et al., 2003), carrots (Solfrizzo et al., 2004), tomato products (Andersen and Frisvad, 2004; da Motta and Valente Soares, 2001; Ozcelik et al., 1990) and grains (Broggi et al., 2007; Scott, 2001). *Alternaria alternata* can grow at low temperatures so that contamination of refrigerated foodstuffs during transport and storage is possible (Ozcelik et al., 1990). *Alternaria* toxins are connected to certain health disorders (Woody and Chu, 1992). The acute toxic effects of AOH and AME are quite weak: their LD_{50} is higher than 400 mg/kg of bodyweight for mice. However, AME is cytotoxic and shows synergistic effects with AOH (Pero et al., 1973). There are only a few studies which investigate the effects of *Alternaria* toxins in mammalian cells, but Brugger et al. (2006) showed that AOH is mutagenic in cultured cell lines. Furthermore Pfeiffer et al. (2007) demonstrated *in vitro* the DNA strand-breaking ability of AOH and AME. Because of the oncogenic potential of AOH (Liu et al., 1992) a connection to the incidence of oesophageal cancer in Linxian, China was suggested by Dong et al. (1987).

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Tenuazonic acid is produced not only by *Alternaria* species but also by *Phoma* and *Pyricularia* species (Iwasaki et al., 1972; Meronuck et al., 1972; Steyn and Rabie, 1976). Davies et al. (1977) reported the toxicity of TA to chicken embryos and rats where it caused haemorrhage and death. It is thus essential to investigate factors which induce or inhibit mycotoxin production. An increasing number of studies have demonstrated the role of environmental factors in mycotoxin production in many filamentous fungi (Georgianna and Payne, 2009; Schmidt-Heydt et al., 2008). Schmidt-Heydt et al. (2008) demonstrated that abiotic factors such as temperature, pH and water activity exert influence at the transcriptional level and enhance the expression of the biosynthetic genes. Water activity and temperature in particular are shown to influence the production of *Alternaria* mycotoxins (Hasan, 1995; Oviedo et al., 2010; Pose et al., 2010). Besides abiotic factors nutritional factors seem to affect mycotoxin production as well. Nitrogen-starvation induces the expression of genes for the biosynthesis of fumonisin in *Fusarium proliferatum* and ochratoxin in *Aspergillus* spp. (Kohut et al., 2009; Medina et al., 2008) whereas sufficient amounts of nitrogen caused a repression of fumonisin production (Shim and Woloshuk, 1999). In the same way other culture conditions such as composition of culture media, trace elements and carbon source markedly influenced ochratoxin production in *Aspergillus* species (Medina et al., 2004, 2008; Mühlencourt et al., 2004). Some authors studied the influence of carbon sources on ochratoxin production but the result depended on strain and medium used (Ferreira and Pitout, 1969; Lai et al., 1970; Medina et al., 2008). Nevertheless Medina et al. (2008) showed that differences in ochratoxin production can be related to the nature of the sugar.

Although AOH is thought to be formed by a polyketide synthase (PKS) (Gatenbeck and Hermodsson, 1965; Light, 1970; Wittowski, 1984), the biosynthetic genes are not known and the enzymatic pathways have still to be elucidated. AME is formed by methylation of AOH. The putative AOH-O-methyltransferase was partially purified and characterised by Stinson and Moreau (1986). The corresponding gene is still unknown. The tetramic acid TA is proposed to be formed from acetyl-CoA and isoleucine (Gatenbeck and Sierankiewicz, 1973). As well as the lack of knowledge of genetic controls, there are few studies on the influence of culture conditions on *Alternaria* toxin formation. The scope of this study is to examine the influence of nitrogen and carbon sources and different culture conditions on *Alternaria* toxin production in a semi-synthetic medium. This may be helpful in providing more information about the physiology and the metabolic pathways of mycotoxin production in *A. alternata* and it could provide a basis for the development of a process to produce AOH in larger amounts.

2. Materials and methods

2.1. Reagents and standards

Standards of alternariol, alternariol monomethyl ether, altenuene and tenuazonic acid were purchased from Sigma-Aldrich (Germany). All reagents, fine-chemicals and solvents were obtained from Roth (Germany), Becton, Dickinson and Company (BA, Germany) or Sigma-Aldrich (Germany).

2.2. Fungi and medium

A. alternata DSM 12633 was obtained from the DSMZ culture collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). All cultures of *A. alternata* were routinely grown on PDA (39 g/l potato dextrose agar, pH 5.6). Conidia were harvested from plates that were incubated 7 days at 28 °C with 25% glycerol and filtered through Miracloth (Calbiochem). Conidia were counted in a Thoma counting chamber and diluted to a concentration of 1×10^6 conidia per ml. Aliquots were stored at −80 °C. For the experiments modified Czapek-Dox medium was used (modified after Gatenbeck and Hermodsson, 1965): 10 g/l glucose (anhydrous), 0.06 g/l NH_4Cl, 0.25 g/l $NaNO_3$, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4\cdot7H_2O$, 0.25 g/l NaCl, 0.25 g/l KCl, 0.01 g/l $FeSO_4\cdot7H_2O$, 0.01 g/l $ZnSO_4\cdot7H_2O$, 1 g/l yeast extract, pH 5.5. Carbon sources and nitrogen sources were prepared separately and added after autoclaving. For the carbon test glucose was replaced by other carbon sources (see Section 2.4) and for the nitrogen test the mixture of ammonium chloride and sodium nitrate was replaced by other nitrogen sources (see Section 2.3). Carbon and nitrogen tests were both prepared in shaken and in static culture. For both culture conditions a volume of 20 ml of the respective medium was used. All cultures were inoculated with 850 conidia per ml medium. Static cultures were performed in standard disposable Petri dishes (Ø 85 mm), and the shaken cultures in 100 ml glass shake-flasks with baffles. For both culture conditions a time course was performed. Static cultures were kept for 5–12 days in the dark at 28 °C. Shaken cultures were cultivated on a rotary shaker at 140 rpm for 5–8 days at 28 °C in the dark. All conditions were performed in triplicate. Each sample was prepared in an individual flask or Petri dish and the entire contents used for analysis. Results were presented for the static cultures after 8 days, and for the shaken cultures after 7 days.

For the fermentation experiments 1.5 l of the basal modified Czapek-Dox medium at pH 5.5 was used. The process was operated in the small-scale bioreactor (vessel volume 2.0 l) Minifors (Infors, Switzerland) for 14 days at 28 °C in the dark. The medium (1.5 l) was inoculated with 1×10^6 conidia. Stirrer speed was increased from 400 rpm to 900 rpm after 48 h, and aeration rate was 0.013 vvm. For titration 0.2 M sodium hydroxide and 0.2 M phosphoric acid were used.

2.3. Nitrogen sources

For the nitrogen tests the nitrogen sources ammonium chloride and sodium nitrate of the modified Czapek-Dox medium were replaced. In these tests glucose was added as carbon source. The basal medium had a total nitrogen concentration of 56.8 mg/l. To ensure the same conditions for all samples the amount of all tested nitrogen sources was adjusted to a total nitrogen concentration of 56.8 mg/l. Apart from ammonium and nitrate salts several amino acids and urea were investigated: $NH_4Cl+NaNO_3$ (0.06 g/l + 0.25 g/l), NH_4Cl (0.217 g/l), $NaNO_3$ (0.345 g/l), NH_4NO_3 (0.162 g/l), $(NH_4)H_2PO_4$ (0.467 g/l), NH_4CH_3COO (0.313 g/l), KNO_3 (0.411 g/l), glycine (0.305 g/l), serine (0.427 g/l), proline (0.467 g/l), phenylalanine (0.671 g/l), arginine (0.177 g/l), asparagine (0.305 g/l), aspartate (0.54 g/l), glutamate (0.597 g/l) and urea (0.122 g/l). To elucidate the influence of yeast extract in the medium a control sample without additional nitrogen source was performed.

For the determination of ammonium and nitrate the photometrical assays “Ammonium-Test” (Spectroquant®, Merck, Germany) and “Nitrat-Test” (Spectroquant®, Merck, Germany) were used.

2.4. Carbon sources

For the carbon tests glucose was replaced by other carbon sources and the mixture of ammonium chloride and sodium nitrate was added as nitrogen source. In all samples the total carbon amount was 4 g/l and the concentration of all carbon sources was adjusted to this. A wide range of different carbon sources was tested: D-glucose (10 g/l), L-arabinose (10 g/l), D-xylose (10 g/l), D-galactose (10 g/l), L-rhamnose (10.11 g/l), D-fructose (10 g/l), D-maltose (10 g/l), D-sucrose (9.5 g/l), lactose monohydrate (10 g/l), starch (10 g/l), cellulose (10 g/l), glycerol (10.22 g/l), D-sorbitol (10.11 g/l) and sodium acetate trihydrate (22.66 g/l). In shaking flask experiments a combination of glucose/sucrose and glucose/sodium acetate was tested additionally. The total carbon concentration remained constant; the ratio of applied carbon was 1:1 (5 g/l glucose + 4.25 g/l sucrose) and 25:1 (9.62 g/l glucose + 0.91 g/l sodium acetate). To