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Hydroxamate siderophores of *Scedosporium apiospermum*

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Abstract *Scedosporium apiospermum* is an emerging pathogen colonizing the airways of patients with cystic fibrosis and causing severe infections in immunocompromised hosts. In order to improve our knowledge on the pathogenic mechanisms of this fungus, we investigated the production of siderophores. Cultivation on CAS medium and specific assays for different classes of siderophores suggested the secretion of hydroxamates. A maximal production was obtained by cultivation of the fungus at alkaline pH in an iron-restricted liquid culture medium. Siderophores were then extracted from the culture filtrate by liquid/liquid extraction, and separated by reverse phase high performance liquid chromatography. Two siderophores, dimerumic acid and N^{α}-methyl coprogen B, were identified by electrospray ionization-mass spectrometry and MS–MS fragmentation. Finally, comparison of various strains suggested a higher production of N^{α}-methyl coprogen B by clinical isolates of respiratory origin. Studies are initiated in order to determine the potential usefulness of these siderophores as diagnostic markers of scedosporiosis.

Keywords *Scedosporium apiospermum* · Hydroxamate-type siderophores · N^{α}-methyl coprogen B · Dimerumic acid

SB, GL, AL and JPB are members of the ECMM (European Confederation of Medical Mycology)/ISHAM (International Society for Human and Animal Mycology) Working group on *Pseudallescheria/Scedosporium* infections, and of the ISHAM working group on fungal respiratory infections in cystic fibrosis.

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Introduction

Iron is an essential element involved in many important cellular processes such as DNA synthesis, respiration and detoxification of free radicals. Although it is widely distributed on Earth, iron is not easily accessible to microorganisms because it is mainly under the ferric state (Fe^{3+}) which is poorly soluble in water. In order to survive, most of the microorganisms have developed numerous mechanisms to scavenge iron (Howard 1999). One of them

is the secretion of siderophores which occurs when microorganisms grow in an iron-restricted environment. These small organic molecules have a very high affinity for iron and can create very stable complexes with ferric iron. These complexes are more soluble in water and therefore make iron available for fungi or bacteria. In pathogenic microorganisms, siderophores have been suggested as virulence factors since they can interact with some host iron-containing proteins like transferrin. Siderophores are able to remove iron from these proteins directly (Hissen et al. 2004) or in combination with a proteolytic process (Wolz et al. 1994). Furthermore, it has been demonstrated that siderophore biosynthesis is crucial for virulence in several human, animal or plant pathogenic microorganisms (Haas et al. 2008; Johnson 2008), including the opportunistic fungus *Aspergillus fumigatus* (Schrettl et al. 2004; Hissen et al. 2005).

Scedosporium apiospermum, formerly considered as the anamorph state of *Pseudallescheria boydii*, is a filamentous fungus encountered in polluted soil and water which may cause various infections resulting from traumatic inoculation of some soil fungal elements such as cutaneous/subcutaneous mycetoma, or from the inhalation of some airborne spores such as sinusitis and pulmonary mycetoma (Guarro et al. 2006). Besides, a large study on patients suffering from cystic fibrosis (CF) showed that it ranks the second among the filamentous fungi colonizing the respiratory tract of these patients (Cimon et al. 2000). More recently, it has been described as an emerging pathogen causing severe and often lethal infections in immunocompromised patients, particularly lung transplant patients (Guarro et al. 2006). Equally, numerous cases of disseminated scedosporiosis with cerebral involvement have been reported after near-drowning, resulting from haematogenous spread from a primary pulmonary site of infection (Katragkou et al. 2007). Additionally, *S. apiospermum* is a therapy-refractory pathogen since it is resistant or poorly susceptible to almost all current antifungals (Hennequin et al. 1997). For all these reasons, *S. apiospermum* is a challenging pathogen and there is an urgent need for a better understanding of the pathogenesis of scedosporiosis. Potential virulence factors have been identified in *S. apiospermum*, including a subtilisin-like serine-protease (Larcher et al. 1996) and a Cu, Zn superoxide dismutase (Lima et al. 2007). This study was therefore designed to identify siderophores in *S. apiospermum* since there is now accumulated evidence for their role as virulence factors in numerous pathogens.

Materials and methods

Microorganisms and culture conditions

Scedosporium apiospermum 90.4595, originally isolated from a broncho-alveolar fluid in the Laboratory of Parasitology-Mycology of Angers University Hospital, was used throughout this study. This strain was previously used for purification and characterization of a subtilisin-like serine protease (Larcher et al. 1996) and a Cu, Zn superoxide dismutase (Lima et al. 2007) and it was deposited at the IHEM (Institute of Hygiene and Epidemiology-Mycology section) culture collection (Scientific Institute of Public Health, Brussels, Belgium) under the accession number 15155. According to recent taxonomic changes (Gilgado et al. 2005, 2008), this strain was identified as *S. apiospermum* sensu stricto by sequencing of the internal transcribed sequence (ITS) regions (ITS1 and ITS2) of the RNAr genes. Other strains of the *S. apiospermum* complex, listed in Table 1, were also used for some experiments. All strains were routinely maintained on yeast extract-peptone-dextrose-agar (YPDA) plates containing 0.05% chloramphenicol with incubation at 37°C for 10–15 days.

Unless otherwise specified, the inocula used in this study were obtained from 7-day-old cultures on YPDA by scraping off the agar plates in sterile ultra-pure water (MilliQ Plus, $R = 18.2$ mOhm cm^{-1} at 25°C, Millipore), followed by three washes (15,000*g*, 5 min) of the mycelium in ultra-pure water.

Siderophore detection

A modification of the universal chemical assay of Schwyn and Neilands (1987) was used to detect siderophore production in an agar-based culture medium. Chrome azurol S (CAS) agar plates (Sigma Chemical) were prepared with 1/10 CAS indicator solution and 9/10 yeast morphology agar (YMA; Difco Laboratories). Plates were inoculated by

Table 1 Strains of *S. apiospermum* used in this study

IHEM number	Origin	Source	Date of isolation	Country of isolation
14263	Human (CF patient)	Sputum	09/01/98	Angers, France
14451	Human (CF patient)	Sputum	24/02/98	Giens, France
14268	Human (CF patient)	Sputum	08/01/98	Giens, France
14462	Human (CF patient)	Sputum	04/03/98	Tours, France
15155	Human	Bronchial aspiration	19/09/90	Angers, France
3817	Human	Cerebrospinal fluid	1986	Rotterdam, The Netherlands
6908	Human	Cutaneous/subcutaneous mycetoma	1992	Huy, Belgium
13947	Environment	Feathers of European starlings	27/12/93	Locarn, France
20812	Environment	Soil of potted plant	17/06/05	Angers, France
20828	Environment	Soil of potted plant	16/06/04	Angers, France

central spotting and then incubated for 2 weeks at 37°C. A positive reaction consisted in a color change of CAS reagent from blue to orange, leading to a clear visible red-orange halo around the fungal colony. Siderophore production was determined by measuring the diameters of the red-orange halo and of the fungal colony. Results are expressed as the CAS rates which correspond to the ratio between these two diameters.

Siderophore production

For study of the kinetics of siderophore production, *S. apiospermum* IHEM 15155 was cultivated in a liquid culture medium (LCM) consisting in a copper- and iron-depleted yeast nitrogen base (YNB)-glucose 2% (BIO101™, QBiogene) supplemented with amino acids (Complete supplement mixture, CSM, QBiogene) according to Lesuisse et al. (2001), except that pH was adjusted to different values (pH 4, 7 or 8).

Cultures were performed in 200-ml flasks containing each 50 ml of LCM. Agitation was achieved using a magnetic stirrer if indicated, and cultures were incubated at 37°C for 2–10 days. After centrifugation at 50,000*g* for 30 min at 4°C, the supernatant was clarified through 0.45-µm-pore size membranes, lyophilized and finally solubilized in 1 ml ultra-pure water for chemical assays for siderophore detection and characterization. Results were normalized to the mycelial dry weight determined after lyophilization of the fungal pellets. All experiments were performed in triplicate and mean values (±SD) were calculated.

Chemical assays for siderophore detection and characterization

Chemical assays were performed on concentrated culture filtrates. Production of phenolate/catecholate-type siderophores was investigated by the Arnow test (Arnow 1937) and absorption at 318 nm (Young et al. 1967). Hydroxamate-type siderophores were quantified by the periodate oxidation assay (Emery and Neilands 1962) and the ferric perchlorate test (Atkin et al. 1970). Catechol, 2,3-dihydroxybenzoic acid, acetohydroxamic acid and deferoxamine B mesylate (DFB) were obtained from Sigma and used as controls.

For the ferric perchlorate test, siderophore concentration was normalized as DFB equivalent. Standard curve was obtained with DFB, using serial twofold dilutions ranging from 0.25 to 0.004 mM. Results are expressed in µmol DFB equivalent per g of fungal biomass.

Siderophore extraction

The culture filtrate was supplemented with ferric chloride 0.1% and ammonium sulfate to reach 80% saturation. After stirring overnight at 4°C, the suspension was filtered through Celite® 521 (Aldrich) which gave the crude extract.

Extraction of siderophores was performed according to the methodology of Neilands (Neilands 1952). Siderophores were extracted 6 times with 50 ml of benzyl alcohol from 1 l of crude extract. The benzyl alcohol extract was then washed with 50 ml of an

ammonium sulfate saturated solution and finally dried by anhydrous sodium sulfate. To 300 ml of the organic phase, 3 l of diethylether were added and the siderophores were extracted 10 times with 100 ml of ultra-pure water. The aqueous phase was then washed with 100 ml of diethylether. Finally, water was evaporated under reduced pressure to obtain the siderophore extract.

HPLC–UV–Vis–ESI–MS identification

Siderophores were identified by high performance liquid chromatography (HPLC) which was carried out according to McCormack et al. (2003) using a quaternary pump (Separations Module, Waters 2795) and a Polymerex® RP-1 (40 × 4.00 mm, 5 μm, 100 Å, Phenomenex) stationary phase under a flow rate of 1 ml min^{-1}. The siderophore extract was solubilised in sterile ultra-pure water (1 g l^{-1}) and 20 μl of the obtained solution were injected in the column. The mobile phase consisted in a combination of 0.1% aqueous formic acid (eluent A), and 0.1% formic acid methanol (eluent B). Elution was conducted using a linear gradient starting from a mix of 95% eluent A/5% eluent B and ending after 20 min by a mix of 5% eluent A/95% eluent B, followed by a 10-min elution with 5% eluent A/95% eluent B. Siderophores were detected by UV–visible spectrophotometry at 254 and 435 nm (Dual λ Absorbance Detector, Waters 2487) and identified by positive electrospray ionization mass spectroscopy (ESI+-MS or ESI+-MS–MS; Esquire 300^{plus}, Bruker). The flow rate into the mass spectrometer was 150 ml min^{-1}.

Production of siderophores by the different strains studied was quantified on chromatograms obtained at 435 nm. Results were normalized to the mycelial dry weight.

Results

Detection of siderophores on solid culture medium

Scedosporium apiospermum IHEM 15155 was cultivated on CAS medium. After incubation for 2 weeks, a red/orange halo appeared around the fungal colony, attesting the chelation of iron in the culture medium and the secretion of siderophores by the fungus (Fig. 1). A red/orange halo was also observed around colonies for all the other stains tested on CAS medium. Slight variations were seen in the CAS rates which ranged from 1.5 to 5.8, and *S. apiospermum* IHEM 15155 ranked the second among all the strains tested with a CAS rate value of 5.3 (data not shown).

Siderophore type determination

Concentrated culture filtrate from *S. apiospermum* IHEM 15155 grown in LCM gave a negative reaction for the Arnow test and absorption at 318 nm which are specific for phenolates/cathecolates-type siderophores. Conversely, the periodate oxidation test and the ferric perchlorate test were positive, thus suggesting the secretion of hydroxamate-type siderophores in the culture medium, and identical results were obtained for all the other strains tested.

Determination of optimum conditions for siderophore production

In order to obtain an optimal siderophore production by *S. apiospermum*, different factors potentially influencing its production were studied: pH, incubation time

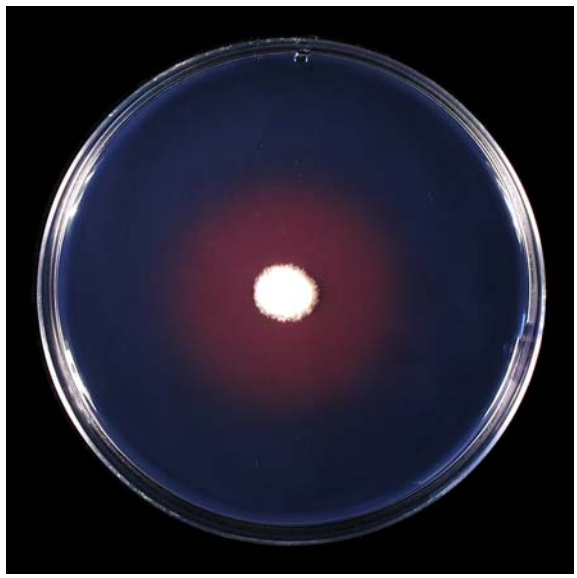

Fig. 1 Growth of *S. apiospermum* IHEM 15155 on CAS medium after 2 weeks of incubation. Note the large *red-orange halo* around the colony indicating iron complexation, and therefore the secretion of siderophores

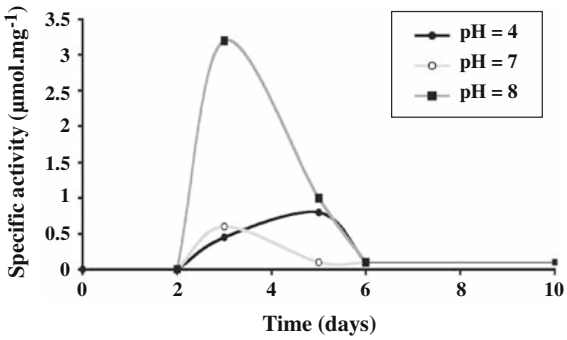

Fig. 2 Influence of culture conditions on siderophore production by *S. apiospermum* IHEM 15155

and agitation. Figure 2 shows that siderophore secretion by *S. apiospermum* was largely influenced by pH and the duration of incubation. Increasing the pH induced a significant increase in siderophore production which was maximal at pH 8. Study of the kinetics of siderophore production showed a maximum production after 4 days of incubation, which decreased drastically at day 5.

A very weak siderophore specific activity was observed when the fungus was cultivated under agitation (0.133 µmol mg^{-1}). Cultivation of the fungus without agitation appeared to be suitable for siderophore production since it resulted in a 20-fold higher specific activity (2.7 µmol mg^{-1}).

Siderophore identification

After extraction, siderophores were analyzed by LC–MS. The chromatogram at 435 nm showed two peaks with retention times of 3.1–4.2 and 5.7–6.2 min (Fig. 3A) corresponding to $m/z = 537.9$ and $m/z = 793.9$ (Fig. 4). The chromatograms of the extracted masses from the total ionic current fitted well with the 435 nm detection (Fig. 3). A bibliographic search for fungal siderophores showed that only two siderophores have been described with these molecular masses: dimerumic acid ($[M + Fe\text{-}2H]^+ = 537.9$) and methyl coprogen B ($[M + Fe\text{-}2H]^+ = 793.9$) (Fig. 5). MS–MS fragmentation of the $m/z = 537.9$ and $m/z = 793.9$ (Fig. 6) confirmed their identification as dimerumic acid and methyl coprogen B, respectively (Fig. 7). For this last metabolite, MS^3 fragmentation of the $m/z = 648.9$ molecular ion ($m/z = 519.9$ and $m/z = 537.9$) allowed us to determine the position of the methyl substituent and to identify it as N^{α}-methyl coprogen B.

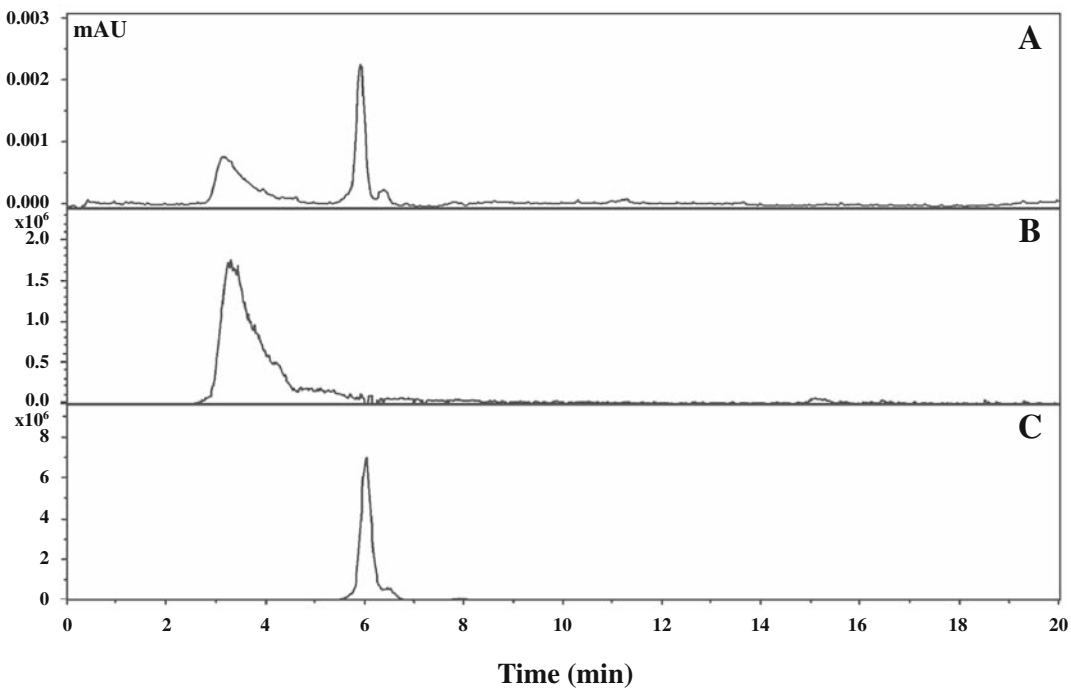

Fig. 3 RP–LC chromatograms of culture filtrate from *S. apiospermum* IHEM 15155. **A** Detection at 435 nm; **B** extracted mass for $m/z = 537.9$; and **C** extracted mass for $m/z = 793.9$

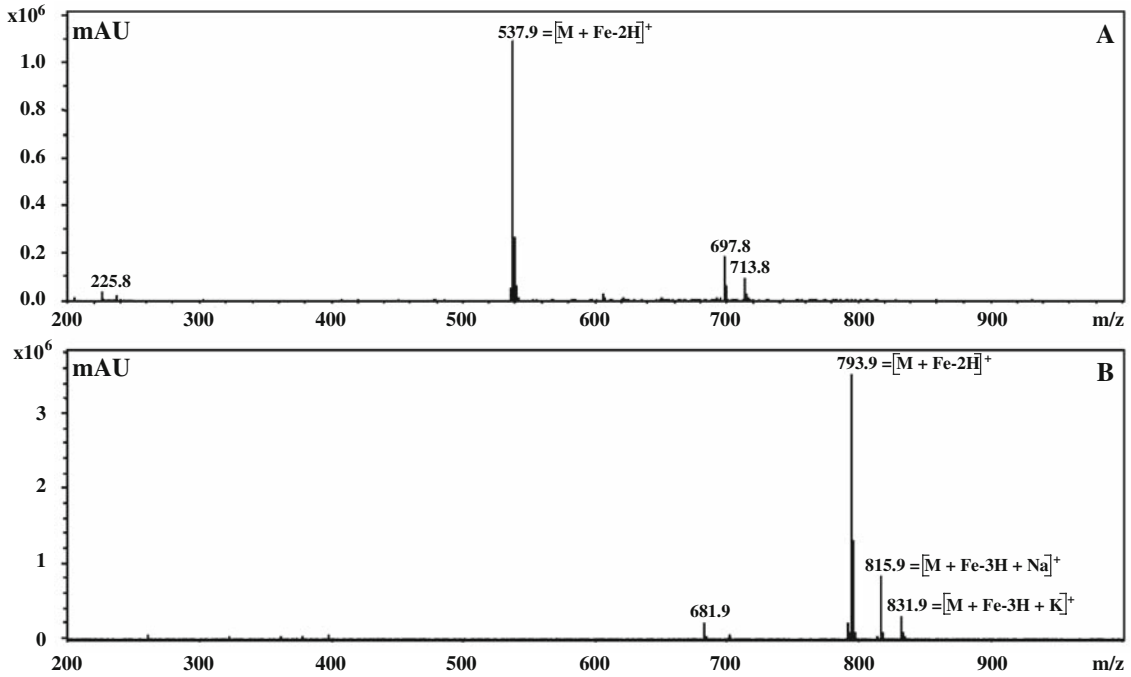

Fig. 4 ESI+-MS profiles according to peak retention time. **A** 3.1–4.2 min; and **B** 5.7–6.2 min

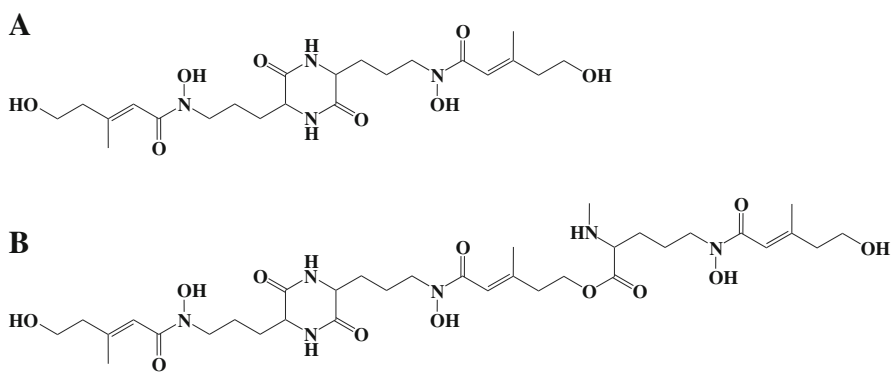

Fig. 5 Chemical structure of siderophores from *S. apiospermum*. **A** Dimerumic acid; and **B** N^{α}-methyl coprogen B

Comparison of siderophore production by different strains of *S. apiospermum*

The influence of the origin of the strain on siderophore production was investigated by cultivating ten strains of the *S. apiospermum* complex with various clinical or environmental origins in LCM adjusted to pH 7. Culture supernatants were analyzed by LC–UV after liquid/liquid extraction of the siderophores. Whereas only two strains (IHEM 14263 and 20812) were found to produce dimerumic acid, N^{α}-methyl coprogen B was detected in the culture supernatant for all the strains studied. In addition, the highest production of N^{α}-methyl coprogen B was seen for strains isolated from respiratory specimens and for strain IHEM 3817 which was recovered from a cerebrospinal fluid (Fig. 8).

Discussion

After a long period where the *S. apiospermum* complex was considered as a causative agent of cutaneous/subcutaneous or pulmonary mycetoma almost exclusively, the occurrence of this fungus in CF (Cimon et al. 2000) and a worrisome number of

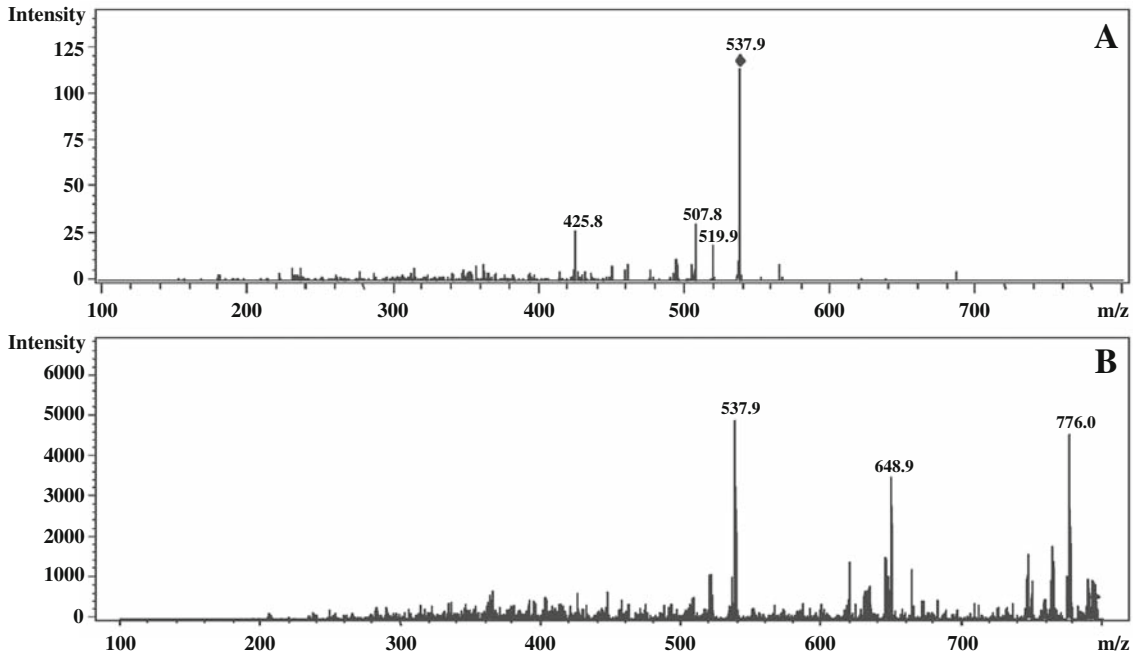

Fig. 6 ESI+-MS–MS profiles of $m/z = 537.9$ (**A**) and $m/z = 793.9$ (**B**)

problematic infections in case of corticosteroid-induced diabetes (a common complication of CF) (Guignard et al. 2008) or lung transplantation (which remains the ultimate treatment of CF patients) (Symoens et al. 2006), have generated a new interest in basic research on this fungus. However, very little information are still available on the pathogenesis of scedosporiosis. Only two putative virulence factors have been identified for *S. apiospermum*, i.e. an alkaline serine-protease belonging to the subtilisin subfamily (Larcher et al. 1996) and a Cu, Zn superoxide dismutase (Lima et al. 2007) which may help the fungus to invade the host tissues and to evade the host immune response, respectively.

Iron is required for many crucial processes of the fungal physiology and knowledge in the mechanisms developed by *S. apiospermum* for iron uptake is still in infancy. As far as we know, a unique publication, mainly focused on taxonomic purposes, suggested the secretion of siderophores for *S. apiospermum* (De Hoog et al. 1994). Our results allowed a definitive demonstration of the production of siderophores by this fungus by cultivation of various strains on CAS medium and the production of a red/orange halo around the colonies which attested the iron complexation by small molecules diffusing in the agar-based culture medium.

The optimal conditions for siderophore production by strain IHEM 15155 were then investigated. Our results showed that it was largely influenced by pH of the culture medium. Siderophore production monitored during fungal growth by the Arnow assay and normalized to the mycelial dry weight was fivefold higher at pH 8 compared to that observed at neutral or acid pH. This phenomenon is well known in other microorganisms (Neilands 1984). Under basic conditions, ferric iron is poorly soluble and forms ferric hydroxide precipitates (Raymond et al. 1984; Kraemer 2004), thus increasing iron stress and stimulating siderophore secretion. Conversely, acid conditions favor the solubility of ferric iron, facilitating iron acquisition by direct reduction mechanisms. Besides, some microorganisms release in their environment small acid molecules in order to decrease the pH and subsequently to increase iron solubility (Winkelmann 2007). At acid pH (like pH 4), iron stress is therefore lower, thus resulting in a reduced siderophore production. Agitation of the culture medium also largely influenced siderophore production since cultivation of the fungus without

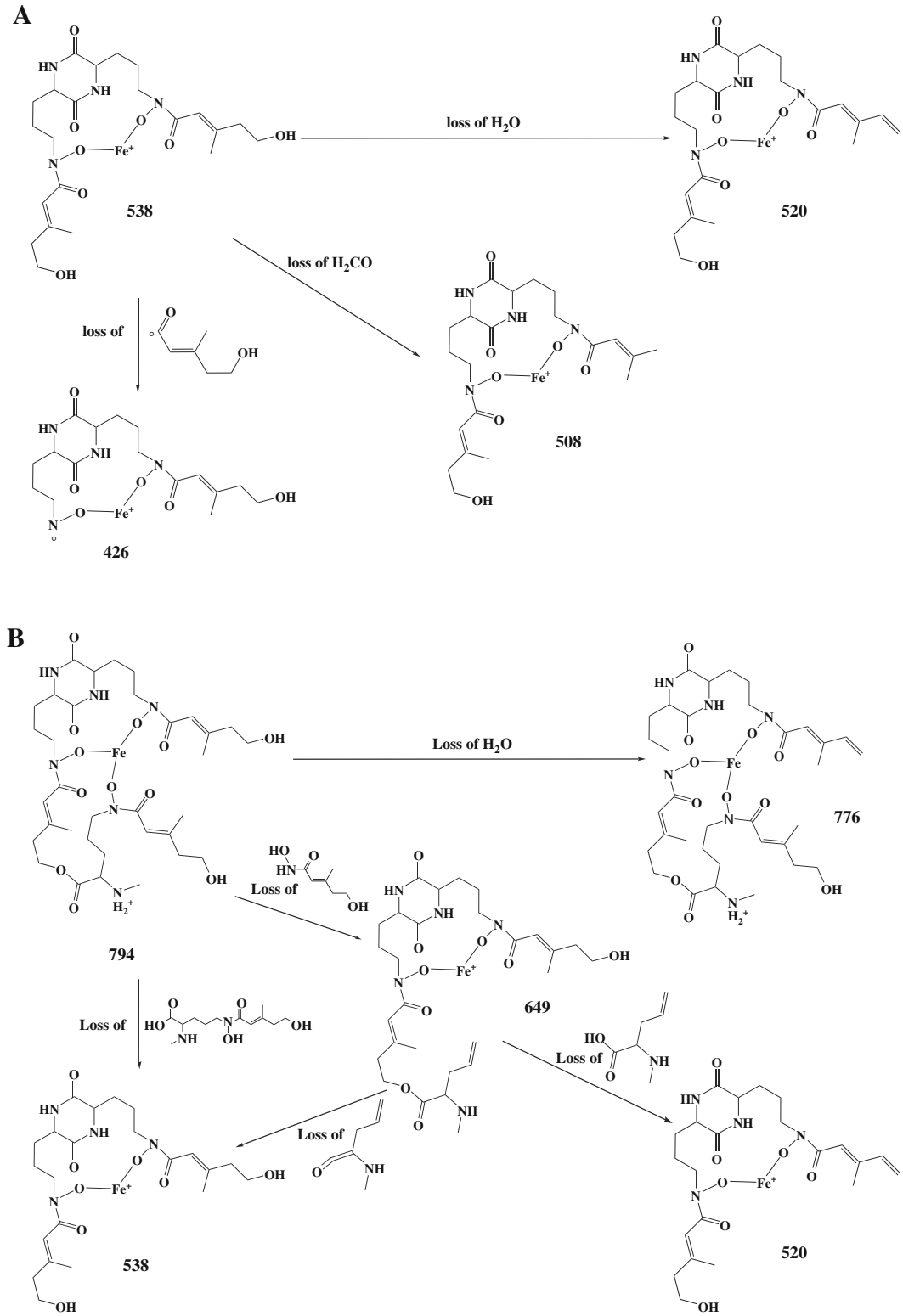

Fig. 7 Fragmentation scheme of iron chelate of dimerumic acid (**A**) or N^{α}-methyl coprogen B (**B**) by ESI+-MS

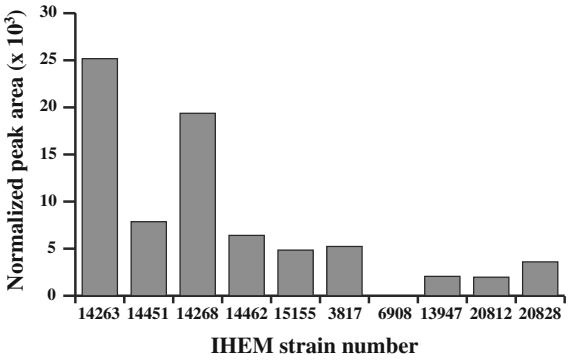

Fig. 8 Production of N^{α}-methyl coprogen B by different strains of the *S. apiospermum* complex as measured by HPLC–UV after culture in LCM adjusted to pH 7 and liquid/liquid extraction

agitation resulted in a 20-fold higher production compared to that observed with continuous stirring. Same results have been reported for other putative virulence factors of *S. apiospermum* such as the subtilisin-like serine proteinase (Larcher et al. 1996). This may be related to the different metabolic pathways used for fungal growth under aerobic or anaerobic culture conditions. Under aerobic conditions, the fungal metabolism requires iron-dependent proteins (Neilands 1984), such as cytochromes of the mitochondrial respiratory chain and Fe/S proteins. In contrast, agitation results in a reduced oxygen pressure, leading to fermentating metabolism. Under these conditions, the need for iron is markedly lower, thus resulting in a low siderophore production.

Optimal conditions for siderophore production were defined as cultivation of the fungus without any agitation for 4 days at 37°C in LCM adjusted to pH 8. This result is in agreement with previous studies on other fungal pathogens showing a maximal siderophore secretion for an incubation time ranging from 2 to 7 days (Neilands 1984). Compared to the mycelial dry weight, secretion of siderophores drastically decreased after 4 days of incubation, indicating a fast growth of *S. apiospermum* after siderophore secretion. It has been observed in some bacteria belonging to the genius *Frankia* that siderophores are secreted prior to the production of extracellular proteins (Boyer et al. 1999).

Growth conditions during lung colonization in CF patients are related to the optimum culture conditions for production of siderophores by *S. apiospermum*, including low iron availability (Lamont et al. 2009), neutral pH (Kodric et al. 2007) and aerobic conditions (stirring or not). The CF lung is also characterized by secretions with a high viscosity and a high sodium chloride concentration (Davis 2006). However, little information is available on the influence of these parameters on siderophore secretion by microorganisms colonizing the respiratory tract of CF patients. For *Bacillus subtilis*, it has been observed that an increase in sodium chloride concentration leads to a higher production of siderophores (Hoffmann et al. 2002). Moreover, siderophores of *Pseudomonas aeruginosa* can be detected in sputum samples from CF patients (Haas et al. 1991). Therefore, we can hypothesize that altered lung physiology in CF patients constitutes favorable conditions for siderophore production by *S. apiospermum*.

These culture conditions allowed us to characterize further the siderophores secreted by *S. apiospermum* IHEM 15155 which was conducted first by LC–MS. A bibliographic search for fungal siderophores revealed two already known siderophores for the masses detected, i. e. dimerumic acid for $m/z = 537.9$ and *N*-methyl coprogen B for $m/z = 793.9$ (Howard et al. 2000). These two siderophores belong to the hydroxamate class and more precisely to the family of coprogens which are secreted by several other fungi (Renshaw et al. 2002), including the Dematiaceous *Stemphylium botryosum* (Manulis et al. 1987) and the dimorphic pathogenic fungus *Histoplasma capsulatum* (Howard et al. 2000). Chemical identification of siderophores from *S. apiospermum* was in agreement with the proposed hydroxamate structure suggested from specific assays for the different classes of siderophores, since only chemical tests specific for hydroxamates were positive on concentrated culture medium. In order to confirm the identification, fragmentation of the molecules corresponding to the two masses was realized and results were compared to the proposed structures. Fragmentation patterns confirmed the identification of these siderophores as dimerumic acid and N^{α}-methyl coprogen B. Detection of hydroxamate-type siderophores in *S. apiospermum* is not really surprising since it is well known that siderophores secreted by fungi principally belong to the hydroxamate class (Renshaw et al. 2002). Polycarboxylates are exclusively found in Zygomycetes (van der Helm and Winkelmann 1994) and strikingly, phenolates/catecholates have never been reported in

fungi. In our study, specific assays for phenolates/catecholates were negative for all the strains tested, in agreement with the scientific literature (Holzberg and Artis 1983; Renshaw et al. 2002).

Siderophore production by *S. apiospermum* was also found to be dependent on the strain. Despite slight variations in the CAS rate, comparison of ten strains of the *S. apiospermum* complex with various environmental or clinical origins revealed important differences in the production of N^{α}-methyl coprogen B when grown at pH 7 which is usually the pH in respiratory secretions (Kodric et al. 2007). Two groups were found in the ten strains studied. The first group was related to lung colonization or pulmonary infection since it was composed of strains IHEM 14263, 14451, 14268 and 14462 recovered from sputum samples from patients with CF and of strain IHEM 15155 isolated from a bronchial aspiration from a patient with unknown predisposing factor. The second group comprised three environmental strains, IHEM 13947, 20812 and 20828 isolated from soil or feathers of European starlings, and the strain IHEM 6908 which was recovered from a case of cutaneous/subcutaneous mycetoma. Although preliminary, these results suggest a specialization or adaptative process in strains of the *S. apiospermum* complex. A high production level of N^{α}-methyl coprogen B seemed to be a common feature of strains capable of chronic colonization of the airways in patients with CF or involved in lung diseases. Curiously, a high production level of this siderophore was also seen for strain IHEM 3817 which was recovered from a cerebrospinal fluid. However, CSN involvement is commonly seen in disseminated scedosporiosis following a primary pulmonary site of infection or colonization, for examples after lung transplantation in chronically colonized CF patients (Symoens et al. 2006) or after near-drowning (Katragkou et al. 2007). A retrospective analysis of a large number of cases of scedosporiosis after near-drowning always revealed preceding clinical and/or radiological evidence of lung disease indicating the mode of invasion (Katragkou et al. 2007). Interestingly, strains of the *S. apiospermum* complex were also divided into three groups by means of DNA–DNA reassociation techniques methods (De Hoog et al. 1994). Most strains belonging to group I were recovered from pulmonary colonization or infections, whereas group II mainly comprised clinical strains isolated from the ear, nose and throat area (principally fungal sinusitis) and group III was composed of environmental strains.

Together these results provide new insights into the mechanisms of iron uptake by *S. apiospermum*. Further studies are needed to confirm the possible link between production of N^{α}-methyl coprogen B and lung colonization or infection. Additionally, this hydroxamate-type siderophore has never been described for the other microorganisms colonizing the airways of CF patients. Studies are now conducted in order to determine whether or not N^{α}-methyl coprogen B could be detected directly from sputum samples and if it could be used as a biological marker for pulmonary infection or colonization by *S. apiospermum*.

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