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Isolation and characterization of yeasts associated with plants growing in heavy metal- and arsenic-contaminated soils

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Research Article

**Isolation and characterization of yeasts associated with plants growing in heavy
metal- and arsenic-contaminated soils**

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37 **Short title:** Heavy metals, arsenic-resistance yeasts.

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Abstract

Yeasts were quantified and isolated from the rhizospheres of five plant species grown at two sites of a Mexican region contaminated with arsenic, lead, and other heavy metals. Yeast abundance was about 10^2 CFU g^{-1} soil and 31 isolates were obtained. Based on the phylogenetic analysis of 26S *rRNA* and ITS fragment, six species within five genera were identified, including *Cryptococcus* (80.64%), *Rhodotorula* (6.45%), *Exophiala* (6.45%), *Trichosporon* (3.22%), and *Cystobasidium* (3.22%). *Cryptococcus* spp. was the predominant group. Pectinases (51.6%), proteases (51.6%), and xylanases (41.9%) were the enzymes most common, while poor siderophores (16.1%) and Indole Acetic Acid (IAA) (9.67%) production was detected. Isolates of *Rhodotorula mucilaginosa* and *Cystobasidium sloffiae* could promote plant growth and seed germination in bioassay with *Brassica juncea*. Resistance of isolates by As and heavy metals was as follows: $As^{3+} \geq 100$ mM; $As^{5+} \geq 30$ mM; $Zn^{2+} \geq 2$ mM; $Pb^{2+} \geq 1.2$ mM, and $Cu^{2+} \geq 0.5$ mM. Strains of *Cryptococcus albidus* were able to reduce Arsenate (As^{5+}) into Arsenite (As^{3+}), but no isolate was capable of oxidizing arsenite. This is the first study on the abundance and identification of rhizosphere yeasts and in a HM and arsenic-contaminated soil and in arsenate reduction by the species *C. albidus*.

Keywords: Yeast, Rhizosphere, Reduction, Arsenic, Speciation.

Introduction

Arsenic (As) is a common toxic contaminant produced by industry extraction or anthropogenic input in nature, but it is also released as a consequence of rock weathering or volcanic explosions (Donahoe et al. 2004; Wang and Mulligan 2006). In different environments, As is present as inorganic (As^{5+} and As^{3+}) or organic [Monomethylarsonic Acid (MMA), DimethylArsonic Acid (DMA), and TrimethylArsine Oxide (TMAO)] forms, which can be transformed by microbial activities, such as oxidation-reduction and methylation-demethylation (Turpeinen et al. 2002).

Many bacteria, archaea, and fungi have developed mechanisms for avoiding arsenic toxicity, including the following: (i) reduction of Arsenate (As^{5+}) to Arsenite (As^{3+}) and expelling the latter outside of the cells (Čerňanský et al. 2009); (ii) arsenic complexation with other molecules inside the cells, such as glutathione and vacuolar sequestration (Rosen 2002); (iii) methylation of arsenic to the less toxic organic forms (Qin et al. 2006); (iv) oxidation of arsenite to the less toxic arsenate (Gihring and Banfield 2001), and (v) metalloid immobilization by absorption and accumulation in biomass (Wang and Xhao 2009).

Similar to other microorganisms in the soil and rhizosphere, yeasts play an important ecological role, such as recycling nutrients, aggregating soil particles, assimilating secondary products from bacteria and other fungi, exhibiting different interactions (amensalism, predation, and competition) with other microorganisms and plants, and possessing versatile metabolisms for utilizing and transforming nitrogen compounds

(Botha 2011). Some yeasts, such as *Trichosporon* spp., *Rhodotorula* spp., and *Candida tropicalis*, are known for their capability to resist and accumulate arsenic and heavy metals (Olasupo et al. 1993; Ilyas et al. 2014; Ilyas et al. 2015). The biotransformation of arsenic is well studied in the yeast *Saccharomyces cerevisiae*, and its related pathways and the genes and mutants involved have been characterized (Menezes et al. 2004; Shah et al. 2010; Todorova et al. 2010). Other yeasts, such as *Schizosaccharomyces pombe*, *Trichosporon* spp., *Rhodotorula* spp., and *Cryptococcus humicolus* have been studied in terms of the accumulation, biosorption, and quantification of the oxidation and reduction process (Button et al. 1973; Salgado et al. 2012; Ilyas 2014). However, no information exists about the yeast population associated with HM- and arsenic-resistant plants or their characterization.

The aim of this work was to evaluate the diversity of rhizospheric yeasts associated with HM- and arsenic-resistant plants and to evaluate their ability to biotransform As. In order to accomplish this, we isolated and characterized the rhizosphere yeasts associated with five plant species grown at two sites in a small town, namely Villa de la Paz, in the State of San Luis Potosí, Mexico, a traditional mining region with mine tailings, soils, and plants contaminated with high concentrations of arsenic and HM (Franco-Hernández 2010). To date, no study on microbial diversity and the HM and arsenic detoxification potential in this region has been reported.

Materials and Methods

Sampling sites

The sampling sites are located in Villa de la Paz in the State of San Luis Potosí (23.7 N, 178.7 W), a zone with a mean annual temperature of 18°C and an average annual precipitation of 486 mm. The two sampling sites comprise a mine tailing with an altitude of 1,557 m, and a natural hill with an altitude of 1,830 m, with a distance of about 5 km between them. In a previous study, it was reported that this soil is contaminated with risk elements (Franco-Hernández et al. 2010). Soil analysis revealed the following: Arsenic (As) >3,574 mg.kg⁻¹, lead (Pb) >555.45 mg.kg⁻¹, Copper (Cu) >2,012 mg.kg⁻¹, and Zinc (Zn) >1,337 mg.kg⁻¹ have been detected in the mine tailing; at the hill site, the data were the following: As >1,071.50 mg.kg⁻¹; Pb >5,478.60 mg.kg⁻¹; Cu >749.30 mg.kg⁻¹, and Zn, >1,672.85 mg.kg⁻¹ (data shown in Supplementary Material). In this work, the physicochemical parameters of the mine and hill site were analyzed using the methods described by Vásquez-Murrieta et al. (2006).

Plant identification and soil sampling characterization

At both sites, four individual plants of the most abundant plant species were sampled randomly by extracting plants with their roots together with their soils. The samples were maintained in plastic bags, transported immediately to the laboratory, and stored thereafter at 4°C. All of the sampled plants were mature and appeared healthy without the presence of parasites. The plants were identified by Botanists in the *Plant Ecology Laboratory* of the National School of Biological Science (ENCB) of the National Polytechnic Institute (IPN) in Mexico City, based on typical morphology and taxonomic features depending on the plant species (Calderón de Rzedowski and Rzedowski 2001; McVaugh 1984; McVaugh 1987). Rhizosphere soils were prepared by brushing off the

soils adhering to the root surface of the plants, as previously described (Trujillo-Cabrera et al. 2013), and these were used for yeast isolation. Soils sampled together with the plants of each species at the same site were compiled in the same volume, which was then employed for analysis of the contents of HM and arsenic and nutrients, as well as the physicochemical features, as reported previously (Franco-Hernández et al. 2010; Vásquez-Murrieta et al. 2006).

Yeast isolation

For isolation of yeasts, 0.5 g of rhizospheric soil from each plant species was suspended in 4.5 mL of sterile saline solution (NaCl 0.85%). Serial dilutions were prepared up to 10^{-3} , and an aliquot of 0.1 mL from each dilution was spread on the following media in Petri dishes: Yeast-Peptone-Dextrose (YPD) agar (Yeast extract, 10 g; Peptone, 20 g; Dextrose, 20 g; Agar, 15 g; Distilled water, 1 L, added with Streptomycin, 20 mg) and Rose-Bengal Agar (DIFCO) (Chloramphenicol, 0.1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; KH_2PO_4 , 1.0 g; Dextrose, 10 g; Rose-Bengal, 0.05 g; Soy peptone, 5.0 g; agar 15 g, and Distilled water, 1 L). The inoculated Petri dishes were incubated at 28°C for 7 days. Single colonies were selected according to their morphology and were purified by repeated cross-striking on YPD plates of until all of the colonies in the same isolate presented similar morphology (Al-gabr et al. 2014). Purity of the isolates was verified microscopically, and the pure isolates were conserved at -70°C in microtubes (1.5 mL) half filled with YPD broth supplied with glycerol (50%, w/v).

Amplification and sequencing of yeasts 26S rRNA gene and ITS fragment

Genomic DNA extracted from each yeast isolate using the protocols of Allers and Lichten (2000) was used as template to amplify the *26S rRNA* and ITS fragments by PCR with a thermocycler (MaxyGene Thermal Cycler THERM 1061; AxyGen Scientific), following the subsequent protocol: an initial 10-min denaturing step at 94°C, followed by 30, 1-min cycles at 94°C, 1 min of annealing at 54°C, 1 min extension at 72°C, and a final 8-min polymerization step at 72°C. The PCR mixture (50 µL) contained 50–100 ng of DNA template, 3 mM MgCl₂, 2.5 U *Taq* DNA polymerase (Invitrogen, USA), 1X PCR buffer, 20 pmol of primers for *26S rRNA*: NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') (Redecker 2000), and the following for the ITS fragment: ITS1 (5'-TCC CGT AGG TGA CCT GCG G 3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC 3') under the same thermal condition (Gardes and Bruns 1993) and 200 µM of each dNTP. Amplification products were visualized after electrophoresis in agarose gel (1%, w/v) in 0.5X TAE buffer by staining with an aqueous solution of ethidium bromide (0.5 µg.mL⁻¹). A PureLink™ 310002 commercial kit (Invitrogen) was utilized for purifying the PCR products, which were then sequenced under Big Dye™ terminator cycling conditions with the same primers using the Automatic Sequencer 3730XL in Macrogen (Korea).

Phylogenetic analysis

The D1/D2 regions of the *26S rRNA* sequences obtained and the ITS fragment were employed to estimate the taxonomic affiliation and genotype designations of the yeast isolates. BLASTn searches for *26S rRNA* and ITS sequence data in the National Center

for Biotechnology Information (NCBI) GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to extract the sequences for related taxa and were utilized as references for the subsequent phylogenetic analysis. All of the sequences obtained in this study were manually edited with the reference alignment editor BIOEDIT and aligned together with the reference sequences using CLUSTAL X ver. 1.7 software (Thompson 1997). The phylogenetic tree was constructed by the Maximum Likelihood method (Guindon and Gascuel 2003) using the best models: (Kimura 2 Parameter + I + G) for 26S rRNA and Tamura 3 parameter + I + G for ITS fragments selected by using jModelTest ver 3.06 software, based on the Akaike Information Criterion (AIC) (Posada 2008). Statistical validation at each node was determined by 1,000 bootstrap replicates. A zygomycete, *Rhizopus microsporum*, was employed as out-group for tree rooting.

Determination of enzymatic activities

The productions of the following enzymes were analyzed: amylase; pectinase; xylanase; cellulase; protease, and pectinase. Enzymatic activities were performed by initially growing the isolates in YPD broth for 24 h at 28°C. Afterward, 100 µL aliquots were inoculated on the specific culture media for the investigation of each enzyme. The plates were incubated at 28°C during 72 h. Screening for amylases and pectinases was performed in Castañeda medium (Castañeda-Agullo 1956), into which 2% (w/v) of starch or 1% (w/v) of pectin was added, correspondingly. For xylanase and cellulase screening, the Congo red medium was produced (Hankin and Anagnostakis 1977; Suto et al. 2002). For protease detection, the skim-milk medium was utilized (Atlas 1997). Positive

amylase activity was revealed by the presence of a clear zone around a colony after immersing the yeasts colonies in Lugol solution. Positive pectinase activity was recognized by the presence of a clear ring around a colony following the immersion in Cethyl Trimetyl Ammonium Bromide (CTAB) solution (CTAB 5%, w/v). Positive degradation of cellulose, xylan, or casein was identified in the corresponding medium by the presence of a clear ring around a colony. The Enzymatic Index (EI) was determined within 72 h of incubation, which was expressed as the relationship between the average diameter of the transparent (degradation) ring and the average diameter of the colony (Hankin and Anagnostakis 1975).

Determination of the potential of plant growth- promoting traits for isolates

Phosphate solubilization capacity was evaluated with Pikovskaya medium (Paul and Sundara Rao 1971) and was considered positive if a clear ring appeared around the colony. Polyamine production was detected using the Long Asthon Decarboxylase (LAD) medium (Arena and Manca de Nadra 2001), with the color change of the medium from yellow to red considered as a positive test. Siderophore production was evaluated according to Schwyn and Neilands (1987) using Cromo-Azurol S (CAS) medium, and the color change of the medium to yellow, orange, or purple around the colony was considered as a positive test. IAA production was evaluated in YPD broth according to Nassar et al. (2005) modified by Limtong and Koowadjanakul (2012); the appearance of a pink color within 30 min after treatment with Salkowski reagent was recorded as a positive test.

Quantification of IAA

Quantification of the IAA produced by the yeast isolates was carried out using the Salkowski method (Glickmann and Dessaux, 1994). One pre-inoculum of each yeast was prepared in YPD at 28°C for 24 h. One mL of this culture was employed to inoculate a second flask with YPD medium and was incubated at 28°C at 150 rpm, until reaching about 10^8 cell/mL⁻¹, at which time tryptophan 1% (SigmaTM) was added. One- mL aliquots were taken every 24 h; these were placed in an Eppendorf tube and centrifuged at 1,500 rpm/5 min. The supernatant was transferred into a new tube, and the same amount of Salkowski reagent was added to this (12 g.L⁻¹ FeCl₃, 7.9 M H₂SO₄), the reaction was left for 45 min at 28°C under conditions of darkness, and the reaction was read in a spectrophotometer at 540 nm. The quantity of IAA was determined using a standard curve with a range of concentrations of 1, 2, 3, 5, 6, 8, 9, 10, 15, and 20 µg.mL⁻¹. The standard curve was prepared using a stock solution of IAA (JT Baker[®]) with a final concentration of 1 mg.mL⁻¹, effecting proper dilutions in YPD medium. Each determination was carried out in triplicate. Statistical analysis by means of two-way Analysis of variance (ANOVA) was conducted using the method of Duncan with $p < 0.05$ to determine significant differences.

Effect of IAA produced by rhizospheric yeast on *Brassica juncea* seed germination

Five hundred µL of sterile, filtered supernatants from the cultures described previously and containing 4 µg of IAA were allowed to be absorbed onto Petri dishes containing water agar. Ten seeds of *Brassica juncea* disinfected with 2% sodium hypochlorite were placed in each Petri dish. The negative control had 10 seeds per Petri dishes without IAA.

All Petri dishes were incubated at 28°C and germination percentage was determined at 24 and 48 h. All assays were performed in triplicate. Kruskal-Wallis statistical analysis was performed to evaluate the germination percentage and the one-way ANOVA were conducted to evaluate plant growth. The means of each experiment were compared using the Duncan multiple range test. All analyses were carried out using SigmaPlot ver. 12.0 statistical software (Copyright© 2006; Systat Software, Inc.), and we considered $p < 0.05$ acceptable for statistical significance.

Resistance of the isolates to HM and arsenic

Minimum Inhibitory Concentration (MIC) was evaluated using resistance to HM and arsenic in MES-Buffered Minimal Medium (MBMM) proposed by Rathnayake et al. (2013). HM and As were supplemented at concentrations of 0.05, 0.1, 2.0, 4.0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mM) in the forms of CuSO₄, ZnSO₄, Pb (NO₃)₂, NaH₂AsO₄, and NaAsO₂. Inoculation and incubation conditions were the same as those for resistance to salinity.

Oxidation and reduction of arsenic compounds by the isolates

The ability to oxidize the arsenite or reduce the arsenate of the yeast isolates was estimated by utilizing the KMnO₄ qualitative test (Salmassi et al. 2002). The yeasts were cultured in liquid Chemically Defined Medium (CDM) (Weeger et al. 1999) medium containing NaH₂AsO₄ or NaAsO₂ at a final concentration of 100 mg.L⁻¹ with an initial Optical Density (OD)₆₀₀ of 0.4. The culture was incubated for 5 days with shaking (120 rpm). Then, 20 µL of KMnO₄ solution (0.01 mol.L⁻¹) was added to 1 mL of the culture.

The presence of pink color in the broth indicates positivity for arsenite oxidation and generation of yellow color indicated arsenate reduction. The isolates positive in the qualitative test were evaluated with the quantitative method of Molybdenum blue (Hu et al. 2012).

Results

Plant Identification and Soil Characterization

A total of five plant species were collected in the region of Villa de La Paz. At the mine tailing site, the plants *Sphaeralcea angustifolia*, *Prosopis* spp., *Flaveria angustifolia*, and *Thitonia diversifolia* were the most abundant, while *Bahia absinthifolia*, *Sphaeralcea angustifolia*, and *Prosopis* spp. comprised the main plants on the hill. Soils at both sampling sites were seriously contaminated by multiple HM and As, especially As, Cd, Cu, Mn, Pb, and Zn, which presented at concentrations (mg.kg^{-1}) of 3,574, 129, 2,012, 1,382, 555, and 1,337 in the mine tailing, and of 1,071, 62, 749, 3,054, 5,488, and 1,672 on the hill, respectively (detailed information available in Supplementary Table S1). In addition, greater values related with nutrient features were observed in the hill soil, such as Cation Exchange Capacity (CEC), Organic Carbon (OC), Total Nitrogen (TN), and Total Phosphorous (TP), were 77 cmolc.kg^{-1} , 7.7%, 0.32 mg.kg^{-1} , and 69 mg.kg^{-1} , respectively, while corresponding values were 38, 1.9, 0.09, and 33 at the mine tailing.

Yeast Isolation and Identification

During the isolations, we found that the abundance of yeasts in rhizosphere soils was around 200 CFU.g⁻¹ of soil. In total, 31 rhizospheric yeasts representing different colony morphology were isolated from the sampled plants (Table 1). The D1/D2 fragment of the *26S rRNA* gene was amplified from all of the isolates, with the expected size of 600 pb. Additionally, the ITS fragment was amplified with the same expected size. The nucleotide sequences acquired in this study have been deposited in GenBank under accession numbers KP455389-KP455419 for *26S rRNA* and KT716400-KT716430 for the ITS fragment.

Phylogenetic analyses of the D1/D2 sequences grouped the isolates within the phyla *Basidiomycota* (29 isolates) and *Ascomycota* (two isolates), with 25 isolates belonging to the genus *Cryptococcus* (80.64%), two to *Rhodotorula* (6.45%), two to *Exophiala* (6.45%), one to *Trichosporon* (3.22%), and one to *Cystobasidium* (3.22%) (Fig. 1A). The majority of the isolates exhibited high sequence coverage and identity (100 and 99–100%) with the defined species (data available as Supplementary Table S2). Only two isolates, YR30 and YR31, presented 100% coverage and 97% identity with *Exophiala pisciphila*. Twenty four isolates were identified as *Cryptococcus* spp. related with the species *Cryptococcus albidus*/*Cryptococcus kuetzingii*/*Cryptococcus adeliensis*; YR14 was designed as *Cryptococcus uzbekistanensis*, isolate YR11 was identified as *Cystobasidium slooffiae*, and finally, isolate YR20 was designated as *Trichosporon* spp. and was related with the group *Trichosporon japonicum*/*Trichosporon coremiiforme*/*Trichosporon insectorum*.

The ITS sequences demonstrated the following information: The *Cryptococcus* genus was the most common and was represented mainly by the species *C. albidus* and *C. uzbekistanensis* (25 isolates exhibited an identity range of 99–100%). The genus *Rhodotorula mucilaginosa* was represented by three isolates (YR24, YR29, and YR11). The *Exophiala* genus was represented by isolates belonging to the species *Exophiala capensis* with low identity of 87%. Finally, isolate YR20 was again designated as *Trichosporon*, related with the group *T. japonicum*, *T. insectorum*, *Trichosporon faecale*, and *Trichosporon asteroides*, as depicted in Fig. 1B.

Enzymatic Activity and Potential Plant Growth- promoting Features of Rhizospheric Yeast Isolates

Results of the enzymatic characterization demonstrated that 51.6% (16/31), 51.6% (16/31), 41.9% (13/31), 12.9% (4/31), and 0% of the rhizospheric yeasts presented pectinase, protease, xylanase, cellulase, and amylase activities, respectively (Table 1). For the plant growth promoting features, only siderophore and IAA productions were detected among some isolates at the proportions of 16.1% (5/31) and 9.67% (3/31), respectively. The species *R. mucilaginosa* (two isolates) and *C. sloffiae* (one isolate) were positive for AIA production, and both genera were selected for the IAA quantification. Polyamine production and phosphate solubilization were absent in all isolates.

IAA Quantification of the Two Isolated Yeasts

Three yeasts are capable of producing IAA, two of them belonging to *R. mucilaginosa* (YR29 and YR24,) with auxin produced concentrations of 9.61 and 9.02 mg.mL⁻¹ in 7

days respectively, while *C. slooffiae* produced only 6.8 mg.mL^{-1} , with a significant difference in IAA production between strains and between incubation times ($p < 0.05$). In addition, the concentration of IAA increased as time passed from incubation (Fig. 2).

Germination Promotion of *Brassica juncea* Seed by IAA Secreted by Rhizospheric Yeasts

A significant increase was observed in seed germination of *B. juncea* with treatments containing filtrates of rhizospheric yeasts (YR24, YR11, and YR29) compared with the control. Seed germination at 48 h of incubation increased by $>70\%$ in all treatments including the control. Only the treatment with the YR24 strain filtrate demonstrated a significant difference ($p < 0.05$) from the rest of these, with 96.6% germination (Fig. 4). In the case of seedling growth, in all treatments supplemented with filtrate yeast, seedling growth exhibited a significant increase compared with the control ($p < 0.05$) (Figs. 3 and 4). These results suggest that the IAA produced by the rhizospheric yeasts promotes germination and plant growth under the conditions tested.

Heavy-metal Resistance and Arsenic Oxidation/Reduction

The isolated yeasts demonstrated high resistance to Arsenic (As^{3+} and As^{5+}), but were sensitive to the remaining HM, especially to Cu. The MIC of the 31 isolates in MBMM was 0–5 mM for ZnSO_4 , 0–1.2 mM for $\text{Pb}(\text{NO}_3)_2$, 0–0.5 mM for CuSO_4 , 0–30 mM for NaH_2AsO_4 , and 0–100 mM for NaAsO_2 (Table 2). The order of toxicity of RE to the isolates was $\text{Cu} > \text{Zn} > \text{Pb} > \text{As}^{5+} > \text{As}^{3+}$. Higher resistance was observed among yeasts isolated from the mining tailing. Arsenic oxidation/reduction assays showed that 51.6%

(16/31) of the rhizospheric yeasts can reduce arsenate, but none of these oxidized arsenite; all positive isolates belonged to the species *C. albidus* and exhibited a reduction percentage within the range of 10–40% in samples with 0.15 mM of As^V (Table 2),

Discussion

In the present study, the rhizospheric yeasts were isolated and characterized, to our knowledge for the first time, from the plants grown in a region that were seriously contaminated with multiple HM and arsenic. Of the two sampling sites, both the hill and the mining tailing could be classified as extreme environments based on their low nutrients and high As, Pb, Zn, Mn, Cd, and Cu (see data in Supplementary Table S1). The mine tailing possesses high concentration of HM and arsenic (As, Pb, Zn, Mn, Cd, and Cu), poor organic matter, low nitrogen, and is slightly salty and slightly acidic to neutral pH, while the hill also possesses high concentrations of HM and arsenic; however, the concentration of nitrogen and organic matter presented a normal-to-high range and was slightly acid to neutral pH. The parameters of these soils are important because they could affect the bioavailability of HM and arsenic and their toxicity for plants and microorganisms (Jasim et al. 2014).

Yeasts have been isolated from different environments, including extreme environments and from the rhizosphere of some plants (Hong et al. 2002; Hong et al. 2006), and are employed as biocontrol agents or plant growth promoters (El-Mehalawy 2004; Mestre et al. 2011). However, little information is available on yeasts associated with HM- and arsenic-resistant plants. Deng and contributors (Deng et al. 2012) characterized an HM-resistant endophytic yeast, *Cryptococcus* spp. CBSB78, which was resistant to 20 mM

Cd²⁺, 20 mM Pb²⁺, 10 mM Zn²⁺, and 7 mM Cu²⁺, and could improve the growth and heavy-metal extraction of inoculated *Brassica* plants. Castro-Silva et al. (2003) isolated and analyzed 12 HM-resistant yeasts from water, sediment/soil, and plant resources, which exhibited HM resistance to 1 mM CdCl₂ and/or 1–20 mM ZnCl₂, but the authors did not identify the isolates.

Currently, molecular identification employing the large-subunit rDNA D1/D2 domain and ITS fragment sequence analysis has been utilized to estimate the taxonomic affiliation of the yeasts (Kurtzman and Robnett 1998; Linton et al. 2007; Gardes and Bruns 1993). In the present study, these techniques could successfully identify the isolates into five genera (Figs. 1A and 1B) (Table 1). However, species affiliations were uncertain in the following one case: the isolate YR20 possessed a sequence nearly identical to those of *T. japonicum*, *T. insectorum*, and *T. coremiiforme* on 26S rRNA analysis and was related with the group including *T. japonicum*, *T. insectorum*, *T. faecale*, and *T. asteroides* on ITS. These cases indicated the inability of two molecular markers for differentiating closely related species; therefore, the exact species definition of *Trichosporon* spp. isolates requires additional molecular analysis, such as multi-locus sequencing (Bovers et al. 2008; Bernhardt et al. 2014; Linton et al. 2007).

Both the abundance and the taxon number detected in this study were rather low, which might be related with the extreme environmental factors, especially the high concentrations of HM and arsenic. Botha (2006) revealed that the pH and Cu concentration in soil were the principal factors for decreasing yeast number. In our study, Cu concentration reached 2,012 and 749 mg.kg⁻¹ soil at the mining tailing and at the hill

soil, respectively, much greater than their MIC values ($0\text{--}0.5\text{ mM} \approx 0\text{--}32.8\text{ mg.kg}^{-1}$) for the isolates (Table 2). These results suggest a difference in the rhizospheric Cu concentration and in the soil surrounding the plants, suggesting positive interaction between yeast and plants. These possible interactions require further investigation.

Previously, *Cryptococcus*, *Rhodotorula*, and *Trichosporon* have been isolated from phylloplane and rhizosphere of sugar cane (Biswas et al. 2001; Insuellas de Azeredo et al. 1998) and from bulk and rhizosphere soils in forest region (Hong et al. 2002; Mestre et al. 2011). The isolation of these in the present study further confirmed their ubiquitous distribution.

Identification of *Cryptococcus* as the main group in our study was similar to previous reports that *Cryptococcus* comprised the cosmopolitan yeasts in different soils (Slavikova and Vadkertiova 2003; Slavikova et al. 2007; Vishniac 1995) and in rhizosphere/endophytic environments (Deng et al. 2012; Insuellas de Azeredo et al. 1998; Mestre et al. 2011). The *C. albidus* clade, which covered the majority of the isolates obtained in the present study, is the most important organism in sandy and desert soils, described as dominant in Mexican soils (Vishniac 2006). However, all of the three species in this clade that is, *C. albidus*, *C. adeliensis*, and *C. kuetzingii* (synonym for *Cryptococcus albidus* var. *kuetzingii*) have rarely been isolated as pathogens (Liu et al. 2013; Tintelnot and Losert 2005).

In the past decade, *Rhodotorula* species, including *R. mucilaginosa*, which covered two isolates in this study, are found to be ubiquitous in soil and rhizosphere (Hong et al.

2002). Similar to *Cryptococcus*, *Rhodotorula* species belonged to the capsulated yeast, but presented an orange or red color. Both capsule and pigments were able to protect these from different stress conditions, such as radiation (Molinè et al. 2010).

The genus *Trichosporon* contained species that are widely distributed in different environments. It has rarely infected immunocompromised persons as a pathogen (Colombo et al. 2011). Some of the saprophytic species possessed capacities for bioremediation and biotechnology (Santos et al. 2001). *T. insectorum* is a killer yeast isolated from the gut of insects and artisanal cheese (Fuentefria et al. 2008), while *T. coremiiforme* could degrade corn cob acid and produce diverse lipids (Huang et al. 2013).

Previously, little information on *Exophiala* and *Cystobasidium* from the environments was available. In the present study, two isolates were identified as members of *Exophiala*, classified as a Dark Septate Endophyte (DSE); there are some reports concerning the extreme Cadmium (Cd) tolerance of *Exophiala pisciphila*, including metal ion binding and transportation, and organic acid metabolism (Zhao et al. 2015). Because DSE are typical root endophytes, their potential effects on host plants should also be considered. Under heavy-metal stress, these fungi can affect heavy metal uptake and the tolerance of the host plants, e.g., *Zea mays* L. (Li et al. 2011). *Cystobasidium* is a genus described as living in different associations with other fungi, lichens, and woods; in addition, some isolates have clinical relevance (Yurkov et al. 2015). To our knowledge, this is the first time that *Cystobasidium* yeast has been isolated from rhizosphere of HM- and arsenic-resistant plants. Detection of *Cystobasidium* and *Exophiala* in the present study enlarged

the diversity of rhizosphere yeasts, especially in HM-contaminated regions, and implied that they may play some ecological role in HM-contaminated soils.

In tests for enzymatic and potential plant growth- promoting activities (Table 1), considerable proportions of the isolates presented pectinases (51.6%), proteases (51.6%), and xylanases (42.9%), but only 12.9% of the isolates presented cellulases. These enzymes are involved in the degradation of the plant cell wall (Riou et al. 1991; Schulz and Boyle 2005). Therefore, the presence of these enzymes may help these rhizospheric yeasts to colonize the rhizosphere.

Previously, it was reported that some fungi, including the *Rhodotorula* yeast, could produce siderophore (such as rhodotorulic acid) under iron-deficient conditions, which exerts an antagonistic effect against other filamentous fungi (Calvente et al. 2001). However, the soils involved in the present study are iron-rich (about 35 g Fe kg⁻¹ soil); thus, siderophore production should not be related with iron deficiency, but rather linked with other functions. It has been reported that the siderophore could increase producer-resistance to HM or metalloids (Schalk et al. 2011), increase As (Shah et al. 2010), improve soil fertility, and inhibit fungal pathogens (Ali and Vidhale 2013; Matthijs et al. 2007; Shah et al. 1992). The six siderophore-producing yeasts were defined as members of four genera and were isolated from rhizosphere of three plant species grown at both sites: *Cryptococcus* spp. YR6, YR12, and *Trichosporon* spp. YR20 from *Flaveria angustifolia*, *Sphaeralcea angustifolia*, and *Prosopis* plants, respectively, grown at the mine tailing, *Rhodotorula* spp. YR24 and *Exophiala* spp. YR30 and YR31 from *Prosopis* plants grown at the hill. However, the low proportion (16.1% of the isolates) and the low

index implied that siderophore production might not comprise determinants for the colonization and survival of yeasts in the rhizosphere of plants grown in the tested environments.

IAA production was detected in three isolates, associated with three plant species grown at the mine tailing or at the hill. These were members of *Cystobasidium* spp. YR11 (from *F. angustifolia*, mine tailing) *Rhodotorula* spp. YR24 (from *S. angustifolia* at the hill), YR29 (from *Prosopis* spp. at the hill). The genera *Rhodotorula* and *Cystobasidium* produced IAA in amounts of 6.8–9.61 µg/mL, and these concentrations are considered low in comparison with other genera in *Ascomycota*, such as *Candida maltosa*, *Candida glabrata*, and *Candida tropicalis* (Limtong and Koowadjanakul 2012). Their low proportion in this study demonstrated that this feature was not selected by the rhizosphere in the soils tested. However, IAA production was sufficient for promoting seed germination and plant growth. Polyamines and inorganic phosphate solubilization were not detected among the yeasts isolated in this study, perhaps based upon the same reason estimated for IAA production.

HM and As resistance has been reported for some yeasts (Olasupo et al. 1993, Vadkertiová and Sláviková 2006). In our work, the yeast isolates demonstrated poor-to-intermediate resistance to HM and As, compared with the results of other authors (Vadkertiová and Sláviková 2006). However, *C. albidus*-related yeasts exhibited high resistance to arsenic: up to 30 mM for arsenate and 100 mM for arsenite. In general, arsenate is less toxic than arsenite, and greater resistance to arsenate than arsenite has been reported in some bacteria and fungi. Thus, it could be estimated that these 13 yeast

isolates may possess a different mechanism for arsenite resistance. A mechanism related with this estimation has been well studied in *Saccharomyces cerevisiae*, in which the arsenate was reduced to arsenite and was expelled from the cell (Čerňanský et al. 2009). In nature, arsenite can be also detoxified by methylation, which transforms the arsenite into a volatile form (Su et al. 2011). Further study is required on our isolates in order to ascertain whether they possess the methylation function or another. To date, scarce information is available on As transformation by yeasts, and there is no report on As transformation by *Cryptococcus*. Therefore, our study was, to our knowledge, the first report on arsenate reduction by this yeast.

In conclusion, yeasts associated with the rhizosphere of *S. angustifolia*, *Prosopis* spp., *Thitonia diversifolia*, *F. angustifolia*, and *Bahia absinthifolia* plants grown in soils seriously contaminated with arsenic and other HM presented low abundance (10^2 CFU g⁻¹ of soil) and low diversity, covering only five species in five genera. The *Cryptococcus* group comprised the major yeast found in all of the five plants grown at both sampling sites. Many yeast isolates produce pectinases, proteases, and cellulases and have a low production of siderophore and IAA. The majority of the yeasts demonstrated resistance to salinity, alkaline conditions, and also resistance to multiple HM and arsenic at high concentrations. Some isolates presented higher resistance to arsenite than to arsenate and they reduced arsenate to arsenite. Future study to evaluate the capacity for transforming As³⁺ into methyl-compounds and the potential for bioremediation is needed.

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Table and Supplementary Table Titles

Table 1. Enzymatic activities and IAA production of the yeasts isolated of different plant collected in the region of Villa de La Paz, San Luis Potosí, Mexico

Table 2. MIC of risk elements and capacity for oxidize or reducing arsenic of rhizospheric yeasts

Supplementary Table S1. Physicochemical characteristic of the soil of the two sampling sites in Villa de la Paz, San Luis Potosí, Mexico

Supplementary Table S2. Yeasts identified based upon *26S rRNA* and ITS sequence analysis

Figures Legends

Fig. 1A. Phylogenetic tree showing the taxonomic affiliation of the rhizospheric yeasts isolated from two soils seriously contaminated by HM and arsenic. The tree was constructed by Maximum Likelihood by Kimura 2 parameter + I model with the sequences of *26S rRNA* D1/D2 domain. Bootstrap values >50% are presented alongside the branch. Isolate numbers are presented in boldface and sequence accession numbers are indicated in parentheses.

Fig. 1B. Phylogenetic tree showing the taxonomic affiliation of the rhizospheric yeasts isolated from two soils seriously contaminated by HM and arsenic. The tree was constructed by Maximum Likelihood by the Tamura 3 parameter + I + G model with sequences of ITS fragment. Bootstrap values >50% are presented alongside the branch. Isolate numbers are presented in boldface and sequence accession numbers are indicated in parentheses.

Fig. 2. Kinetics of IAA production by yeasts *C. slooffiae* (YR11) and *R. mucilaginosa* (YR24 and YR29). A statistical analysis of two-way ANOVA using the method of Duncan with a $p < 0.05$ to determine significant differences.

Fig. 3. Percentage of seed germination at 24–48 h of incubation in water-agar supplemented with yeast filtrates. YR24 = *R. mucilaginosa*, YR11 = *C. slooffiae*, and YR29 = *R. mucilaginosa*. $P < 0.05$. Bar = one times the Standard Error of the Mean (SEM). Different letters indicate statistical significance.

Fig. 4. Plant growth (cm) in water-agar supplemented with yeast filtrates. YR24 = *R. mucilaginosa*, YR11 = *C. slooffiae*, and YR29 = *R. mucilaginosa*. $P < 0.05$. Bar = one times the Standard Error of the Mean (SEM). Different letters indicate statistical significance.

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254x190mm (72 x 72 DPI)

Kinetics of IAA production by yeasts *C. sloffiae* (YR11) and *R. mucilaginosa* (YR 24 and YR29). A statistical analysis of two-way ANOVA using the method of Duncan with a $p < 0.05$ to determine significant differences.

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254x190mm (72 x 72 DPI)

Table 1. Yeast isolates with their relevant information and enzymatic/plant growth promoting activities

Strain No.	Taxonomic affiliation	Index of activity for*				Production	
		Cellulases	Xylanases	Pectinases	Proteases	Siderophore [#]	IAA ug/ml
From <i>Thitonia diversifolia</i> in mine tailing							
YR1	<i>Cryptococcus albidus</i>	ND	ND	ND	ND	ND	-
YR2	<i>Cryptococcus albidus</i>	ND	ND	2	1	ND	-
YR3	<i>Cryptococcus albidus</i>	ND	ND	ND	1.33	ND	-
YR4	<i>Cryptococcus albidus</i>	ND	ND	ND	2	ND	-
YR5	<i>Cryptococcus albidus</i>	ND	ND	1	ND	ND	-
From <i>Flaveria angustifolia</i> in mine tailing							
YR6	<i>Cryptococcus albidus</i>	ND	ND	1.4	1.38	ND	-
YR7	<i>Cryptococcus albidus</i>	ND	ND	ND	ND	1.83	-
YR8	<i>Cryptococcus albidus</i>	ND	1.5	3.91	1.43	ND	-
YR9	<i>Cryptococcus albidus</i>	ND	1.5	2.48	1.52	ND	-
YR10	<i>Cryptococcus albidus</i>	ND	1.18	3.18	1.38	ND	-
YR11	<i>Cystobasidium sloffiae</i>	ND	1.2	ND	ND	ND	6.8
From <i>Spharealcea angustifolia</i> in mine tailing							
YR12	<i>Cryptococcus albidus</i>	ND	ND	ND	ND	1	-
YR13	<i>Cryptococcus albidus</i>	ND	ND	1.66	1.77	ND	-
YR14	<i>Cryptococcus uzbekistanensis</i>	ND	ND	1.29	ND	ND	-
From <i>Prosopis</i> sp in mine tailing							
YR15	<i>Cryptococcus albidus</i>	ND	ND	ND	ND	ND	-
YR16	<i>Cryptococcus albidus</i>	ND	ND	ND	ND	ND	-
YR17	<i>Cryptococcus albidus</i>	ND	ND	ND	ND	ND	-
YR18	<i>Cryptococcus albidus</i>	ND	ND	ND	1.94	ND	-
YR19	<i>Cryptococcus albidus</i>	ND	ND	ND	1.52	ND	-
YR20	<i>Trichosporon</i> sp	ND	1.52	ND	ND	1.34	-
From <i>Bahia absinthifolia</i> in hill							
YR21	<i>Cryptococcus albidus</i>	ND	1.5	2.16	1.88	ND	-
YR22	<i>Cryptococcus albidus</i>	ND	2	1.66	1.33	ND	-
From <i>Spharealcea angustifolia</i> in hill							
YR23	<i>Cryptococcus albidus</i>	ND	ND	ND	ND	ND	-
YR24	<i>Rhodotorula mucilaginosa</i>	1.5	2.11	3.75	ND	2.53	9.02
YR25	<i>Cryptococcus albidus</i>	ND	1.4	3.16	1.63	ND	-
YR26	<i>Cryptococcus albidus</i>	ND	1.63	2.33	2.16	ND	-
YR27	<i>Cryptococcus albidus</i>	ND	1.5	4.66	1	ND	-
YR28	<i>Cryptococcus albidus</i>	ND	ND	2.16	2.22	ND	-
From <i>Prosopis</i> sp in hill							
YR29	<i>Rhodotorula mucilaginosa</i>	1.75	ND	3.2	ND	1	9.61
YR30	<i>Exophiala pisciphila</i>	1.97	3.13	ND	ND	1	-
YR31	<i>Exophiala pisciphila</i>	2.16	2.38	ND	ND	ND	-
Proportion (%) of positive isolates		12.9	41.9	51.6	51.6	16.1	
Number of positive isolates		4	13	16	16	5	3

*. Index of rhizospheric yeast

ND- Not Detected

Table 2. MICs of risk elements and capacity for oxidize or reducing arsenic of rhizospheric yeasts

Strain	Taxonomic affiliation	MIC (mM)					Arsenate Reduction Percentage (%)
		Zn ⁺²	Pb ⁺²	Cu ⁺²	As ⁺⁵	As ⁺³	
From <i>Thitonia diversifolia</i> in mine tailing							
YR1	<i>Cryptococcus albidus</i>	0.5	0.6	NG	2	30	10.24
YR2	<i>Cryptococcus albidus</i>	0.5	1.2	NG	1	30	10.24
YR3	<i>Cryptococcus albidus</i>	0.5	1.2	NG	NG	NG	–
YR4	<i>Cryptococcus albidus</i>	NG	1.2	NG	0.5	1	–
YR5	<i>Cryptococcus albidus</i>	1	0.6	NG	2	50	23.21
From <i>Flaveria angustifolia</i> in mine tailing							
YR6	<i>Cryptococcus albidus</i>	1	1.2	NG	2	100	30
YR7	<i>Cryptococcus albidus</i>	1	0.6	NG	1	20	–
YR8	<i>Cryptococcus albidus</i>	NG	1.2	NG	NG	30	15
YR9	<i>Cryptococcus albidus</i>	NG	1.2	NG	0.5	1	–
YR10	<i>Cryptococcus albidus</i>	1	1.2	0.5	NG	20	20
YR11	<i>Cystobasidium sloffiae</i>	2	NG	NG	2	10	–
From <i>Spharealcea angustifolia</i> in mine tailing							
YR12	<i>Cryptococcus albidus</i>	1	1.2	NG	2	30	20
YR13	<i>Cryptococcus albidus</i>	1	1.2	NG	30	100	40
YR14	<i>Cryptococcus uzbekistanensis</i>	2	1.2	0.5	NG	NG	–
From <i>Prosopis</i> sp. in mine tailing							
YR15	<i>Cryptococcus albidus</i>	2	0.6	0.5	10	30	16
YR16	<i>Cryptococcus albidus</i>	1	1.2	NG	30	30	15
YR17	<i>Cryptococcus albidus</i>	1	1.2	NG	4	60	15
YR18	<i>Cryptococcus albidus</i>	0.5	1.2	NG	20	90	30
YR19	<i>Cryptococcus albidus</i>	0.5	1.2	0.5	4	100	20

YR20	<i>Trichosporon japonicum</i>	0.5	NG	NG	NG	2	–
From <i>Bahia absinthifolia</i> in hill							
YR21	<i>Cryptococcus albidus</i>	1	1.2	NG	0.5	30	20
YR22	<i>Cryptococcus albidus</i>	NG	1.2	NG	0.5	4	–
From <i>Spharealcea angustifolia</i> in hill							
YR23	<i>Cryptococcus albidus</i>	1	1.2	NG	0.5	30	10
YR24	<i>Rhodotorula mucilaginosa</i>	0.5	NG	NG	NG	NG	–
YR25	<i>Cryptococcus albidus</i>	1	1.2	NG	0.5	30	–
YR26	<i>Cryptococcus albidus</i>	2	1.2	NG	0.5	30	–
YR27	<i>Cryptococcus albidus</i>	1	1.2	NG	0.5	20	10
YR28	<i>Cryptococcus albidus</i>	2	1.2	NG	NG	20	–
From <i>Prosopis</i> sp in hill							
YR29	<i>Rhodotorula mucilaginosa</i>	1	1.2	NG	NG	1	–
YR30	<i>Exophiala pisciphila</i>	5	0.3	NG	NG	1	–
YR31	<i>Exophiala pisciphila</i>	5	1.2	NG	NG	1	–
Range or percentage		0.5-2	0.6-1.2	0-0.5	0.5-4	1-100	51.6%

*, Oxidation of arsenite was negative for all the isolates.

NG- Not growth