Growth and mycotoxin production by *Aspergillus fumigatus* strains isolated from a saltern

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Abstract

Different microfungi were isolated from salty soil and hypersaline water samples, taken from the extreme environment of operating marine solar salters. The filamentous fungus most frequently and repeatedly isolated was identified as the pathogen *Aspergillus fumigatus*. Because of its great morphological variability and mycotoxin production ability, 50 *A. fumigatus* isolates were selected for additional characterisation by their secondary metabolites profile, which was determined by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). At standard conditions 45% of the isolated strains produced different mycotoxins and in ecological conditions prevailing in the saltern (high salinity and temperature) production of important mycotoxins (verruculogen, fumitremorgins B and C, fumigaclavine A) was lowered or inhibited.

Keywords: *Aspergillus fumigatus*; Mycotoxin; Saltern; Low water activity; Halotolerance

1. Introduction

The most extreme natural hypersaline environments are inland lakes and salters. In the Mediterranean natural park Seča, salt for human consumption is still being produced with a natural procedure of sea water evaporation, with the help of solar energy and wind. This extreme environment, originating from the 13th century, is protected under UNESCO Ramsar convention. The prevailing environmental factors present in summer are salinities that may range from the salinity of sea water (3%) to saturated solutions (32%) and high temperature [1]. Few investigations have been made regarding the biodiversity and occurrence of fungi in natural high salt environments and descriptions in the literature of xerophilic and xerotolerant fungi are mainly limited to those causing food spoilage [2].

Using different techniques and selective media, we have isolated filamentous fungi from numerous soil and water samples, taken at different sites in the abandoned and still active salters, during different seasonal and weather conditions. At lower water potentials species of the genera *Aspergillus, Penicillium* and *Cladosporium* dominated the active mycobiota [3]. *Aspergillus fumigatus* has been isolated repeatedly and with high frequency from salty soil samples as well as from hypersaline water samples, with salinities ranging from 3–15% NaCl.

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A. fumigatus is a widespread thermophilic and xerotolerant species, known to occur naturally on decomposing, self-heating plant material and organic debris from which it releases a high number of spores into the atmosphere [4]. Its importance as a pathogenic fungus has been stressed repeatedly. It causes allergic bronchopulmonary aspergillosis (ABPA), aspergillomas and invasive pulmonary aspergillosis [4,5]. It also produces several mycotoxins, the most known being fumitremorgins A and B and verruculogen, that act on the central nervous system, causing sustained tremors, convulsions and death in animals. Verruculogen is the most toxic, causing tremors in swine and sheep [5,6]. Even the most toxic of chemicals have little toxicological implications for man or animal unless they can be shown to occur in the environment or in food and are therefore a potential threat to human and animal health. From the literature, it is known that clinical A. fumigatus isolates often differ quite markedly from food borne or soil isolates [7]. Little data exist on secondary metabolite production of A. fumigatus isolates from natural habitats and so far growth, mycotoxin production and isolation of A. fumigatus strains from salterns has not yet been described.

2. Materials and methods

2.1. Strains

Forty A. fumigatus strains were isolated from salty soil, with salt concentration from 0.5–1 M NaCl and average pH from 7.0–7.9, using agar (MEA+0.5 M NaCl) baits, incubated in soil for 30 days and agar (MEA+0.5 M NaCl) soil dilution method [8]. Ten strains were isolated from hypersaline water (3–15% NaCl), pH 7.0–8.0, by filtering through sterile membrane filters (pore width 0.45 mm). Some of the samples were pretreated by incubation at 80°C for 30 min. Baits, diluted soil or filters were placed on the following selective media: GYMA and MEA+0.5 M NaCl, MEA+3 M NaCl, with added antibiotics (chloramphenicol, oxytetracycline) and incubated at 30°C and 50°C, up to 40 days. All isolated A. fumigatus strains have been included in the Slovenian Fungal Culture Collection (MZKI).

2.2. Taxonomic identification

For the taxonomic identification isolates were grown on 2% malt extract agar, oatmeal agar and Czapek agar and incubated for 7 days at 25°C and 37°C. The strains were identified using Raper and Fennell’s key for the genus Aspergillus [9].

2.3. Media

Growth rate and morphological characteristics were observed on the following media: Malt extract agar, MEA (aw = 0.999), MEA+0.5 M NaCl (aw = 0.984), MEA+3 M NaCl (aw = 0.878), MEA+4 M NaCl (aw = 0.828), yeast extract sucrose agar, YES (aw = 0.976), 50% glucose yeast extract maltose extract agar, GYMA (aw = 0.884) and malt extract 50% glucose agar, MY 50G (aw = 0.878). aw of the media were determined by the water activity system CX-1, Campbell Scientific Ltd.

2.4. Growth rate

Plates were inoculated in the centre with spores or with a mycelial fragment and incubated at 30°C and 50°C up to 40 days. Growth was measured with Mitutoyo (500-341) electronic meter. The diameters of all the 50 colonies were measured and mean diameters were calculated. The growth rate was expressed in mm/h [10]. Cultures were examined for conidia visually and with a light microscope.

2.5. TLC analyses

For TLC analyses 50 A. fumigatus strains were first inoculated on MEA and incubated for 7 days at 25°C, followed by inoculation on YES for the stimulation of extracellular metabolites (EM) and to Czapek yeast autolysate (CYA) medium for the stimulation of intracellular metabolite (IM) production, for 7 days at 25°C. For the determination of EM and IM production at conditions prevailing in the saltern, the strains were after growing for 7 days at 25°C on MEA inoculated on MEA+0.5 M NaCl and MEA+3 M NaCl and incubated for 14 days at 30°C and 50°C. The TLC agar plug method was used for screening for metabolite production [11]. The TLC (0.2 mm silicagal Merck 60 F 254) plates
were eluted for the detection of EM in toluene/ethyl-acetate/90% formic acid 5:4:1 (TEF) and after visualisation at normal light, 365 and 254 nm light followed by 0.5% (v/v) anisaldehyde in methanol/acetic acid/conc. sulphuric acid 17:2:1 spraying and heating for 8 min at 105°C. For the detection of IM the plates were eluted in chloroform/aceton/iso-propanol 85:15:20 (CAP) and after visualisation sprayed with Ce(SO₄)₂ in 3 M sulphuric acid and treated at 110°C for 8 min [11,12].

In certain cases the plates were sprayed also with 50% ethanol sulphuric acid [12]. Griseofulvin dissolved in chloroform/methanol 2:1 was used as standard in all cases and relative Rf values to griseofulvin were calculated as RfR. Mycotoxins were identified by descriptions in literature [12,13] and comparison with the available standards (helvolic acid, gliotoxin, fumagillin) was made.

2.6. HPLC analysis

Based on TLC results certain strains were selected for further identification of secondary metabolite production by high performance liquid chromatography (HPLC). Isolates were grown on CYA, YES, Sigma yeast extract sucrose agar (YES), and MEA for two weeks at 25°C. Three Petri dishes, one of each CYA, YES and SYES were placed in a Stomacher bag and extracted with chloroform methanol (2:1) for 3 min on a Colworth Stomacher. Further procedures were as described by Frisvad and Thrane [14]. The resulting extracts were analysed by a Hewlett Packard HP 1090 M HPLC with diode array detection DAD system [16].

3. Results and discussion

The combination of reduced a_w, type of solute present (NaCl), supported by solar heating at 40°C or more exert natural selection pressures on microorganisms living in the saltern and enable growth only of those developing adaptive mechanisms and physiological responses. Because it seems that fungal growth in the presence of high concentrations of solute is determined primarily by the water potential of the medium and not by the chemical nature of the solute [15], we were interested whether isolated A. fumigatus strains are physiologically adapted to the hypersaline environment in terms of growth rate and preference of solute lowering the a_w. All strains were grown on different media with a_w adjusted with NaCl or glucose from 0.99 to 0.828 at 30°C and 50°C. Although the minimal a_w for A. fumigatus growth is reported to be from 0.85–0.95 [2], some of them were able to grow even on 4 M NaCl, with a_w = 0.828, after 15 days of incubation at 30°C. The majority of strains had a higher growth rate on media with NaCl as controlling solute (Fig. 1). At a_w = 0.999 the sporulation started the 2nd or 3rd day, lowering of a_w to 0.878 delayed sporulation up to 18 days and at a_w = 0.828 the colonies did not sporulate at all. Effects of high temperature were more expressed on media with a_w below 0.976. Sporulation appeared after 7 days and was very scarce. Cultures that have been inoculated with mycelium instead with spores, had a higher growth rate both at low a_w and 50°C.

A. fumigatus isolates were morphologically more variable than described by Raper and Fennell [9], particularly differences in conidial ornamentation, occasional septation of the phialides and somewhat radiate shape of the heads were repeatedly observed. When water isolates were morphologically compared to soil isolates, the latter have been often less floccose, with more conidia, but could not be otherwise separated from soil isolates. The specificity of A. fumigatus production of secondary metabolite suggested an additional chemotaxonomic approach [16]. The intracellular and extracellular metabolite profiles of the 50 isolated A.
Fig. 2. HPLC trace of *Aspergillus fumigatus*. Metabolite 1 is fumigaclovine A, 2 is a puercatin, 3 is TR-2, 4 is trypoquinol, 5 is fumitremorgin C, 6 is trypacatin, 7 is monomethylsulochrin, 8 is a trypoquinol, 9 is an antraquinone, 10 is vescularlogen and 11 is fumagillin. Metabolites were in the 10 tested strains very similar.

*fumigatus* strains were compared, using the TLC agar plug method, at standard conditions. The strains were generally good producers of EM and had very homogenous profiles, ranging from 25–36 EM. TLC analyses have shown that 40% of strains produced verruculogen, 24% fumagilline and 18% fumitremorgin B. 12% of the strains synthesised verruculogen and fumagilline and 2% fumitremorgin and fumagillin. None of the tested strains synthesised all three mycotoxins at the same time in TLC detectable quantities. All isolates, that were additionally characterised by HPLC, produced fumagatin, fumigaclovine A, fumagillin, trypacatin, monomethylsulochrin, several trypoquinolins, trypoquinol, verruculogen, fumitremorgin C, a puercatin and an antraquinone. Gliotoxin and helvolic acid were not detected (Fig. 2). Although the growth rate was higher on media with NaCl as solute, the production of metabolites was more intense on media with glucose, at *a*$_w$ from 0.976–0.984.

At *a*$_w$ below 0.878, the production of secondary metabolites was considerably lowered and none of the mycotoxins investigated ( verruculogen, fumitremorgins, fumagillin) were detected, even after prolonged incubation.

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References


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