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A low-cost and environmentally-friendly potential procedure for inorganic-As remediation based on the use of fungi isolated from rice rhizosphere

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ABSTRACT

Rice, a staple food for over half of the world's population, represents a significant dietary source of inorganic arsenic. Thus, it is of great importance to reduce the levels of inorganic arsenic in rice with minimal cost and efficiency. Based on this, the present study focused on the isolation and identification of fungi present in soil rhizosphere to be used as a low-cost and environmentally-friendly procedure for inorganic arsenic remediation. Soil samples, from rice production areas, were evaluated in matter of toxic and essential elements (Pb, Cd, Ni, Mn, Se, Co, Cr, Ba, Rb, U, Cs, V, Cu, Fe, Mg, Zn) by inductively coupled mass spectrometry (ICP-MS). The isolated and identified filamentous fungi were *Penicillium* sp., *Aspergillus* sp., *Trichoderma* sp., *Cladosporium* sp., *Rhizopus* sp. and *Westerdykella* sp. Fungi were submitted to As^{3+} exposure in Czapeck growth media, and the biomass and broth of each fungus were analyzed by high performance liquid chromatography coupled to ICP-MS. The results have shown capacity of arsenic biotransformation and accumulation. Arsenic-tolerance test (0–50 mg l^{-1} As^{3+}) has shown that the most arsenic-tolerant genera were *Penicillium* sp. and *Aspergillus* sp. Our data led us to believe that filamentous fungi may influence on arsenic biogeochemistry in rice paddy soils and further these microorganisms might be potentially used for bioremediation.

1. Introduction

Arsenic (As) is a high priority toxic element for the Agency for Toxic Substances and Diseases Registry [1]. Food and water are the main source of As in matter of human exposure. Once this element enters in blood stream, it can cause several kinds of cancers such as bladder, skin and lung. In addition, vascular diseases are associated to As exposure [1].

In general, As is widely scattered in the environment and can be absorbed and metabolized by several types of plants and animals, producing a variety of As species with different degrees of toxicity. The most common species in increasing scale of toxicity are: arsenobetaine (AsB), arsenocholine (AsC), di-methyl arsenic acid (DMA), mono-methyl arsenic acid (MMA), arsenate (As^{+5}) and arsenite (As^{+3}), where the latter two As-species (inorganic As) are the most dangerous for humans [2,3].

Rice, a staple food for over half of the world's population, represents a significant dietary source of inorganic arsenic. Rice, unlike other cereals, is cultivated generally in flooded conditions (irrigated cultivation type) which leads to an efficient mobilization of As and hence increases the accumulation in the plant [4]. The most toxic form of arsenic, As^{3+}, presents high solubility in water,

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therefore it has higher mobility in paddy soils in comparison to methylated forms, MMA and DMA, that are less toxic species [5]. In the plants, these methylated species are absorbed slower than the inorganic compounds. As^{3+} and As^{5+} are mainly absorbed by the silicate and phosphate transporters in roots, respectively, due to molecular size similarities [5–9]. For this reason, inorganic arsenic is efficiently absorbed by roots, and consequently it reaches the grains [5].

Therefore, to minimize the risks for rice consumers, the content of inorganic arsenic in grains must be reduced. Concerning this fact, some studies have been undertaken to understand and minimize/mitigate the contamination of rice:(i) phyto-extraction: use of hyperaccumulator plants in contaminated soils [5], (ii) phyto-stabilization: use of tolerant plants to avoid the dispersal of contaminated soil [10], (iii) phyto-immobilization: use of plants that reduce the mobility and bioavailability of arsenic in soil or plants that adsorb arsenic or form precipitates with lead (Pb) or iron (Fe) [11], (iv) phyto-volatilization: use of common or genetic modified plants that produce volatile forms of arsenic [12]. However, most of these procedures have limitations including high cost, lack of simplicity, as well as problems related to time and the disposal of generated residues [13].

Microorganisms play an important role in the metabolism of soluble and non-soluble compounds, as well as toxic and non-toxic chemical species in soil [13,14]. Thus microorganisms, mainly those naturally present in plants rhizosphere, seems to be a low-cost and environmentally-friendly alternative to reduce or even eliminate the most toxic As species present in the soil used for rice crop. The ability of fungi to tolerate, absorb and metabolize toxic elements is well known and it has been explored in several previous studies [14–17]. For example, species of the genera *Penicillium* and *Aspergillus* are in general the most tolerant to toxic elements, and presents high growth even when they are exposed to different toxic elements at high concentrations such as 2000 mg l^{-1} [17]. Moreover, these microorganisms can methylate As to mono-methylarsonic acid (MAA), di-methylarsinic acid (DMA) and tri-methylarsine oxide (TMAO) [14–16].

Therefore, the present work aimed to evaluate rice soil-rhizosphere's microorganisms as a cost effective method for bioremediation of As uptake by rice plants. In this regard, we isolated and identified fungi from rice rhizosphere and their *in vitro* activity for biotransformation (uptake/oxidation/methylation) and tolerance to As were evaluated.

2. Material and Methods

2.1. Reagents, solutions and instrumentation

Deionized water (resistivity 18.2 MΩ cm) was used in all experiments (MilliporeRiOs-DI TM, Bedford, MA, USA). All reagents and media used were of analytical grade, and all the solutions were stored in amber bottles at −20 °C. Plastic bottles (Nalgen®), vials and glassware were cleaned by soaking in 15% (v/v) HNO_3 for 24 h, rinsing five times with ultra-pure water and then drying in a class 100 laminar flow hood before use. Total chemical elements and As speciation were determined by using an inductively coupled plasma mass spectrometer (ICP-MS ELAN DRC II, PerkinElmer, USA) and a high performance liquid chromatography (HPLC) coupled to an ICP-MS (HPLC series 200 and ICP-MS PerkinElmer, USA), respectively. Multi-element solution (10 mg l^{-1}) containing As was obtained from PerkinElmer (Shelton, CT, USA) and As species were prepared using reagents purchased from Sigma–Aldrich (St. Louis, USA). Moreover, a biological safety cabinet (Pachane 410, Piracicaba, Brazil), optical microscope (Olympus CX31, Center Valley, USA), microbiological incubator (Nova Ética 410/7NDR, Brazil), disposable sterile plastic material (Petri dishes, pipettes and microplates were purchase from J Prolab, Brazil) and autoclave (Phoenix AV 50, Brazil) were also used in some experiments.

2.2. Rice soil-rhizosphere sampling

Soils were sampled from two different cities with a tradition of rice cultivation: Alegrete (29° 46′ 59″ S 55° 47′ 31″ W) state of Rio Grande do Sul, Brazil and Guaratinguetá (22° 48′ 57″ S 45° 11′ 34″ W) state of São Paulo, Brazil. Samples from Alegrete were collected from 4 specific sites: Caiboaté (sample 1), experimentation field of Universidade do Pampa (sample 2), Training Center of the Rice Producers' Cooperative (sample 3), and Tigre Farm (sample 4). Samples from Guaratinguetá were collected from Bresolin Farm (samples 5 to 12) (Fig. S1).

The sampling was performed according to Coelho et al. [18], with modifications. Briefly, 1 cm layer of soil (top soil) was removed. Then, a 30 cm in length plastic tube (previously sterilized) was inserted into the soil. The soil containing rice roots was withdrawn. Three samples were obtained from each site, and then passed through a sieve (2 mm, cleaned with 70° GL alcohol), and thoroughly mixed by agitation. The soil that remained attached to the roots was considered the rhizosphere [18]. Finally, this rhizosphere was removed, homogenized, packed in sterilized plastic bags containing air and stored at 4 °C prior to isolation of microorganisms and the determination of organic matter and chemical elements.

2.3. Determination of chemical elements and organic matter in rhizosphere samples

Rhizosphere samples (2 g) were dried using oven air circulation (55 °C), passed through a sieve (<0.25 mm), and homogenized. Furthermore, 100 mg of this soil sample was weighed (in triplicate) in a 50 mL conic tube followed by the addition of 2 mL of HNO_3 (14 mol l^{-1}). This mixture was allowed to stand for 48 h (pre digestion). After this, 4 mL of H_2O_2 (30% v/v) was added and the mixture was digested in a microwave oven using a stepwise heating program proposed by Nardi et al. [19]. The digested contents were diluted to 50 mL with ultra-pure water and the total amounts of the elements As, Cd, Pb, Ni, Mn, Se, Co, Cr, Ba, Rb, U, Cs, V, Cu, Fe, Mg and Zn were determined by ICP-MS. For quality control purposes the reference material of soil NCS ZC73007 (from China National Analysis Center for Iron and Steel, China) was analyzed. Additionally, the concentrations of organic matter in all soil samples were determined based on the extraction procedure using sodium pyrophosphate [20].

2.4. Isolation and identification of filamentous fungi from the rhizosphere

One gram of each collected soil was added to 10 mL of sterile deionized water. This mixture was homogenized during 1 min and serial dilutions (1:10) in sterile deionized water was performed, until achieving a 1:10,000 diluted sample. Then, 100 μL of each sample dilution was inoculated on a potato dextrose agarose (PDA), spread with a sterile Drigalski spatula and incubated at 28 °C in petri dishes (90 Ø × 15 mm).

The fungal growth was observed daily until achieving the formation of asexual reproductive structures. The classical identification of the most common isolated fungi was performed based on their macroscopic and microscopic characteristic assessed by microculture technique [21]. The asexual reproductive structure from each colony was observed under the optical microscope at 400x (data not shown). Fig. S2 show some