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Fungi and Arsenic: Tolerance and Bioaccumulation by Soil Saprotrophic Species

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Abstract: Increasing arsenic environmental concentrations are raising worldwide concern for its impacts on human health and ecosystem functionality. In order to cope with arsenic contamination, bioremediation using fungi can represent an efficient, sustainable, and cost-effective technological solution. Fungi can mitigate arsenic contamination through different mechanisms including bioaccumulation. In this work, four soil saprotrophic fungi *Absidia spinosa*, *Purpureocillium lilacinum*, *Metarhizium marquandii*, and *Cephalotrichum nanum*, isolated from soils with naturally high arsenic concentrations, were tested for their ability to tolerate different sodium arsenite concentrations and accumulate As in different cultural conditions. pH medium after fungal growth was measured to study pH variation and metabolic responses. Arsenic bioaccumulation and its influence on the uptake of other elements were investigated through multi-elemental analysis using hydride generation atomic fluorescence spectrometry (HG-AFS), inductively coupled plasma mass spectrometry (ICP-MS) and inductively coupled plasma optical emission spectroscopy (ICP-OES). Considering the increasing interest in siderophore application for metal bioremediation, the production of siderophores and their affinity for both Fe and As were also evaluated. All species were able to tolerate and accumulate As in their biomass in all of the tested conditions and produced siderophores with different affinities for Fe and As. The results suggest that the tested fungi are attractive potential candidates for the bioremediation of As contaminated soil and worthy of further investigation.

Keywords: soil fungi; bioaccumulation; arsenic; arsenite tolerance; *Absidia spinosa*; *Purpureocillium lilacinum*; *Metarhizium marquandii*; *Cephalotrichum nanum*; siderophores; multi-elemental analysis

1. Introduction

Increasing arsenic (As) environmental concentrations are raising worldwide concern due to the impacts on human health and ecosystem functionality [1,2]. In fact, chemical species of As can be toxic for living organisms as arsenate can compete with the essential inorganic phosphate, and arsenite can inactivate many enzymes by binding to protein thiols [3]. In humans, As can cause severe toxic effects to integumentary, cardiovascular, reproductive, and neurological systems, leading in extreme cases to the development of malignant tumors and death [4].

Arsenic is a naturally occurring metalloid that is widely distributed in the Earth's crust [3]. Primarily associated with igneous and sedimentary rocks, As is naturally found in the environment because of natural processes including weathering of As-enriched minerals, volcanic emissions, and biological activities [4–7]. In several locations worldwide (e.g., Bangladesh, India, China, USA) high As concentrations in groundwater, drinking water, and soils are associated with natural geologic

sources [1,5,6]. In many Italian areas, highly natural As concentrations in lakes, volcanic aquifers, drinking waters, rocks, soils, plants, and food have been reported to be associated with geological and environmental factors [8–10]. On the other hand, other important sources contributing to As environmental contamination are anthropogenic activities including, for instance, the use of agricultural pesticides, wood preservatives, and medicines, waste incineration, and mineral ore processing [3,5,11]. Indeed, in some Italian areas, former mining activities have contributed to an increase of As in the environment, leading to its high concentrations [12,13].

In order to cope with As contamination, bioremediation and bioaugmentation using microorganisms can represent an efficient, environmentally-friendly, and cost-effective technological solution. In particular, fungi are promising bioresources in the remediation of As pollution [1–3,11]. Fungi are important geoactive agents, playing very important geological roles in several processes including decomposition, biogeochemical cycling, element biotransformation, metal and mineral transformations, bioweathering, and soil formation [3,14–16]. They are able to tolerate extreme and very limiting environmental conditions such as highly concentrated mixtures of toxic substances. Some species have been reported to accumulate high concentrations of arsenic and volatilize it via methylation [1,2,14,17]. Several soil fungi isolated by As-contaminated soils have been successfully tested for As tolerance and removal [18,19]. Among these, the majority belong to the genera *Aspergillus*, *Fusarium*, *Penicillium*, *Rhizopus*, and *Trichoderma* [2,19–21]. Most of the above-mentioned tested strains were isolated from contaminated sites such as agricultural soils, paddy fields, and mines mainly in China and India. In order to maximize the efficiency in mycoremediation applications, it is very important to focus on saprotrophic fungi isolated from soils with high As concentrations as they can be better equipped to survive and cope with the associated chemical stress [22,23].

In this context, siderophores, which are low-molecular-weight organic compounds, are crucial for many of the above-mentioned roles played by fungi in several processes, especially for soil mineral weathering and biogeochemical cycles [24]. Their main role is to bind extracellular Fe(III), generally not available for organisms, and transport it inside the cell to meet the metabolic requirements [25,26]. However, siderophores are remarkably effective in solubilizing and increasing the mobility not only of Fe, but also of a wide range of elements including Cd, Ni, As, and actinides [24–27]. Consequently, in the last years, there has been an increasing interest in siderophore application for metal bioremediation [24]. Most fungi can produce hydroxamate-type siderophores, but other types have also been observed including rhizoferrin, a polycarboxylate siderophore, in Zygomycetes and phenolate compounds in other fungi [26,28,29]. Evidence has shown that since microorganisms can also acquire Fe from As-bearing minerals (es. Scorodite), consequently increasing the As mobilization, siderophore production can correlate with increased As resistance, even if a direct link to the siderophores' action binding As was not observed [25]. The potential of siderophores in the bioremediation of potentially toxic elements is very high, as siderophore-producing fungi are abundant in soils, and siderophores can strongly influence speciation, bioavailability, and the fate of metals, metalloids, and radionuclides in the environment [26]. Therefore, since siderophores represent a useful tool, in order to deepen the tested species potential in As bioremediation, siderophore production was taken into account.

In this context, four fungal species, *Absidia spinosa* Lendn., *Cephalotrichum nanum* (Ehrenb.) S. Hughes, *Metarhizium marquandii* (Massee) Kepler, S.A. Rehner & Humber, and *Purpureocillium lilacinum* (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson, previously isolated from Italian soils with naturally high As concentrations, were investigated in the presence of As, as As(III) or arsenite, in two different nutritional conditions. In fact, fungi are strongly influenced by nutritional condition in their growth and ecology and therefore also in their ability to tolerate stresses. Furthermore, medium composition may have an effect on metal uptake [30,31]. To our best knowledge, these species have not been previously studied for their ability to tolerate and accumulate As, but their potential in the bioremediation of some toxic metals has been reported [32–39]. In particular, Coles et al. [32] reported on the tolerance and solubilization of zinc compounds by *A. spinosa*, as fungal species associated with *Thlaspi caerulescens*. *A. spinosa*, and *P. lilacinum* adsorbed plutonium onto mycelium and spores [33]. *P. lilacinum* was reported to be tolerant to and accumulate

Pb, Cd, and Cr [34–37], while *M. marquandii* resulted tolerant to Zn and Pb and able to uptake them [38,39]. No species of the genus *Cephalotrichum* was previously tested with metals. However, being oligotrophic fungi, these species inhabit extreme habitats with low availability of nutrients, for example, *C. stemonitis* has been isolated from a closed gold mine with high As concentrations [40,41]. Hence, these four fungi show a great potential for the development of biotechnological applications for As remediation, and consequently the knowledge of their interactions with arsenic deserves to be deepened. Therefore, to shed further light on this topic, this study was aimed to investigate the (1) fungal tolerance to high As concentrations; (2) arsenic bioaccumulation in fungal biomass; (3) influence of As presence in the medium on the uptake and accumulation of other elements; and (4) the production of siderophores and their affinity for As compared to that for Fe.

2. Materials and Methods

2.1. Screening of Fungal Strains for Arsenic Tolerance and Accumulation

Four strains of soil saprotrophic fungi, previously isolated from environments with high natural concentrations of arsenic and currently preserved at the culture collection of the Fungal Biodiversity Laboratory (FBL) (Sapienza, University of Rome), were screened to evaluate growth responses and assess As tolerance and As bioaccumulation. The tested strains, belonging to the Mucoromycota and Ascomycota phyla, were *Absidia spinosa* (FBL 69), *Cephalotrichum nanum* (FBL 73), *Metarhizium marquandii* (FBL 484), and *Purpureocillium lilacinum* (FBL 478). *A. spinosa* and *C. nanum* were isolated from different soils of the geothermal area of Travale-Radicondoli, characterized by high concentrations of potentially toxic elements including As [42], while *P. lilacinum* and *M. marquandii* were isolated from the soils of Latin Valley, which are characterized by high background concentrations of As [43]. The strains were reactivated and maintained at 25 °C in the dark on Malt Extract Agar (MEA), prepared according to the following composition (g/L in distilled water): malt extract, 20; peptone, 1; glucose, 20; agar, 20. All components were purchased from Becton Dickinson (Sparks, MD, USA). Prior to the experiments 7-day old stock cultures of the fungal strains were established. Tolerance screenings to sodium arsenite ($NaAsO_2$) (assay ≥ 90%; antimony (Sb) ≤ 0.5%; Sigma-Aldrich) were carried out in Petri plates containing solid culture medium supplemented with a sodium arsenite solution and mixed to homogenize before solidification. In order to evaluate the influence of nutritional conditions on the strains' ability to tolerate and accumulate arsenic, the plate test was performed on two cultural media, Malt Extract Agar (MEA) and Czapek-Dox Agar (CDA). The composition of CDA was as follows (g/L distilled water): sucrose, 30; $NaNO_3$, 3; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; and $FeSO_4 \cdot 7H_2O$, 0.01. All chemicals were purchased from Merck (Darmstadt, Germany). The pH of CDA was adjusted to 5.8 by introducing HCl, while MEA pH was left unmodified (pH 5.4). To assess a possible dose-dependent response, three different concentrations 10 mg/L, 20 mg/L, and 50 mg/L of $NaAsO_2$ were tested, which were equivalent to 5.8 mg/L, 11.5 mg/L, and 28.8 mg/L of As(III), respectively. The concentrations were selected considering the As concentrations tested in previous papers such as Vala et al. [44] and the environmental As concentrations of the soils from which the fungi were isolated. Controls and chemical blanks were also set up. All assays were carried out in quadruplicate. Growth responses and fungal tolerance to As were investigated by tolerance indexes based on the growth data of diametric extension and dry weights. Two indexes were calculated: Rt:Rc (%) defined as the ratio of the colony extension rates in the presence (Rt) or absence (Rc) of $NaAsO_2$ and the Tolerance Index (T.I.) based on the dry weights (DW) of fungal biomass (T.I. (%) = (DW of treated mycelium/DW of control mycelium) × 100) [23]. To facilitate the recovery of the mycelium, sterile cellophane membranes, allowing the passage of nutrients and metabolites between the medium and the colony, were placed on the surface of the culture medium [22]. Inoculation was carried out placing at the center of the plate a 6-mm-diameter plug taken from the actively growing margin of the stock cultures using a sterile cork borer. During the seven days of incubation, measures of diametric extension were carried out daily. After incubation for seven days at 25 °C in the dark, using a sterile razor blade,

the mycelia were recovered from the membrane and oven dried at 100 °C until reaching a constant weight for at least two days.

Following the biomass removal, the pH of the culture medium was measured at specific intervals across the diameter of the Petri dish using a pH meter HI 99161, fitted with a conical tip FC 202D pH electrode (Hanna Instruments, Woonsocket, RI, USA) to evaluate the pH variation related to metabolic responses.

2.2. Elemental Chemical Analysis of the Tested Species' Fungal Biomass

Concentrations of 18 elements (As, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Pb, S, Sb, Sn, and Sr) in the fungal biomass of *A. spinosa*, *C. nanum*, *M. marquandii*, and *P. lilacinum* were analyzed to evaluate the different accumulation of the elements in the four tested strains grown on MEA and CDA media (control) and on MEA and CDA media added with 10 mg/L, 20 mg/L, and 50 mg/L of sodium arsenite. All treatments, sample preparation, and chemical analyses were carried out in quadruplicate.

Four samples for each of the four fungal species subjected to the eight different treatments (MEA and CDA media added or not with 10 mg/L, 20 mg/L, and 50 mg/L of sodium arsenite), for a total of 128 samples, were prepared and chemically analyzed in accordance with the following procedure. Each sample was transferred to a quartz vessel and subjected to microwave assisted acid digestion (Ethos Touch Control with Q20 rotor, Milestone, Bergamo, Italy) for 30 min, using 2 mL of ultrapure concentrated HNO_3 (assay > 67%; residue < 1 mg/L) and 1 mL of H_2O_2 (assay > 30%; residue < 20 mg/L); HNO_3 and H_2O_2 were high purity solvents for trace analysis and were purchased from Promochem, LGC Standards GmbH (Wesel, Germany). The vessel was irradiated with a maximum power of 1000 W and microwave digestion was carried out at maximum temperature (180 °C) and pressures ≤ 40 bar. The digested solution was then diluted to 50 mL with deionized water (Arioso UP 900 Integrate Water Purification System) and filtered through syringe filters (25 mm diameter, 0.45 µm pore size, GVS Filter Technology, Morecambe, UK).

Concentrations of Ca, Fe, K, Mg, Na, P, and S were determined by inductively coupled plasma optical emission spectrometry (ICP-OES; Vista MPX CCD Simultaneous; Varian, Victoria, Mulgrave, Australia) in axial view mode and equipped with a cyclonic spray chamber. Determination of Cd, Co, Cr, Cu, Mn, Ni, Pb, Sb, Sn, and Sr concentrations was performed by quadrupole inductively coupled plasma mass spectrometer (ICP-MS; model 820-MS; Bruker, Bremen, Germany) equipped with a glass nebulizer (0.4 mL/min; Analytik Jena AG, Jena, Germany). Overall, ICP-OES was used for the determination of elements present in higher concentrations, while ICP-MS was used for the analysis of trace and ultra-trace elements. For both ICP-OES and ICP-MS determinations, external standard calibration curves were performed by serially diluting multi-element standard stock solutions (1000 ± 2 mg/L; Exaxol Italia Chemical Manufacturers Srl, Genoa, Italy). To control the nebulizer efficiency, rhodium was set at 5 µg/L as the internal standard for all of the measurements and was prepared from standard stock solutions (1000 ± 2 mg/L; Panreac Química, Barcelona, Spain). For ICP-MS analysis, a standard solution containing 5 µg/L of Ba, Be, Ce, Co, In, Pb, Mg, Tl, and Th was prepared daily in 1% HNO_3 from a multi-standard stock solution (10.00 ± 0.05 mg/L; Spectro Pure, Ricca Chemical Company, Arlington, TX, USA) to select the best ICP-MS performance. Further details about the ICP-OES and ICP-MS conditions are reported in Canepari et al. [45,46] and in Astolfi et al. [47], respectively. Atomic fluorescence spectrometry with hydride generation (HG-AFS; 8220 Titan HG-AFS, FullTech Instruments, Rome, Italy) was used for the determination of the total As concentration in fungal biomass, since this technique enables avoiding possible interferences in As determination by ICP-MS due to Cl cluster formation and has a higher sensitivity for As with respect to ICP-OES, thus allowing a higher analytical quality of the data to be obtained [48,49]. Calibration standard solutions were obtained using certified standard mono-element solution of As (1000 ± 10 mg/L; Merck, Darmstadt, Germany), 5% HCl (Promochem, LGC Standards GmbH, Wesel, Germany) was used as the carrier and 2% $NaBH_4$ (Sigma-Aldrich Chemie GmbH, St. Louis, MO, USA) in 0.5% NaOH (Carlo Erba Reagenti Srl, Rodano, MI, USA) as the reducing agent. Standards and samples were

prepared in 5% HCl + 1% KI (Sigma-Aldrich Chemie GmbH, St. Louis, MO, USA) + 0.5% ascorbic acid (Fluka Analytical, St. Louis, MO, USA).

The values of blanks, subjected to similar sample preparation and analytical procedures, were deducted from all measurements and the limits of detection were set at three times the standard deviation of 10 replicate blank determinations. Finally, the obtained values were divided by the dry weight of each sample to obtain the element concentrations [50–52] in the fungal biomass of the tested species at the different examined conditions. The obtained element concentrations are reported in Supplementary Materials Table S1. The certified reference materials BCR 482 lichen (IRMM, Geel, Belgium) and NIST 1515 apple leaves (National Institute of Standards and Technology) [51,53] were used to test the accuracy of the measurements and to validate the entire analytical process (Supplementary Materials Table S1). Results were in good agreement with certified values (95% confidence level); recovery percentages (≥80%) and standard deviations (≤20%, except for Sn) were satisfactory and indicated a good precision of the measurements.

2.3. Siderophore Production Screening

Siderophore production was screened using the Chrome Azurol CAS Assay. The assay was performed both for arsenic (As-CAS) and iron (Fe-CAS), according to the protocol modified by Mehnert [27]. Cultures of the four fungal strains were inoculated in 100 mL Erlenmeyer flasks, containing 50 mL of Malt Extract Broth (MEB), using four plugs of 6 mm diameter of mycelium taken from seven day old stock cultures on MEA using a cork borer. The cultures, established in triplicate, were incubated in the dark at 25 °C with constant shaking at 100 rpm on a rotatory shaker (ASAL 711/D). Samples of culture medium from the established cultures were collected at 7, 14, and 21 days of growth. To avoid mycelium fragments possibly altering the spectrophotometric measurements, the culture medium was filtered using a 33 mm diameter sterile syringe filter with a 0.45 µm pore size made of mixed cellulose esters (ClearLine). The test was performed in a 96 multi-well plate in order to screen all the biological replicates, performing multiple readings of each one. In each well, culture filtrate and CAS solution were added in a 1:1 ratio, in the amount of 100 µL of each one. A negative control was arranged using sterile culture media and CAS solution, while a positive control was arranged adding 180 mM Ethylenediaminetetraacetic acid (EDTA) from a 500 mM stock (pH = 8) to the MEB and CAS solution. Absorbance was read at 620 nm using a Multiskan™ FC Microplate Photometer (Thermo Scientific™) after four hours of incubation.

2.4. Statistical Analysis

All statistical analyses were carried out using the statistical software R (version 3.5.2) [54] under the R-studio environment (version 1.1.463) [55].

The Shapiro–Wilk test was used to evaluate the normality of the data (package stats) [56]. To test data homoskedasticity, as appropriate, the Levene test (package lawstat) [57] and Bartlett test (package stats) [58] were used.

Hereafter, analysis of variance was performed using the Welch test (package stats) [59], followed by the all-pairs comparison post-hoc test Tamhane's T2 (package PMCMRplus) [60].

Principal component analysis (PCA) was performed on the element concentrations determined in the fungal biomass of the tested species grown on MEA and CDA media (control) and on MEA and CDA media added with 10 mg/L, 20 mg/L, and 50 mg/L of sodium arsenite (Supplementary Materials Table S1) to cluster the samples (scores) according to the different accumulation of the elements (loadings). The matrix of the data, composed of 120 samples (four samples for each of the four fungal species subjected to the eight different treatments; eight outlier samples were excluded from the multivariate statistical computation) and 18 variables (As, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Pb, S, Sb, Sn, and Sr), was transformed by performing column mean centering and row and column autoscaling. This allowed correcting variations of the data due to the different scaling and units of the examined variables. Data were then analyzed by using PCA.

3. Results

3.1. Evaluation of Growth and Tolerance of Tested Fungi in the Presence of Arsenic and Culture Medium pH Analysis

The values of diametric growth, biomass production, and tolerance indexes of the tested species in the presence of different As concentrations are shown in Tables 1–3. In all tested conditions, As did not inhibit the growth of the tested fungal species, which showed normal colony development and sporulation. Moreover, growth differences among the tested species, also in relation to the tested media, could be appreciated (Tables 1 and 2). In fact, on CDA, *A. spinosa* showed greater growth than other species, while the lowest values of diameter and dry weights in both nutritional conditions were observed in *C. nanum*. *M. marquandii* like *C. nanum* showed greater diametric growth on CDA than on MEA, and biomass production of *C. nanum* on CDA was higher than on MEA.

Most of the calculated values for the tolerance indexes were higher than 80%, disclosing a strong tolerance of the tested strains (Table 3). *A. spinosa* on MEA with 20 mg/L and 50 mg/L of sodium arsenite represented the only exception, showing both Rt:Rc and T.I. indexes lower than 70%. As the arsenic concentration increased in the medium, the values of both indexes reduced up to 50.3% for Rt:Rc and 52.8% for T.I. This trend was observed only on MEA, as the tolerance indexes for *A. spinosa* on CDA were >90%. It is worth mentioning that in some cases, the tolerance indexes were higher than 110%. In fact, the Rt:Rc for *C. nanum* grown on MEA amended with 50 mg/L sodium arsenite was 116.7%, while the T.I. for *C. nanum* on MEA with 10 mg/L and 50 mg/L were 110.6% and 111.4%, respectively (Table 3). However, the diametric values and biomass production of *C. nanum* on MEA for all tested As concentrations were not significantly different from that of the control (Tables 1 and 2). Regarding *M. marquandii*, the T.I. was 113.5% on MEA with 20 mg/L, even if biomass production on MEA control was not significantly different from that on MEA with 20 mg/L (Tables 2 and 3).

The addition of different concentrations of sodium arsenite to both media did not change the medium pH (Table 4). Regardless of As concentration, *A. spinosa* lowered the pH of both tested media, while *P. lilacinum*, *M. marquandii*, and *C. nanum* increased CDA pH (Table 4). No variations of MEA pH were observed in *M. marquandii* and *C. nanum*, while *P. lilacinum* was able to slightly acidify MEA (control, 10, and 20 mg/L of sodium arsenite) (Table 4).

Table 1. Diametric values of fungal colonies after seven days of growth at 25 °C. Data are expressed as the mean ± standard error of independent biological replicates [a].

Diameter (mm)									
Media		**MEA**				**CDA**			
	$NaAsO_2$ Concentration	**Control**	**10 mg/L**	**20 mg/L**	**50 mg/L**	**Control**	**10 mg/L**	**20 mg/L**	**50 mg/L**
Species	***A. spinosa* FBL 69**	86.0 ± 0.0	76.9 ± 1.4	59.0 ± 0.9 **	45.8 ± 1.5 *	86.0 ± 0.0	84.6 ± 0.8	86.0 ± 0.0	85.5 ± 0.5
	***P. lilacinum* FBL 478**	38.8 ± 0.3	38.0 ± 0.0	38.1 ± 0.4	37.7 ± 0.7	39.1 ± 0.5	38.5 ± 0.3	38.0 ± 0.4	36.1 ± 0.4
	***M. marquandii* FBL 484**	32.0 ± 1.0	30.1 ± 0.7	33.1 ± 0.8	31.0 ± 0.7	34.6 ± 1.6	35.3 ± 0.5	36.9 ± 0.6	34.6 ± 0.4
	***C. nanum* FBL 73**	14.8 ± 0.3	15.4 ± 0.9	15.3 ± 0.1	16.4 ± 1.0	24.0 ± 0.2	24.5 ± 0.6	23.9 ± 0.1	23.4 ± 0.4

[a] Asterisks denote a significant difference (post-hoc Tamhane's T2-test) between the treatment and the control (* $P < 0.1$; ** $P < 0.05$).

Table 2. Dry weight values of fungal biomass after seven days of growth at 25 °C. Data are expressed as the mean ± standard error of independent biological replicates [a].

Dry Weight (g)									
Media		**MEA**				**CDA**			
$NaAsO_2$ Concentration		**Control**	**10 mg/L**	**20 mg/L**	**50 mg/L**	**Control**	**10 mg/L**	**20 mg/L**	**50 mg/L**
Species	***A. spinosa* FBL 69**	0.0880 ± 0.0011	0.0783 ± 0.0013	0.0550 ± 0.0014 ***	0.0465 ± 0.0027 *	0.1021 ± 0.0042	0.0956 ± 0.0039	0.0966 ± 0.0024	0.1119 ± 0.0039
	***P. lilacinum* FBL 478**	0.0423 ± 0.0011	0.0395 ± 0.0006	0.0421 ± 0.0014	0.0409 ± 0.0004	0.0415 ± 0.0015	0.0437 ± 0.0019	0.0422 ± 0.0015	0.0403 ± 0.0009
	***M. marquandii* FBL 484**	0.0241 ± 0.0014	0.0231 ± 0.0010	0.0274 ± 0.0010	0.0243 ± 0.0029	0.0236 ± 0.0007	0.0241 ± 0.0016	0.0245 ± 0.0014	0.0240 ± 0.0014
	***C. nanum* FBL 73**	0.0105 ± 0.0001	0.0116 ± 0.0007	0.0106 ± 0.0002	0.0117 ± 0.0007	0.0152 ± 0.0022	0.0155 ± 0.0008	0.0160 ± 0.0011	0.0146 ± 0.0006

[a] Asterisks denote a significant difference (post-hoc Tamhane's T2-test) between the treatment and the control (* $P < 0.1$; ** $P < 0.05$; *** $P < 0.01$).

Table 3. Tolerance indexes of the tested species exposed to arsenic (Rt:Rc and T.I.) on Malt Extract Agar (MEA) and Czapeck Dox Agar (CDA) [a].

MEA							
	Tolerance Index	**Rc:Rt (%)**			**T.I. (%)**		
	$NaAsO_2$ Concentration	**10 mg/L**	**20 mg/L**	**50 mg/L**	**10 mg/L**	**20 mg/L**	**50 mg/L**
Species	***A. spinosa* FBL 69**	88.7	66.7	50.3	88.9	62.5	52.8
	***P. lilacinum* FBL 478**	97.8	98.1	96.8	93.4	99.4	96.5
	***M. marquandii* FBL 484**	93.1	104.2	96.3	95.8	113.5	100.7
	***C. nanum* FBL 73**	106.4	105.1	116.7	110.6	101.2	111.4
CDA							
	Tolerance Index	**Rc:Rt (%)**			**T.I. (%)**		
	$NaAsO_2$ Concentration	**10 mg/L**	**20 mg/L**	**50 mg/L**	**10 mg/L**	**20 mg/L**	**50 mg/L**
Species	***A. spinosa* FBL 69**	98.3	100	99.4	93.6	94.6	109.6
	***P. lilacinum* FBL 478**	98.2	96.7	91.2	105.5	101.9	97.2
	***M. marquandii* FBL 484**	102.1	107.6	100	102.4	104.2	102.0
	***C. nanum* FBL 73**	102.6	99.3	96.7	101.9	105.4	96.3

[a] Rt:Rc is defined as the ratio of the colony extension rates in the presence (Rt) or absence (Rc) of arsenic. T.I. is defined as the ratio of the dry weight of the fungal biomass in the presence or absence of arsenic.

Table 4. pH values of the medium after growth of the fungal species for seven days at 25 °C. Data are expressed as the mean ± standard error of independent biological replicates [a].

		Medium pH							
	Medium	**MEA**				**CDA**			
	$NaAsO_2$ Concentration	**Control**	**10 mg/L**	**20 mg/L**	**50 mg/L**	**Control**	**10 mg/L**	**20 mg/L**	**50 mg/L**
	Blank	5.4 ± 0.1	5.3 ± 0.0	5.3 ± 0.1	5.6 ± 0.1	5.7 ± 0.0	5.7 ± 0.0	5.6 ± 0.1	5.8 ± 0.1
Species	***A. spinosa* FBL 69**	3.5 ± 0.1 ***	3.6 ± 0.1	3.5 ± 0.1	3.6 ± 0.1 ***	4.4 ± 0.0 *	4.5 ± 0.1	4.6 ± 0.1 **	4.4 ± 0.0 ***
	***P. lilacinum* FBL 478**	4.9 ± 0.2	4.6 ± 0.1	4.8 ± 0.1	5.2 ± 0.1	6.3 ± 0.0 ***	6.2 ± 0.0	6.0 ± 0.1	6.2 ± 0.0
	***M. marquandii* FBL 484**	5.3 ± 0.0	5.4 ± 0.0	5.3 ± 0.1	5.4 ± 0.1	6.4 ± 0.1	6.2 ± 0.1	6.3 ± 0.1	6.1 ± 0.0
	***C. nanum* FBL 73**	5.3 ± 0.1	5.3 ± 0.1	5.3 ± 0.1	5.5 ± 0.1	6.7 ± 0.0 **	6.3 ± 0.1	6.5 ± 0.0 **	6.3 ± 0.0

[a] Asterisks denote a significant difference (*post-hoc* Tamhane's T2-test) between the blank and the species for the respective treatments (* $p < 0.1$; ** $p < 0.05$; *** $p < 0.01$).

3.2. Evaluation of As Bioaccumulation in Fungal Biomass

All of the tested species under all of the tested conditions were able to accumulate As (Table 5). The highest value of As concentration was observed in *C. nanum* on both MEA with 50 mg/L of sodium arsenite and CDA with 50 mg/L, while *A. spinosa* on CDA and MEA with 50 mg/L showed the highest values of As content. The statistical analysis showed that for all tested species, arsenic concentrations in the samples of all tested conditions were statistically different when compared with those of the control samples (Table 5). Cultural conditions, at least in some cases, influenced the concentration and content of As in fungi.

3.3. Evaluation of Element Concentrations in Fungal Biomass

To evaluate the different accumulation of the elements in the fungal biomass of *A. spinosa*, *C. nanum*, *M. marquandii*, and *P. lilacinum* grown on MEA and CDA media (control) and on MEA and CDA media added with 10 mg/L, 20 mg/L and 50 mg/L of sodium arsenite, the concentration variability of As, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Pb, S, Sb, Sn, and Sr was assessed (Supplementary Materials Table S1).

Principal component analysis performed on the obtained data allowed us to cluster the samples (scores) according to the different accumulation of the elements (loadings) in the four tested strains in the different experimental conditions. Five significant components accounting for 71.47% were obtained (the scores and loadings are shown in Supplementary Materials Table S2); the variance explained by each component was 22.3%, 18.2%, 15.3%, 8.9%, and 6.7%. The first component (PC1), which explains 22.3% of the total variance, well separated the samples of the fungal species in which higher concentrations of As were accumulated from the others. On the other hand, PC2, which explains 18.2% of the total variance, well clustered the elements, depending on their concentration variability among the samples and separated the samples of the fungal strains grown on MEA medium from those grown on the CDA medium. Therefore, PC1 and PC2, which explain 40.5% of the total variance, are represented in the score plot and loading plot of Figure 1.

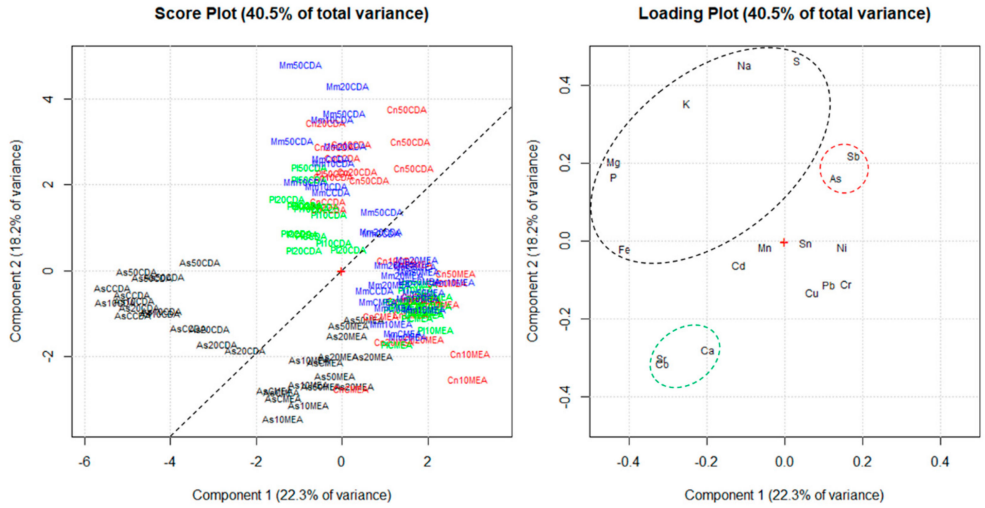

Figure 1. Score plot and loading plot of PC1 and PC2 obtained by the Principal Component Analysis (PCA) performed on the element concentrations determined in the fungal biomass of the tested species *A. spinosa* (As), *C. nanum* (Cn), *M. marquandii* (Mm), and *P. lilacinum* (Pl) grown on MEA and CDA media (C) and on MEA and CDA media added with 10 mg/L, 20 mg/L and 50 mg/L of sodium arsenite, respectively. Sample labels: AsCMEA, As10MEA, As20MEA, As50MEA, AsCCDA, As10CDA, As20CDA, As50CDA (black color); MmCMEA, Mm10MEA, Mm20MEA, Mm50MEA, MmCCDA, Mm10CDA, Mm20CDA, Mm50CDA (blue color); PlCMEA, Pl10MEA, Pl20MEA, Pl50MEA, PlCCDA, Pl10CDA, Pl20CDA, Pl50CDA (green color); CnCMEA, Cn10MEA, Cn20MEA, Cn50MEA, CnCCDA, Cn10CDA, Cn20CDA, Cn50CDA (red color).

In the score plot in Figure 1, we can observe that all of the samples of the fungal species grown on CDA medium were separately clustered from the samples grown on MEA due to their different variability in element concentrations. In fact, the fungal strains grown on CDA showed higher concentrations (Supplementary Materials Table S1) of Fe, K, Mg, Na, P, and S (because of the higher accessibility of nutrients contained in CDA medium; composition is reported in Section 2.1), which are plotted on the left upper part of the corresponding loading plot. Among the four species, *A. spinosa* samples were separately clustered from the others, this can reasonably be ascribed to the different variability in concentrations of Ca, Co, Fe, Mg, P, and Sr, plotted on the left part of the corresponding loading plot. Indeed, as can be seen from Supplementary Materials Table S1, *A. spinosa* was found to be the strain with the highest concentrations of Ca, Co, Fe, Mg, P, and Sr. On the other hand, *P. lilacinum* appeared to accumulate the highest concentration of K, and *C. nanum* and *M. marquandii* were found to be the species with the highest concentrations of Na and S.

Cadmium, Cu, Cr, Mn, Ni, Pb, and Sn were plotted on the central part of the loading plot, since their concentration values had poor variability among the samples. Therefore, As addition in both culture media did not appear to have affected the accumulation of these elements by the different fungal species. On the contrary, from the score plot of Figure 1, we can observe that within each cluster, samples grown on culture media added with 50 mg/L of sodium arsenite, were plotted in the direction of As and Sb on the right upper part of the loading plot, since they showed different concentration variability of these two elements. In fact, from Supplementary Materials Table S1, we can observe that these samples accumulated the highest concentrations of As and Sb. The accumulation of As and Sb increased as the addition of sodium arsenite in the culture media increased, thus revealing a dose-dependent response for all of the tested fungal strains.

3.4. Evaluation of Siderophore Activity of Tested Fungi in the Presence of Arsenic and Iron.

Optical density values, reported in Table 6, show the tested species' siderophore activity for Fe and As. EDTA (180 mM), which showed complete chelation activity and therefore decolorization, was used as a reference. For Fe-CAS, *A. spinosa* and *P. lilacinum* both showed high chelation activity after 14 growth days. *C. nanum* and *M. marquandii*, despite to a lower extent, showed decolorization, reaching the maximum chelation activity at 7 and 21 growth days, respectively (Table 6). The statistical analysis revealed that the OD values for Fe-CAS of each tested species were significantly lower than that of the control (Table 6). The tested species generally showed modest Fe-chelating activity, but a very low or absent affinity for As. In fact, for As-CAS, *A. spinosa*, *C. nanum*, and *M. marquandii* showed only a slight absorbance reduction after 21 days. The OD values for As-CAS of *C. nanum* after 21 days were the lowest and statistically different from the control (Table 6).

Table 5. As content and As concentration in the tested species' fungal biomass after growth of the fungal species for seven days at 25 °C. Data are expressed as the mean ± standard deviation of independent biological replicates [a].

As Content in Fungal Biomass (ng)									
Medium		**MEA**				**CDA**			
	$NaAsO_2$ Concentration	**Control**	**10 mg/L**	**20 mg/L**	**50 mg/L**	**Control**	**10 mg/L**	**20 mg/L**	**50 mg/L**
Species	***A. spinosa* FBL 69**	11.1 ± 4.5	2066 ± 57 ***	3378 ± 254 ***	5305 ± 209 ***	6.2 ± 5.4	1538 ± 991	3634 ± 222 ***	9996 ± 1135 **
	***P. lilacinum* FBL 478**	4.2 ± 4.7	1264 ± 34 ***	2351 ± 138 ***	5453 ± 474 *	≤ LOD [b]	1513 ± 58	2457 ± 522	6360 ± 2501
	***M. marquandii* FBL 484**	1.9 ± 2.9	746 ± 39 ***	1377 ± 347	3103 ± 709 *	5.2 ± 3.6	878 ± 77 ***	1668 ± 382 *	3504 ± 621 **
	***C. nanum* FBL 73**	1.8 ± 3.5	683 ± 24 ***	1278 ± 155 **	2577 ± 1164	2.3 ± 3.6	832 ± 54 ***	1333 ± 584	3170 ± 221 ***
As concentration in Fungal Biomass (µg/g)									
Medium		**MEA**				**CDA**			
	$NaAsO_2$ Concentration	**Control**	**10 mg/L**	**20 mg/L**	**50 mg/L**	**Control**	**10 mg/L**	**20 mg/L**	**50 mg/L**
Species	***A. spinosa* FBL 69**	0.13 ± 0.0052	26 ± 1.1 ***	62 ± 7.9 **	115 ± 12 ***	0.06 ± 0.05	16 ± 10	38 ± 2 ***	90 ± 13 **
	***P. lilacinum* FBL 478**	0.10 ± 0.11	32 ± 0.91 ***	56 ± 6.6 **	133 ± 9.3 **	≤ LOD [b]	35 ± 2	58 ± 11	160 ± 69
	***M. marquandii* FBL 484**	0.11 ± 0.18	32 ± 1.9 ***	51 ± 15	129 ± 8.2 ***	0.22 ± 0.16	37 ± 7.8 *	68 ± 10 **	146 ± 22 **
	***C. nanum* FBL 73**	0.17 ± 0.34	59 ± 5.2 ***	120 ± 11 ***	218 ± 94	0.13 ± 0.21	54 ± 4 ***	81 ± 26	217 ± 14 ***

[a] Asterisks denote a significant difference (post-hoc Tamhane's T2-test) between the treatment and the control (* $p < 0.1$; ** $p < 0.05$; *** $p < 0.01$). [b] value below detection limit (LOD).

Table 6. Optical density (OD) measurements for the tested species in the Fe-CAS and As-CAS assays. Data are expressed as the mean ± standard error of independent biological replicates [a].

Optical Density							
		Fe-CAS			**As-CAS**		
Control		0.523 ± 0.003			0.399 ± 0.006		
180 mM EDTA		0.067 ± 0.000 ***			0.047 ± 0.003 ***		
	Days of Fungal Growth	7 d	14 d	21 d	7 d	14 d	21 d
Species	***A. spinosa* FBL 69**	0.221 ± 0.003 ***	0.202 ± 0.003 ***	0.220 ± 0.004 ***	0.386 ± 0.010	0.373 ± 0.010	0.358 ± 0.012
	***P. lilacinum* FBL 478**	0.272 ± 0.006 ***	0.202 ± 0.001 ***	ND	0.390 ± 0.014	0.438 ± 0.011	0.468 ± 0.008 ***
	***M. marquandii* FBL 484**	0.496 ± 0.003 ***	0.479 ± 0.001 ***	0.424 ± 0.019 *	0.414 ± 0.012	0.415 ± 0.009	0.378 ± 0.017
	***C. nanum* FBL 73**	0.380 ± 0.004 ***	0.412 ± 0.005 ***	0.413 ± 0.002 ***	0.330 ± 0.017	0.335 ± 0.025	0.318 ± 0.008 ***

[a] Asterisks denote a significant difference (post-hoc Tamhane's T2-test) between the treatment and the control (* $p < 0.1$; ** $p < 0.05$; *** $p < 0.01$).

4. Discussion

This work evaluated the potential of four saprotrophic fungal species to tolerate high sodium arsenite concentrations, accumulate arsenic in their biomass, and release siderophores with an affinity for the complexation of As and Fe. The tested species, even though they have been previously reported for their potential in the bioremediation of other toxic elements [32–39], have never been studied before for their tolerance to As.

The tested species were tolerant to all of the tested As concentrations, as pointed out by the tolerance indexes in almost all experimental conditions (Table 3). In fact, only *A. spinosa* was very sensitive to increasing As concentrations on MEA, despite it growing in all tested conditions (Table 3). Since this sensitivity was observed only on MEA, this is clearly related to the differences in nutritional conditions. In particular, it may be determined by the influence of one or more interacting factors including increased As bioavailability, reduced nutrient bioavailability, formation of toxic As organic species by interaction of arsenite with MEA components, and inhibition of fundamental enzyme activities due to As(III) reactions to thiols [61]. In fact, malt extract generally possesses a rich composition of carbohydrates, proteins, peptides, amino-acids, and vitamins [62,63], which require an efficient extracellular enzyme system to be metabolized. On the contrary, CDA grants a greater accessibility of nutrients as it is characterized by a composition mainly based on salts including sodium nitrate as the N source and sucrose as the main C and energy source. However, the importance of the nutritional conditions in determining the tolerance to As seems to be species specific. In fact, contrary to *A. spinosa*, *C. nanum* was not affected in tolerance to As by the differences in nutritional conditions, despite, in all conditions including the controls, it showing a greater diametric growth and an higher biomass production on CDA than on MEA (coherently with the higher accumulation of Fe, K, Mg, Na, P, and S) (Tables 1 and 2). Different patterns of growth between the different nutritional conditions were observed in *M. marquandii*, which showed a higher diametric growth on CDA than on MEA, but not a significant greater biomass production. However, it was able to tolerate As in both media. Considering the equal biomass production in *M. marquandii* on both media, the greater development of the colony on CDA suggests a greater presence of exploration hyphae with a lower number of branches and, therefore, a lower density. It is also worth mentioning that *M. marquandii* and *C. nanum* were slightly stimulated in the presence of sodium arsenite, as the tolerance indexes were higher than 110% (Table 3). Given that toxic elements can influence the physiology and morphology of fungal mycelia, resulting interactions can lead to different mycelial growth strategies and hyphal aggregation [64,65]. Adaptative fungal growth patterns, observed at high metal concentrations in different fungi, led to growth exceeding the control and tolerance index higher than 1 (100%) [20]. High fungal tolerance to sodium arsenite is even more noteworthy, if it is considered that chemical species of arsenic(III) are thought to be more toxic for these organisms than arsenic(V) ones [61,66]. High fungal tolerance to toxic elements may be related to metabolism-dependent or -independent mechanisms of tolerance/resistance to cope with chemical stress [64,67]. Despite there being no previous data on the As tolerance of the tested species, several fungal species have been reported as being tolerant to very high concentrations of As trivalent and pentavalent chemical species [19,21,44,68–70] including As minerals [3,11]. For instance, Vala [44] reported that *Aspergillus candidus* was very tolerant to 25 and 50 mg/L of sodium arsenite and sodium arsenate, showing a luxuriant growth in all treatments. In another work, Srivastava et al. [19] reported values of tolerance index around 1 for taxa belonging to different genera including *Aspergillus*, *Penicillium*, *Rhizopus,* and *Trichoderma*, which were tested to high concentrations of sodium arsenate up to 10,000 mg/L.

Other mechanisms that result from interactions between fungi and toxic elements include accumulation of metals and metalloids, and the production of extracellular enzymes and other metabolites such as organic acids and siderophores [64,71]. The dominance of As species in the environment is strictly dependent on pH and redox potential (E_h). Under reducing conditions and pH less than 9.2, the arsenite species H_3AsO_3 predominates, while under oxidizing conditions, the arsenate species $H_2AsO_4^-$ and $HAsO_4^{2-}$ are dominant at low and high pH, respectively [7]. Arsenic addition

can alter the pH of the medium and its presence, influencing the fungal metabolism, which can indirectly determine pH modification during the fungal growth. In this study, none of the tested species at any concentration showed a significant pH modification compared the control, nor did the addition of sodium arsenite change the pH of the media in the chemical blanks (Table 4). Conversely, the pH variations due to the fungal growth were strongly related to the tested medium, with the pH values of all tested conditions on MEA generally more acidic than those on CDA. Once again, this may be explained by the composition of the different media. Moreover, the pH was strongly related to the metabolism of the tested species (Table 4). In fact, *A. spinosa* acidified both media more than the other species. This may be due to the release of organic acids by this species, which can excrete gluconic acid [72]. Other species of the *Absidia* genus can produce organic acids such as *A. coerulea* that can release citric, tartaric, succinic, and glutaric acids [73]. Fungi can produce and release organic acids (e.g., oxalic, citric, and lactic acids) according to the growth conditions including the C and N sources, the availability of nutrients, and the presence of trace elements [3,67,74,75]. The pH is another important factor that can influence organic acid production and can play an important role in the transformations of minerals and elements by fungi, controlling hydrolysis and complexation, mineral dissolution and precipitation, medium acidification or alkalinization [3,67,71,75]. However, acidification can also be due to other mechanisms including proton excretion through plasma membrane ATPase, uptake of cations in exchange for H^+, and CO_2 production for fungal respiration [64]. Unlike the situation for *P. lilacinum*, that on MEA slightly lowered the pH while on CDA it was slightly increased, showing a greater influence of the medium than any other species (Table 4). In fact, the medium pH around 6 for *P. lilacinum*, *M. marquandii*, and *C. nanum* on CDA (Table 4) may be related to different factors such as the buffering effects of CDA components (e.g., phosphate) and ammonium and lipase production [19]. As well as the assimilation of nitrate, the N source in CDA, can result in medium alkalinization for the release of OH^-, as observed with *Penicillum radicum* and *P. cyclopium* [76]. pH modulation by fungi is important in the regulation of several enzymes, as reported, for instance, in the case of pathogenic fungi [77,78]. For example, in the entomopathogenic fungus *Metarhizium anisopliae*, the production of ammonia, increasing the medium pH, allowed the production of subtilisin proteases, which were active only at basic pH [79].

All tested species were able to accumulate high concentrations of As in their biomass during the seven days of growth (Table 5). In *C. nanum*, As concentration was the highest at 50 mg/L sodium arsenite in both media, while *A. spinosa* showed the highest As content. This difference is mainly due to the biomass production, which was higher in *A. spinosa* than in *C. nanum* (Table 2). In general, the arsenic content and concentration in fungal biomass increased as arsenic concentrations increased in media (Table 5). Along with As, Sb concentration also increased. It is important to note that Sb showed the same behavior of As because it was present as an impurity (<0.5%) in the sodium arsenite added in the culture media. This behavior is mainly due to the chemical similarities between the two metalloids, both belonging to Group 15 of the periodic table [80], which usually leads them to follow similar accumulation pathways [81]. However, even if Sb was present at a relatively low concentration, it was highly accumulated by all of the tested species, thus revealing the outstanding Sb accumulation ability of these strains, which merits further investigation. Moreover, increasing As concentrations in both culture media did not affect the accumulation in the fungal biomass of trace and ultra-trace elements (Cd, Cu, Cr, Mn, Ni, Pb, and Sn). Results in Supplementary Materials Table S1 confirmed that in both culture media, *C. nanum* was the species able to accumulate the highest concentrations of As and Sb, while *A. spinosa* was found to be the strain with the lowest accumulation ability of both elements, which was also observable in the plots obtained by principal component analysis.

Bioaccumulation of As was reported in several works in the presence of trivalent or pentavalent arsenic with fungi belonging to different genera (e.g., *Aspergillus*, *Penicillium*, and *Trichoderma* [11,19,44,68–70]. For instance, after five days of growth with 50 mg/L As(V), As bioaccumulation in *Penicillium janthinellum* was 87.0 µg/g [70]. The highest As removal in *Aspergillus candidus* were observed after three days of growth in the presence of 25 mg/L or 50 mg/L of trivalent or pentavalent arsenic [44]. Iron-coated fungal biomass of a *Paecilomyces* sp.

successfully removed As(III) from aqueous solution by biosorption [82]. Arsenic can be transported inside fungal cells via specific or non-specific transport processes (e.g., via specific and non-specific phosphate, glycerol, or hexose transporters) and mechanisms involved in As bioaccumulation in fungi can include surface association (e.g., biosorption to cell wall components), vacuolar compartmentalization, chelation, immobilization, and sequestration by metal-binding peptides (e.g., glutathione) [67,68]. In *Saccharomyces cerevisiae*, the arsenate, taken up by phosphate transporter, is reduced to arsenite inside the fungal cell, conjugated with glutathione, and transported into vacuoles [83]. According to Cánovas and De Lorenzo [84], similar mechanisms were also reported in the arsenate-hypertolerant *Aspergillus* sp. P37. In fungi, As can be methylated, resulting in As volatilization, and As efflux can occur by arsenite extrusion through the arsenite carrier protein in the plasma membrane (e.g., Acr3p channel in *S. cerevisiae*) [2,19,67–69,83]. These mechanisms can influence arsenic mobilization in the environment.

Since the mobilization of As from rocks, minerals, soils, and other substrates by fungi and other organisms can also be caused by the production of siderophores [67], they may represent feasible, environmentally-friendly bioresources for bioremediation application [24,85]. In this study, the tested species showed the production of siderophores with a high affinity for Fe complexation, while low or no affinity for As (Table 6). In *A. spinosa* and *C. nanum*, albeit to a very small extent, siderophore activity in chelating As was observed. In contrast, *P. lilacinum* and *M. marquandii* showed OD values greater than the reference value. This may be explained by the presence of secondary metabolites in the culture filtrates, interfering with the assay and by a low affinity of siderophores for As. With reference to the secondary metabolites, some pigments in *P. lilacinum* and *M. marquandii* have been isolated and characterized [86–88] and may have influenced the assay.

Conversely, all the tested species released siderophores with an Fe affinity. *A. spinosa* along with *P. lilacinum* showed the highest affinity for Fe complexation since the 14 days of growth reading, with *A. spinosa* showing a slight reduction of chelation at 21 days. A reduction in the effectiveness of Fe chelation over growth time was also observed in *C. nanum*, which showed the lowest OD values at seven days. The efficacy of *A. spinosa* in chelating Fe agrees with the fact that is reported in literature, which is to produce bacterioferritin [89]. This special siderophore belongs to the carboxylate type of siderophores, generally produced by Zygomycetes. Despite this varying according to the species, it has been observed for *Petromyces alliaceus* that its siderophores have a better affinity for Fe than for other tested metals and metalloids [27].

The highest ability of *A. spinosa* and *P. lilacinum* in Fe complexation was confirmed by the higher Fe accumulation in the fungal biomass of these strains (Supplementary Materials Table S1), which were found to be the species with the highest concentrations of Fe, thus confirming their efficacy in chelating Fe. Furthermore, in *A. spinosa* biomass were found the highest concentrations of P, this may be explained by the known ability of Mucorales to accumulate P as polyphosphates, which serves as a phosphate store [90] and as chelators for metals and metalloids [91].

In conclusion, the tested fungal species were tolerant to high concentrations of arsenic. In most of the cases, the nutritional conditions did not influence the tolerance to arsenic. Therefore, the tested fungal species are promising novel candidates for future scale-up studies in As mycoremediation. Considering their potential in As bioaccumulation, these species can be new tools in biotechnological applications (e.g., As recovery). Moreover, from our results, it emerged that these species can uptake other potentially toxic elements such as antimony, which have a strategic importance for industrial and high-tech applications. The production of siderophores with high affinity for Fe has been observed. Considering that siderophores may be involved in the process of tolerance and bioaccumulation, further research is needed to evaluate an eventual release of siderophores with As affinity triggered by As exposure. Finally, this study also led to results that have a potential ecological significance with respect to the role of fungi in arsenic geochemistry and its cycle in ecosystems.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/10/9/3218/s1, Table S1: Element concentrations in fungal biomass, Table S2: Scores and loadings of the five significant components obtained by the PCA performed on the element concentrations in fungal biomass of the tested species.

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