Ecotoxicology (2016) 25:367–379
DOI 10.1007/s10646-015-1595-5

Effect of Cd^{+2} on phosphate solubilizing abilities and hydrogen peroxide production of soil-borne micromycetes isolated from *Phragmites australis*-rhizosphere

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Accepted: 20 November 2015 / Published online: 8 December 2015

Abstract The aims of this work were to evaluate the phosphate-solubilization and hydrogen peroxide (H_2O_2) production by the soil-borne micromycetes, *Aspergillus japonicus, Penicillium italicum and Penicillium dipodomyicola*, isolated from *Phragmites australis* rhizosphere and to study the effect of several concentrations of Cadmium (Cd^{2+}) on both variables. Our results showed that *P. italicum* achieved a higher P-solubilization and H_2O_2 production than *A. japonicus* and *P. dipodomyicola*, as only *P. italicum* showed a positive correlation ($R^2 = 0.71$) between P-solubilization and H_2O_2 production. In dose–response assays, *P. italicum* was also more tolerant to Cd^{2+} (0.31 mM) in comparison to *A. japonicus* (0.26 mM). Analysis of the 2^4 factorial experimental design showed that P-solubilization by *P. italicum* was negatively affected by increases in Cd^{2+} ($p = 0.04$) and yeast extract ($p = 0.02$) in the culture medium. The production of H_2O_2 was positively affected only by glucose ($p = 0.002$). Fungal biomass production was reduced significantly ($p = 0.0009$) by Cd^{2+} and increased ($p = 0.0003$) by high glucose concentration in the culture medium. The tolerance and correlation between P-solubilization and H_2O_2 production in the presence of Cd^{2+} was strain and species dependent. The effects of Cd^{2+}, glucose, ammonium sulfate and yeast extract on those variables were evaluated through a two-level factorial design. *P. italicum* is promising for P-solubilization in soils contaminated with Cd^{2+} and may be an alternative for manufacture of biofertilizers to replace chemical fertilizers.

Keywords Ecotoxicology · Dose–response assay · Phosphate-solubilizing fungi · *Penicillium italicum*

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Introduction

Given the negative environmental impact of chemical fertilizers and their increasing costs (Ahmed and Shahab 2009), the use of phosphate (P)-solubilizing microorganisms (PSM) as biofertilizers opens up a new horizon for better crop productivity, as well as for protecting agroecosystems from hazardous agrochemicals (Sharma et al. 2013), and has attracted great attention during the last decade (Morales et al. 2011). The rhizosphere is considered to be a major niche for microbial activity, where a substantial number of microorganisms may exert a beneficial effect on plant growth. In addition, P-solubilization ability by rhizosphere microorganisms is one of the most important features associated with plant nutrition (Morales et al. 2011). Extensive research (Nenwani et al. 2010) has shown that microorganisms with the ability to transform inorganic phosphate are mainly located in the rhizosphere of plants, where they play an integral ecological role and are involved in a wide range of processes that affect

P-solubilization. It has been reported (Chakraborty et al. 2010) that the presence of several types of microorganisms associated with the rhizosphere could have the ability to solubilize phosphate complexes, guaranteeing a phosphorus supply for plants (Tripura et al. 2005).

The rhizosphere microorganisms, such as soil-borne micromycetes (SBM), arbuscular mycorrhizal fungi, soil yeasts, bacteria and actinomycetes (Clark and Zeto 2000; Al-Falih 2005; Rudresh et al. 2005; Khan et al. 2007; Hamdali et al. 2008), are documented phosphate solubilizers, although SBM have certain advantages in P-solubilization over other microorganisms. For example, SBM are more acid tolerant and therefore may have a much better potential as agents to convert insoluble inorganic molecules through environmental acidification (Chuang et al. 2007); usually, P-solubilization by SBM produces more organic acid than bacteria and consequently exhibits greater P-solubilizing activity (Scervino et al. 2011). Furthermore, SBM does not lose P-solubilizing ability upon repeated sub-culturing under laboratory conditions, as occurs with bacteria (Chuang et al. 2007). In addition, SBM can traverse long distances easier and faster than bacteria and hence could be more important for P-solubilization in soils (Khan et al. 2010). Among SBM that solubilize phosphorus, the genera *Aspergillus* and *Penicillium* are the most representative, although some species of *Paecilomyces*, *Trichoderma*, *Rhizoctonia*, *Chaetomium*, *Cylindrocarpon*, *Fusarium*, *Gliocladium* and *Humicola*have have also been reported as phosphate solubilizers (Vazquez et al. 2000; Chen et al. 2002; Pandey et al. 2008; Posada et al. 2013).

Phosphorus (P) is one of the most dynamic elements in the environment and is present in both terrestrial and aquatic environments. However, limited availability of P in agricultural soils has led to the application of fertilizers, which are accumulated in soils due to the formation of insoluble complexes with iron ($FePO_4$) and aluminum ($AlPO_4$) in acidic environments and calcium ($Ca_3(PO_4)_2$) in alkaline environments (Deepa et al. 2010; Naik et al. 2013). These phosphates can reach water bodies through surface runoff, contributing to eutrophication processes. According to Vymazal (2007), in aquatic environments free orthophosphate is the most common form of P that can be directly utilized by plants. Therefore, it represents a link between organic and inorganic phosphorus in wetlands. Additionally, as it occurs with negative charge and tends to bind to several cations, which are poorly soluble, the assimilation of these compounds is reduced, contributing to its accumulation in sediments. According with Maitra et al. (2015), these sediments may act as an important source of P by releasing sediment-bound P through microbial metabolism, which may be an ecologically sustainable manner of supplying this element to enhance the productivity of oligotrophic water bodies.

The use of aquatic plants is one of the most effective methods for removing phosphorus and pollutants from water (Rezaie and Sahlezadeh 2014). The role of aquatic plants in phytoremediation processes has been widely documented (Arthur et al. 2005), especially for invasive plants (Coats and Rumpho 2014), due to its rapid growth, adaptability and phytoremediation capability in heavy metal removal (Rai 2008). Based on this background, in this work we focus our study on SBM isolated from plant rhizosphere of *Phragmites australis.* This macrophyte has a cosmopolitan distribution and can be found in contaminated environments, mainly those with heavy metals, hydrocarbons and pesticides (Hechmi et al. 2014); in this sense, the *P. australis*-rhizosphere is an excellent reservoir of fungal communities and could be a source of new fungal species involved in ecological processes (Jung and Nechwatal 2008). Most studies regarding phosphate solubilization by fungi (Pradhan and Sukla 2005; Sharma et al. 2013) have been focused on the isolation and characterization of microorganisms from agricultural soils, but the presence of symbiotic microorganisms associated with the rhizosphere of aquatic plants and their ecological functions have not yet been fully defined (Stottmeister et al. 2003).

Tricalcium phosphate has not been highlighted as an important source of P-release, although it constitutes about 1–52 % of total P pools in rivers, lakes and wetlands sediments, and it is considered non-exchangeable, unless there is a rapid change in sediment pH and dissolution of metal oxides (Tong et al. 2005). In addition, cadmium is a potentially toxic substance of concern to human health. Cd^{2+} occurs naturally and is associated with phosphate rock (Roberts 2014), a source of fertilizers, and due to its nature, can form complexes with phosphorus and can also precipitate in sediments (Baars et al. 2014; Jacob et al. 2013). However, heavy metals are toxic to SMB, even at low concentrations; Baldrian (2003) and Gadd (2004) documented the fungitoxicity of heavy metals in SBM and white-rot basidiomycetes, respectively, which resulted in growth inhibition. On the other hand, the fungal tolerance to Cd^{+2} in soil is associated with adsorption mechanisms (Zafar et al. 2007) that depend on the functional groups of fungal cell walls, such as carbonyls, hydroxyls and amides, which are responsible for metal binding during biosorption processes (Xu et al. 2012). In the case of toxicity, presence of metals can result in the generation of reactive oxygen species (ROS) that may cause wide-ranging damage to proteins, nucleic acids and lipids, eventually leading to cell death (Baldrian and Gabriel 2003; Li et al. 2009). Non-redox active metals like Cd^{2+} and zinc (Zn^{2+}) can deplete free-radical scavengers, resulting in ROS production, such

as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) (Azevedo et al. 2007).

The main mechanism reported for phosphate solubilization by fungi is related to the pH decrease due to organic acids and phosphatases, as these play an important role in the mineralization of phosphate salts (Gyaneshwar et al., 2002; Naik et al., 2013). For example, *Aspergillus* and *Penicillium* fungi species can produce organic acids with a high potential to produce H^+ and to promote greater phosphate solubilizing activity (Gharieb 2001). Nonetheless, these mechanisms are not the only ones involved in phosphate solubilization (Sharma et al. 2013) and even environmental factors and nutritional conditions can be involved (Relwani et al. 2008). For example, fungi glucose metabolism contributes to organic acid production, whereby glucose oxidase enzymes can generate H_2O_2 (Wong et al. 2008) that might further contribute to a pH decrease (Magnuson and Lasure 2004), favoring phosphate solubilization. Therefore, the aims of this study were: (1) to evaluate the ability of three SBM isolated from *P. australis*-rhizosphere for P-solubilization and H_2O_2 production in liquid culture medium; (2) to study the impact of Cd^{2+} in development growth and P-solubilization in agar culture medium under a dose–response assay and (3) to study the effect of Cd^{2+} and nutritional factors (glucose, ammonium sulphate and yeast extract) on P-solubilization and H_2O_2 production using a 2^4 factorial experimental design.

Materials and methods

Isolation and molecular identification of fungal strains

Three SBM were isolated from soil samples taken in the surrounding rhizosphere area of common reed plants [*Phragmites australis* (Cav.) Trin. exSteud.] collected from a natural wetland area denominated "La Mancha" located in the central coast of Veracruz state in Mexico (96° 23′09″W, 19° 35′19″N). The rhizospheric soil was characterized as a sandy loam soil (clay 8.41 %, sand 89.6 and silt 2 %), organic matter content was low, pH ranged between 6.67 and 6.73, total phosphorus values ranged from 20 to 125 mg/kg, the organic phosphorus was between 15 and 85 mg/kg, total nitrogen in soil ranged from 830 to 1 645 mg/kg. The pH values of the water samples were between 6.5 and 8.0, the chemical oxygen demand for water was between 11.66 and 15 mg O_2/L, total phosphorus values for water ranged between 0.88 and 2.34 mg/L, total nitrogen values for water were around 8.82 mg/L.

Fungal isolation was carried out using serial dilution of rhizosphere soil sample up to 10^{-3} on rose bengal streptomycin agar with tricalcium phosphate [$Ca_3(PO_4)_2$] as an insoluble source of inorganic phosphorus. In this culture media after 3 days of incubation at 25 °C, a halo around the colony indicated P-solubilization. Colonies with clear zones were further purified by replating on potato dextrose agar for maintenance and conservation. For molecular identification, the mycelia of studied strains were grown in yeast extract medium (standard CYM: dextrose, 20 g; peptone, 2 g; yeast extract, 2 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 0.46 g; K_2HPO_4, 1 g and distilled water; 1000 mL) and incubated at 25 °C during five days. The mycelia were harvested and placed in Eppendorf tubes (1.5 mL), and then frozen in liquid nitrogen (−130 °C). Genomic DNA extraction and purification of dried mycelia was carried out according to Challen et al. (1995). DNA purity was determined by comparative agarose gel electrophoresis (1.5 %, w/v) and ethidium bromide staining. The PCR amplification of the internal transcribed spacers (ITS regions) was carried out using the following primers ITS1 (5́-TCCGTAGGTGAACCTGCGG-3́) and ITS4 (5́-TCCTCCGCTTATTGATATGC-3́). The amplification condition was according to Huerta et al. (2010). Purified PCR products were sequenced by Genomic Biotechnology Center—IPN (Reynosa, Tamaulipas, Mexico). Each sample was sequenced in the genomic. DNA sequence analyses were performed using the basic sequence alignment BLAST program, run against the NCBI database (www.ncbi.nlm.nih.gov). The *Index Fungorum* (www.indexfungorum.org) was used as a species authority.

Culture media and inoculation

The ability of three SBM for P-solubilization and H_2O_2 production were evaluated in modified-Pikovskaya culture medium (Pikovskaya 1948), which consisted of (g/L): $(NH_4)_2SO_4$ (0.5), KCl (0.2), $MgSO_4 \cdot 7H_2O$ (0.1), $MnSO_4 \cdot H_2O$ (0.004), NaCl (0.2), D-glucose (10), $FeSO_4 \cdot 7H_2O$ (0.002, Sigma-Aldrich), yeast extract (0.5), $Ca_3(PO_4)_2$ (0.5, Sigma-Aldrich) and distilled water (1000 mL). Culture medium (120 mL) was deposited in 250-mL Erlenmeyer flasks and autoclaved for 15 min at 1.5 atm and 120 °C. Fungi were inoculated into the flasks using 200 µL of a spore solution (1×10^6 spores/mL) of each fungus. The spore solution was made by taking one mycelial disk of agar from 7-days-old culture of SBM, which was deposited in 1 mL of sterile distilled water supplemented with Tween-20® (Sigma-Aldrich, St. Louis, MO, USA) at 0.05 %. The spores in the solution were counted and adjusted using a Neubauer chamber (Hausser Sci., Horsham, PA, USA). Fungal cultures were incubated at 120 rpm at 25 °C during 16 days. Four replicates were established for each fungus, and a control without inoculation was used. Every 2 days, aliquots of 8 mL from each

flask were taken and centrifuged at 7500 rpm during 10 min; the supernatant was used for P and H_2O_2 determinations.

Analytical methods

Phosphorus-solubilization was determined by phosphomolybdenum blue method. This determination is based on the reaction of ammonium heptamolybdate and potassium antimony (III) oxide tartrate in an acid medium with a diluted solution of phosphate to form antimony–phosphomolybdate complex. This complex is reduced to an intensely blue-colored complex by L (+) ascorbic acid (Method 4500-P, Standard Methods, 2005). The complex was measured at 880 nm in a Shimadzu 8000 UV/IS spectrophotometer (Shimadzu Corp Japan). The standard curve was: $y = 0.2173\ c + 0.0433$ ($y = OD_{880}$, c = P-solubilization in mg/L, $R^2 = 0.983$).

Hydrogen peroxide concentrations were determined using the iodide/iodate method. Solutions A and B for the I_3^- method were prepared according to Klassen et al. (1994). Solution A consisted of 33 g of KI, 1 g of NaOH and 0.1 g of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (ammonium molybdate tetrahydrate) diluted to 500 mL with water. Solution A was kept in darkness to inhibit oxidation of I^-. Solution B (an aqueous buffer) contained 10 g of $C_8H_4KO_4$ (potassium hydrogen phthalate) dissolved in 500 mL of water. The I_3^- method consisted of mixing equal weights of A (3 mL) and B (3 mL), followed by addition of fungal supernatant (3 mL). The absorbance of the resulting solution was measured at 351 nm in a 3-cm^3 cuvette. The blank absorbance was determined by substituting the enzyme extract with sterile deionized water in the reaction mixture. Hydrogen peroxide concentrations were calculated by substituting the absorbance in a standard curve with pure H_2O_2 reagent (30 %, J.T. Baker™, Center Valley, PA, USA) at known concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 mg/L). The standard curve was: $y = 0.134c + 0.0164$ ($y = OD_{351}$, $c = H_2O_2$ in mg/L, $R^2 = 0.997$).

Dose–response bioassay

The effect of Cd^{2+} on mycelial growth and reduction in the solubilizing halo by soil fungi was investigated in dose–response bioassays. Pikovskaya-agar culture medium was supplemented with Cd^{2+} introduced as $Cd(NO_3)_2{\cdot}4H_2O$ and at four different Cd^{2+} concentrations (0, 0.075, 0.15, 0.31 and 0.62 mM), and a culture medium without Cd^{2+} was used as a control. Culture media was emptied into Petri dishes (90 mm ø); the fungi were inoculated in the center of plates using 2 µL of a spore solution (1×10^6 spores/mL). Plates were incubated in darkness at 25 °C and 75 % relative humidity (RH). Four replicates of each Cd^{2+} concentration and control were used. The colonies and solubilizing halo were measured daily from the rear of the plates using a millimetric square, the dose–response bioassay finished when the control mycelia filled the Petri dishes.

From the colony diameter, a specific growth characteristic was calculated: the percentage of mycelial growth inhibition, as the percentage of inhibition (% in cm/cm) = $[(C–T/C] \times 100$, where C and T are the mycelial diameter inside the control and treatment plates, respectively. Daily growth rate (DGR), was defined as $DGR = \Sigma (R_1–R_0)/(T_1–T_0)$, where R_0 and R_1 are the colony diameter at times T_0 and T_1, respectively. Both equations were used to calculate the DGR and percentage growth inhibition of the P-solubilization halo. The effective concentration inhibiting mycelial growth rate by 50 % (IC_{50}) was calculated by Probit analysis using the software SAS 8.1. On the basis of the Probit analysis, the most tolerant strain was chosen for studying the effect of Cd^{2+} and nutritional factors on P-solubilization using a factorial experimental design.

Factorial experiment design

To study the Cd^{2+} effect and nutrimental factors on P-solubilization by *P. italicum*, a 2^4 factorial experimental design was applied. The studied factors were: Cd^{2+} concentration and amounts of glucose, $(NH_4)_2SO_4$ and yeast extract. Table 1 shows the factor levels of independent variables. The values of each variable were coded as minus (–1) for lower and plus (+1) for upper limits. The studied responses were: P-solubilization (Y_1), and H_2O_2 (Y_2) and biomass production (Y_3).

The data analysis was carried out through Design Expert 8.0 (Stat-Ease Inc., Minneapolis, MN, USA). The Eq. 1 was used to describe the regression model, which includes interaction terms:

$$y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n} \sum_{j=i+1}^{n} \beta_{ij} x_i x_j + \varepsilon \quad (1)$$

where β_0 is the constant term, β_i and β_{ij} are the regression coefficient, ε is the error, x_i are the variables and n their number.

Table 1 Range and levels of independent variables chosen to study its impact in response *Y*1, *Y*2 and *Y*3

Factors	Unit	+1	0	−1
Cadmium (Cd)	mM	0.45	0.3	0.15
Glucose (G)	g/L	15.0	10.0	5.0
$(NH_4)_2SO_4$ (N)	g/L	0.75	0.5	0.25
Yeast extract (Y)	g/L	0.75	0.5	0.25

Biomass quantification

For fungal dry cell weight analysis, the suspension was then filtered through pre-weighed Whatman filter paper No. 1, the fungal biomass was washed with sterilized distilled water and this was repeated until the filtrate was clear. Then, the fungal pellets were vacuum filtered through a pre-weighed filter. For dry cell weight measurement, the filters were dried in an oven until constant weight and re-weighed after cooling the filters in a desiccator.

Results

Molecular identification

Polymerase chain reaction (PCR) products from genomic DNA of fungal isolates were analyzed using the BLAST program (blast.ncbi.nlm.nih.gov/Blast.cgi). The identified fungi showed similarities between the partial sequences derived from the ITS regions. A first isolate with PCR length 569 bp was *Penicillium italicum* Wehmer and had 100 % similarity with accession DQ991463.1 submitted by Hernández-Montiel and Ochoa (2007). A second isolate with PCR length 534 bp was identified as *Penicillium dipodomyicola* and had 100 % similarity with accession KM458817 submitted by Frisvad et al. (2000). A third isolate with PCR length 594 bp was identified as *Aspergillus japonicus* var. *aculeatus* Iizuka and had 100 % similarity with accession HM140184.1 by Ma et al. (2011).

P-solubilizing and H_2O_2 production by SBM

All the evaluated strains were able to solubilize tricalcium phosphate at different rates (Fig. 1a). *P. italicum* and *A. japonicus* achieved the maximum P-solubilization values with 107.0 and 104.0 mg/L, respectively, and after 16 days of incubation both fungi solubilized 100 % of the initial P content introduced as $Ca_3(PO_4)_2$. Figure 1b shows the H_2O_2 production by the strains tested through 16 d of culture. *P. italicum* had significantly ($p < 0.05$) higher H_2O_2 production than *P. dipodomyicola* in all evaluations. *P. italicum* achieved the highest H_2O_2 production (2.3–4.4 mg/L) compared to the other tested strains during 12–16 days. Pearson's correlation analysis was used to determine the relationship between P-solubilization and H_2O_2 production by SBM (Table 2), in order to verify involvement of H_2O_2 in phosphorus release. *P. italicum* showed a significantly ($p < 0.05$) higher correlation coefficient (0.71) compared to *A. japonicus* (0.63) and *P. dipodomyicola* (0.65). On the basis of CE_{50} and correlation analysis, *P. italicum* was chosen for the factorial experiment to assess the effect of Cd^{2+} and nutritional factors on P-solubilization, H_2O_2 and biomass production.

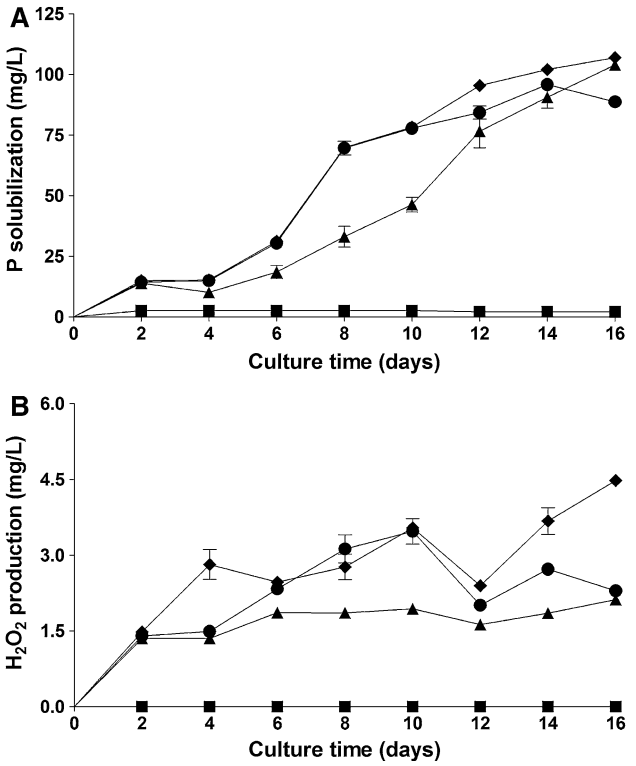

Fig. 1 P-solubilization (**a**) and H_2O_2 production (**b**) by *Aspergillus japonicus* (*filled triangle*), *Penicillium italicum* (*filled diamond*), *Penicillium dipodomyicola* (*filled circle*) and control (*filled square*) in modified-Pikovskaya culture medium

Table 2 Pearson's correlation between P-solubilization and H_2O_2 production in studied strains

SBM Strain	Pearson's correlation	R-square	*p* value
A. japonicus	0.63	38.88	0.060
P. italicum	0.71	50.90	0.047
P. dipodomyicola	0.65	42.44	0.080

Dose–response assay

Penicillium italicum and *A. japonicus*, due to their greater capability of P-solubilization, were selected for the performance of a dose response assay under in vitro conditions (at darkness, 25 °C and 75 % RH) using a Pikovskaya-agar medium added with Cd^{+2}. Toxicological effects on fungal growth and colony morphology were observed at the several Cd^{2+} levels in the culture medium. A wide range of fungal morphological changes such as, discoloration, decrease in mycelial density and low

sporulation were observed during mycelial growth in culture medium when Cd^{2+} was added (Fig. 2). *P. italicum* showed a reduction in DGR per 0.15, 0.31 and 0.62 mM of Cd^{2+} added by 18.3, 39.8 and 61.15 %, respectively. Halo formation due to P-solubilization by *P. italicum* was stimulated by low Cd^{2+} concentration, as the halo DGR was significantly ($p = 0.001$) higher at 0.078 (0.78 cm/d) and 0.155 mM Cd^{2+} (0.73 cm/d) in comparison to controls without Cd^{2+} (0.68 cm/d). Reduction in the halo DGR was only achieved at 0.31 and 0.62 mM Cd^{2+} with 26.3 and 40.5 % reductions, respectively (Table 3). *A. japonicus* was less susceptible to Cd^{2+}, because its mycelial DGR was stimulated by low Cd^{2+} concentrations. Its mycelial DGR was significantly ($p = 0.001$) higher for 0.07 and 0.155 mM Cd^{2+} (0.86 and 0.77 cm/d, respectively) than for controls (0.75 cm/d). Only high Cd^{2+} concentrations reduced the colony DGR by 29.6 and 88.5 % (0.31 and 0.62 mM Cd^{2+}, respectively). However, halo DGR was more affected than colony DGR, with reductions of 9.0, 19.6, 44.6 and 92.4 % for 0.078, 0.155, 0.31 and 0.62 mM Cd^{2+}, respectively (Table 4). A wide range of fungal morphological changes, such as discoloration, decrease in mycelial density and low sporulation were observed during mycelial growth in culture medium with added Cd^{2+}. The same influence was also detected in liquid media for concentrations of 0.25 and 50 mM Cd^{2+}. When compared with other fungal species, such as *Fusarium* sp. and *Alternaria tenuis*, the observed effect on growth and morphology was the same, regardless of Cd^{2+} source and culture media (Gharieb 2001).

Factorial experimental design and mathematical models

On the basis of CE_{50} and correlation analysis, *P. italicum* was chosen for the factorial experiment design to assess the effect of Cd^{2+} and nutritional factors on dependent variables. The results of 20 experiments were used to obtain three mathematical models (Table 5), each obtained from Eq. 1 through the study of the partial coefficients of each factor and interactions between factors. The fitted models were designed to explain the relationship between the independent factors and dependent responses.

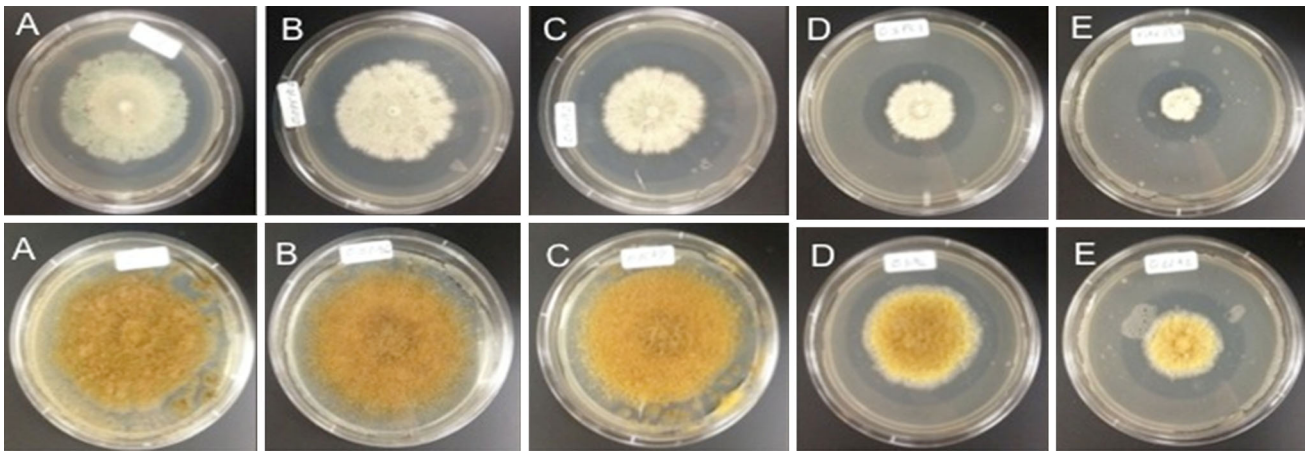

Fig. 2 Dose-response assay in Pikovskaya-agar medium contaminated with Cd^{2+} levels: (*Up*) *Penicillium italicum* and (*Down*) *Aspergillus japonicus*. Control (**a**), 0.07 mM (**b**), 0.015 mM (**c**), 0.031 mM (**d**) and 0.62 mM (**e**)

Table 3 Effect of Cd^{2+} levels on daily growth rate and inhibition percentages in growth in two P-solubilizing soil borne micromycetes

	Penicillium italicum				*Aspergillus japonicus*.			
Cadmium (mM)	DGR colony (cm/day)	DGR halo (cm/day)	Inh. DGR colony (%)	Inh. DGR halo (%)	DGR colony (cm/day)	DGR halo (cm/day)	Inh. DGR colony (%)	Inh. DGR halo (%)
Control	0.55a	0.68b	–	–	0.72b	1.21a	–	–
0.078	0.50a,b	0.78a	8.95c	0	0.86a	1.10a,b	0	9.01c
0.155	0.45b	0.73a,b	18.9c	0	0.77a,b	0.98b	0	19.10c
0.31	0.33c	0.50c	39.80b	26.35b	0.51c	0.67c	29.68b	44.66b
0.62	0.17d	0.40d	68.15a	40.53a	0.08d	0.09d	88.53a	92.44a

Inh inhibition, *DGR* daily growth rate

Means with the same letter in row are not significantly different (LSD, $p = 0.05$)

Table 4 Median effective concentration (EC_{50}) of Cd^{2+} in two P-solubilizing soil borne micromycetes

Isolate	EC_{50}	95 % CI	Probit Eq.	Slope	χ^2	*p*
P. italicum	0.31a	0.29–0.34	Y = 2.02 + 1.0155X	2.02 ± 0.10	345.57	0.0001
A. japonicus	0.26b	0.25–0.27	Y = 2.54 + 4.44X	2.54 ± 0.12	626.84	0.0001

Differences were established according to the overlapping of confidence intervals (CI at 95 %)

Table 5 Values for P-solubilization, hydrogen peroxide, and biomass production by *Penicillium italicum* according 2^4 factorial design

Independent variables				Response types		
Cd^{2+} (*X*1)	G (*X*2)	N (*X*3)	Y (*X*4)	*Y*1	*Y*2	*Y*3
−1	−1	−1	−1	55.23	2.7	1.92
1	−1	−1	−1	56.99	2.61	1.41
−1	1	−1	−1	80.13	2.87	3.63
1	1	−1	−1	75.95	2.88	4.22
−1	−1	1	−1	65.99	2.8	1.5
1	−1	1	−1	100.85	2.73	1.98
−1	1	1	−1	73.86	2.79	5
1	1	1	−1	103.91	2.95	2.95
−1	−1	−1	1	73.38	2.77	2.23
1	−1	−1	1	0	2.75	0.53
−1	1	−1	1	76.11	3.04	5.14
1	1	−1	1	0	3.06	0.68
−1	−1	1	1	88.64	2.16	2.02
1	−1	1	1	48.48	2.84	1.09
−1	1	1	1	80.29	2.96	5.43
1	1	1	1	23.58	3.23	0.95
0	0	0	0	85.43	2.99	2.76
0	0	0	0	98.44	2.95	2.61
0	0	0	0	110.49	2.85	3.68

*Y*1 P-solubilization, *Y*2 H_2O_2 production, *Y*3 biomass production

P-solubilization

The mathematical model for the P-solubilization allowed us to define the optimal media composition and to know the combined effects of studied factors. In addition, the predicted responses (Figs. 3, 4) as well as the residual values between experimental and theoretical data were calculated (Fig. 5a). The ANOVA for phosphate solubilization of *P. italicum* through the 20 assays of the factorial experiment is shown in Table 6. The theoretical data showed a functional relationship with the experimental data, indicating that the predictability of the model was significant at 95 % of confidence level. The coefficient of determination (R^2) value of 80.26 % showed that Eq. 2 was highly reliable. Figure 3 shows observed responses versus those from the statistical model of Eq. 2.

$$Y1_{\text{P-solubilization}} = 68.30 - 11.49^{*}\text{Cd} + 1.52^{*}\text{G} + 10.49^{*}\text{N} - 13.90\ \text{Y} - 19.30^{*}\text{Cd}^{*}\text{Y} \quad (2)$$

The coefficient of determination ($R^2 = 0.8$) indicated a moderately strong relationship between predicted data of P-solubilization from empirical observations and the experimentally obtained data. A normal probability plot of the residuals to assess normality resulted in a straight line

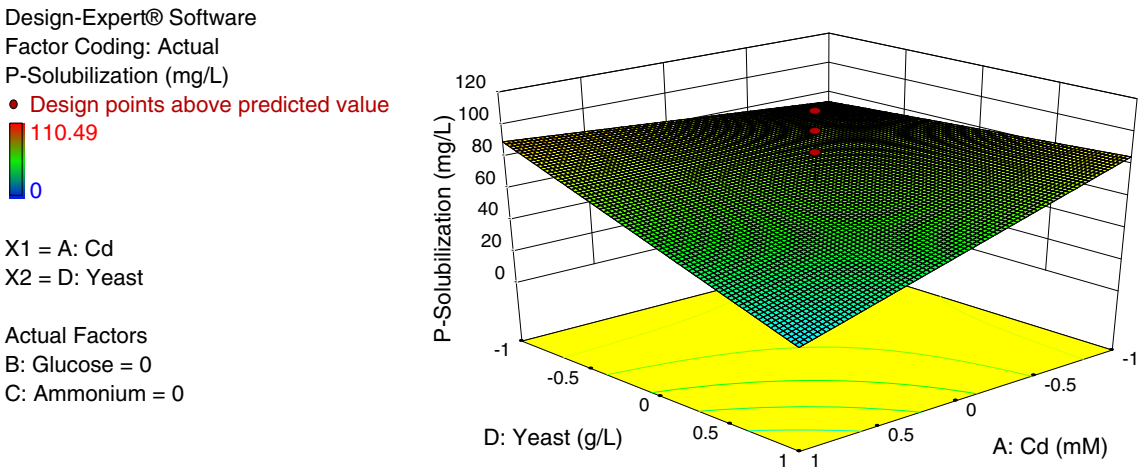

Fig. 3 Graphical analyses of the interaction Cd^{+2} and yeast for P solubilization obtained through the analysis with Design-Expert software

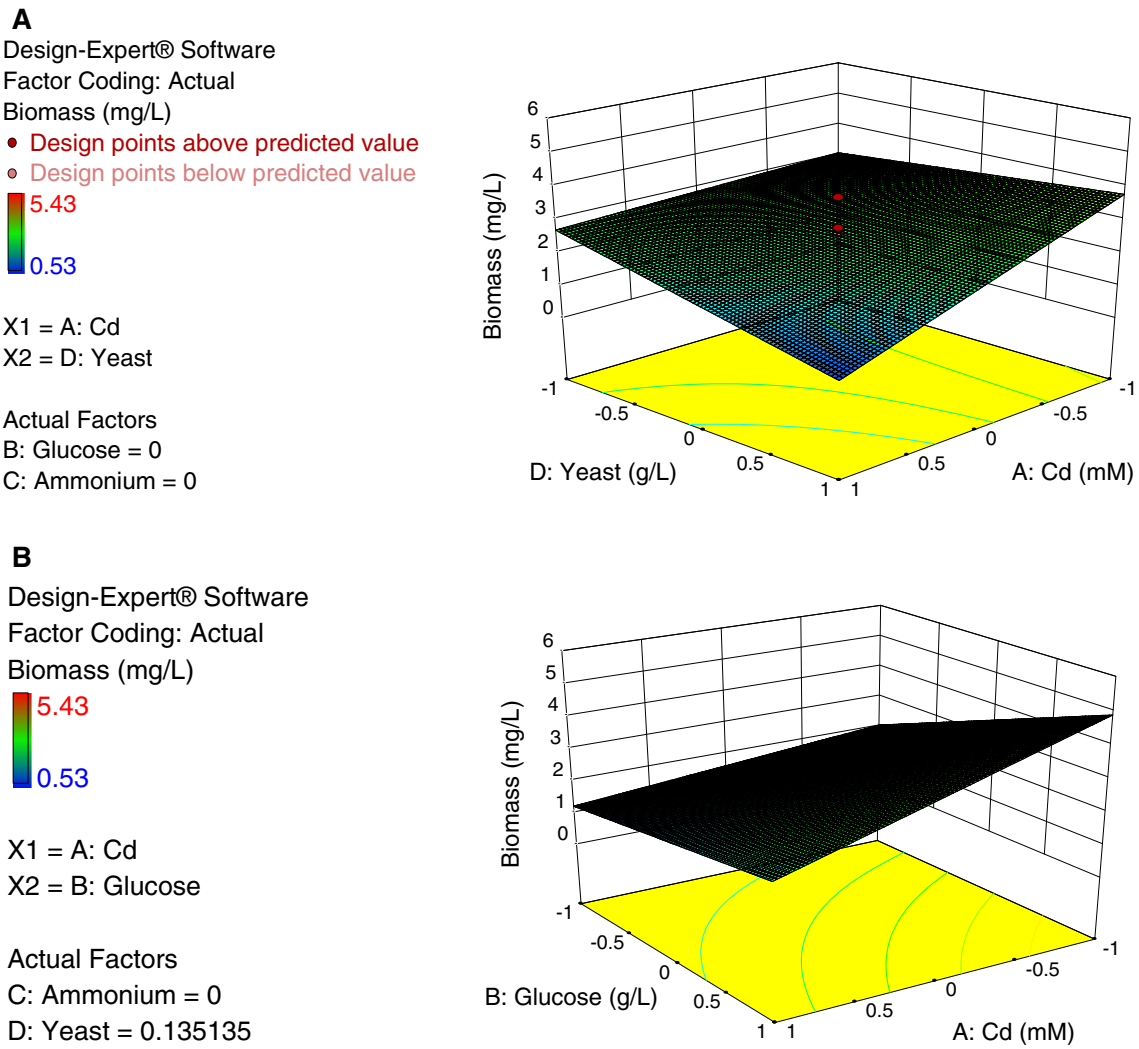

Fig. 4 Interaction analyses **a** Cd^{+2} and yeast and **b** Cd^{2+} and glucose for biomass production obtained through the analysis with Design-Expert Sofware

(Fig. 5b). In addition, the negative coefficients corresponding to Cd^{2+} (−11.49) and yeast extract (−13.90) indicated that P-solubilization decreased with increasing concentration of Cd^{2+} and yeast extract.

Hydrogen peroxide production

According to the ANOVA, the glucose amount has a significant effect on H_2O_2 production ($p = 0.002$); the other studied factors and their interactions did not show significant effects. The highest H_2O_2 production (3.23 mg/L) was found in the experiment with a high concentration of Cd^{2+} (0.45 mM) and a high amount of glucose (15 g/L), $(NH_4)_2SO_4$(0.75 g/L) and yeast extract (0.75 g/L). Assay 17 with low concentration of Cd^{2+} and glucose but high concentration of ammonium nitrate and yeast extract resulted in the lowest H_2O_2 production (2.16 mg/L). The positive coefficient for glucose indicates that the maximum production of H_2O_2 occurred for the highest amount of glucose. Figure 5c contrasts the observed response and the predicted response. The normality test was used to construct a normal probability plot, resulting in a straight line (Fig. 5d). The Pearson's correlation coefficient was high (0.89), indicating a moderately strong relationship between observed and predicted responses.

Biomass production

The model for biomass production (Eq. 3) was significant and reliable ($p = 0.004$ and $R^2 = 0.88$). The factors showing positive effects with a confidence level of 95 % were Cd^{2+} ($p = 0.0009$) and glucose ($p = 0.0003$). Similarly, the interactions of Cd^{2+}*glucose ($p = 0.02$) and Cd^{2+}*yeast ($p = 0.005$) were significant. The ANOVA for biomass production through the 20 assays of the factorial experiment is shown in Table 7.

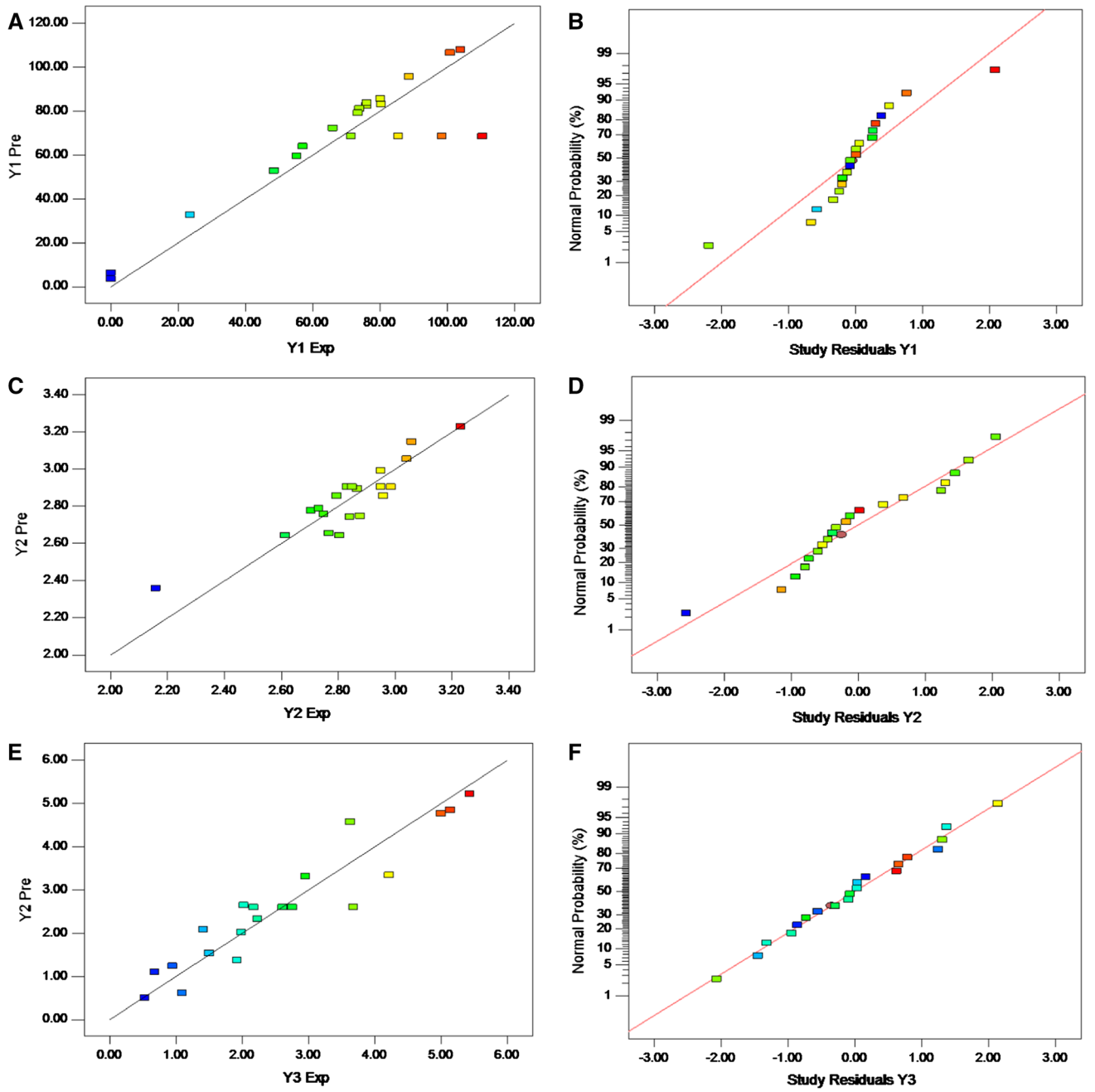

Fig. 5 Graphical analyses of the model fitted for *Y*1 = P solubilization (**a**), *Y*2 = hydrogen production (**c**), and *Y*3 = biomass (**e**). The regression analyses were set between predicted responses and experimental values. Statistically analysis was by normality plot of the residual *Y*1 (**b**), *Y*2 (**d**), and *Y*3 (**f**)

$$Y3_{\text{biomass}} = 2.59 - 0.82^{*}\text{Cd} + 0.96^{*}\text{G} + 0.074^{*}\text{N} - 0.28^{*}\text{Y} - 0.48^{*}\text{Cd}^{*}\text{G} - 0.63^{*}\text{Cd}^{*}\text{Y} \quad (3)$$

The highest value of biomass was achieved in assay 9 (5.43 mg/L), which contained a low amount of Cd^{2+} and high levels of glucose, ammonium nitrate and yeast extract. In contrast, the lowest biomass was for assay 8, which contained high values of Cd^{2+} and yeast extract and low amounts of glucose and ammonium nitrate. The negative coefficient corresponding to Cd^{2+} (−0.82) in Eq. 3 suggested that fungal biomass decreased with increasing Cd^{2+} concentration in the culture medium. The yeast extract (−0.28) showed a similar but weaker effect to Cd^{2+} (Fig. 4a). Glucose played an important role in biomass production by *P. italicum* (Fig. 4b) as shown by the positive coefficient of glucose (0.96) in Eq. 3. Ammonium nitrate was important for biomass production, although less important than

Table 6 ANOVA for P-solubilization by *Penicillium italicum* using 2^4 factorial design

Effects	Estimate	*P* value	SS	Df	MS	F
Main effects						
Cd	*−11.49*	*0.04*	*2113*	*1*	*2113.01*	*44.47*
G	1.52	0.76	36.81	1	36.81	0.77
N	10.49	0.05	1760.01	1	1760.01	37.04
Y	*−13.9*	*0.02*	*3092.19*	*1*	*3092.19*	*65.08*
Two factors interaction						
Cd × N	7.5	0.15	899.25	1	898.25	18.93
Cd × Y	*−19.3*	*0.002*	*5961.77*	*1*	*5961.77*	*125.47*
G × N	−4.31	0.38	296	1	296.79	6.25
G × Y	−5.33	0.29	454.86	1	454.86	9.57
Error			314.16	1		
Total SS			18209.664	18		

The numbers in italic represent the response of the factors that have a significant effect on P-solubilization by *Penicillium italicum*. $R^2 = 0.803$; coefficient of variation (%) = 27.76; Cd = Cd^{2+}

SS sum of squares, *df* degrees of freedom, *MS* square means, *G* glucose, *N* ammonium nitrate, *Y* yeast extract

Table 7 ANOVA for biomass production by *Penicillium italicum* using 2^4 factorial design

Effects	Estimate	*P* value	SS	Df	MS	F
Main effects						
Cd	*−0.82*	*0.0009*	*10.66*	*1*	*10.66*	*22*
G	0.96	0.0003	14.67	1	14.67	30.27
N	0.072	0.6858	0.084	1	0.084	0.17
Y	*−0.28*	*0.1341*	*1.29*	*1*	*1.29*	*2.66*
Two factors interaction						
Cd × G	−0.48	0.02	3.74	1	3.74	7.73
Cd × N	−0.056	0.75	0.051	1	0.051	0.10
Cd × Y	*−0.063*	*0.005*	*6.35*	*1*	*6.35*	*13.10*
G × Y	−0.17	0.3620	0.44	1	0.44	0.91
Error			0.67	2	0.34	
Total SS			42.13	18		

The numbers in italic represent the response of the factors that have a significant effect on Biomass production by *Penicillium italicum*. $R^2 = 0.8850$, coefficient of variation (%) = 26.60, Cd = Cd^{2+}

SS sum of squares, *df* degrees of freedom, *MS* square means, *G* glucose, *N* ammonium nitrate, *Y* yeast extract

glucose. Figure 5e shows the contrast of experimental and predicted responses, and Fig. 5f is a normal probability plot.

Discussion

Fungus genera, such as *Penicillium* and *Aspergillus*, have been shown to have phosphate-solubilizing capacities (Mittal et al. 2008; Naik et al. 2013; Saxena et al. 2013). In this study, three strains isolated from rhizosphere soil of *P. australis* have not been reported for phosphate solubilization under in vitro conditions. Fungal strains *P. italicum* and *A. japonicus* strains presented higher P-solubilization ability and were selected for the implementation of the dose response assay. The IC_{50} calculated by Probit analysis suggested that *P. italicum* was more tolerant to Cd^{+2} than *A. japonicus*, although the latter showed a high DGR under low Cd^{2+} concentrations in a Pikovskaya-agar medium. Some studies have reported the minimum inhibitory concentration (MIC) for some fungal species under semi-solid cultivation; the MICs for Cd^{2+} of *A. japonicus* and *Penicillium* sp. isolated from metal-contaminated soils were 35.5 and 44.4 mM (Zafar et al. 2007). Ahmad et al. (2005) investigated the biosorption of Cd^{2+} by *Rhizopus* and found a MIC of 17.8 mM. These values are high compared to the CI_{50} of *P. italicum* (0.31 mM) and *A. japonicus* (0.26 mM), although the differences could be due to differences in methodology and statistical methods used to determine MICs and CI_{50}. Similarly, the solubilization halo in both *P. italicum* and *A. japonicus* showed differences in percentage inhibition, with the most evident reduction at high Cd^{2+} concentrations (0.31 and 62 mM); however, *P. italicum* showed less reduction in halo solubilization at 0.62 mM Cd^{2+} in comparison to *A. japonicus*. The decrease in mycelial growth was due to the toxic effect of Cd^{2+}. Nonetheless, the solubilization halo present in solid media could suggest that fungi under Cd^{2+} stress can divert energy from growth to cell maintenance functions (Muhammad et al. 2005).

The factorial experiment confirmed that Cd^{2+} decreased the ability of soil fungi to solubilize P and produce biomass. We suggest that the decrease in P-solubilization was correlated with the fungal biomass reduction due to the toxic effect of Cd^{2+} (Vig et al. 2003). Tuason and Arocena (2009) demonstrated that P-solubilization was positively correlated with fungal biomass, glucose oxidation and other intermediates of energy metabolism pathways that are important in the production of organic acids (Chaiharn and Lumyong 2009), corresponding to decreases in pH (Walpola and Yoon 2013). This phenomenon has been directly related to the release of phosphorus of tricalcium phosphate (Johnson and Loeppert 2006; Onthong et al. 2007; Scervino et al. 2010). However, this does not seem to apply to all cases, as there have been reports of positive but non-significant correlations between the amount of soluble P and low molecular weight acids produced by fungi (Pradhan and Sukla 2005). Thus, although organic acid generation is important to fungal metabolism, it may not be the only factor involved in P-solubilization (Rashid et al. 2004). The production of oxalic acid by fungi provides a means of immobilizing soluble metal ions or complexes as insoluble oxalates, thus decreasing bioavailability and increasing

tolerance of these metals (Gadd 2007). In our study, decreasing metal bioavailability could be associated to metal chelation by organic acids under acidic conditions (pH below 5), as it was reported by Gadd (2004), rather than precipitation that occurs at alkaline pH (Hong et al. 2010).

In the presence of Cd^{2+}, organic acids can act as chelating agents, which decrease the metal toxicity (Khan 2005); however, the presence of this metal may in turn influence the generation of organic acids. Nonetheless, this may not be generalized for all species of fungi, as this activity is influenced by metal concentration, nitrogen form and other culture conditions (Sazanova et al. 2015). The mechanisms used to solubilize microbial phosphate include acidification, chelation and exchange reactions, and are affected by nutritional conditions. Most of the previous reports (Pradhan and Sukla 2005) state that calcium phosphates are dissolved by acidification when glucose and ammonium sulphate are used, favoring a decrease in pH. In addition to P-solubilization, we studied H_2O_2 production as a response mechanism to physiological stress due to Cd^{2+} concentration in the culture medium. Although it was possible to determine a correlation between P-solubilization and H_2O_2 production, we did not find a significant effect of Cd^{2+}on H_2O_2 production. It was likely that other physiological responses were involved in the stress induced by Cd^{2+}, such as enzyme activities, organic acid production or another ROS types. There are some reports (Hammel et al. 2000; Tanaka et al. 2006) concerning the production of ROS in several fungal species; however, their role in fungal metabolism is unclear (Bai et al. 2003). Therefore, more detailed study of the particular case of H_2O_2 is necessary to determine its role in Cd^{2+}-induced stress and therefore P-solubilization.

P-solubilization by soil-borne fungi is complex and not completely understood (Narula et al. 2000). In the present study, we found that yeast extract negatively affected the P-solubilization, as yeast extract is a nitrogen source but more complex that other sources, like sulfate ammonium form (Nahas 2007). The increase of Cd^{2+} in Pikovskaya-liquid medium negatively affected P-solubilization; however, high concentrations of glucose reduced the effect of Cd^{2+} on P-solubilization. In addition to nutritional factors (Fomina et al. 2003), the influence of abiotic factors such as pH, temperature, available oxygen and light can affect P-solubilization by soil fungi. The effects of these factors and their interactions with heavy metals should also be considered for P-solubilization by SBM.

Conclusion

Penicillium italicum achieved faster P-solubilization and higher H_2O_2 production than *A. japonicus* and *P. dipodomyicola*, although there was a correlation between P-solubilization and H_2O_2 production only for *P. italicum*; however, no correlation was observed with addition of Cd^{2+}. The Cd^{2+} amount negatively affected both the DGR of mycelium and halo solubilization. *P. italicum* was more tolerant to Cd^{2+} compared to *A. japonicus* and *P. dipodomyicola* in liquid culture medium, where the Cd^{2+} presence and yeast extract negatively affected the P-solubilization by *P. italicum*. Cd^{2+} levels did not influence H_2O_2 production, although a positive effect was observed with high glucose concentration. Fungal biomass decreased in the presence of Cd^{2+} and increased with addition of high glucose concentration in the culture media. Our results confirmed that the Cd^{2+} effect on P-solubilizing fungi was species- and strain-dependent, and we suspect that different mechanisms are involved in Cd^{2+}-induced stress, which should be studied in more detail.

Acknowledgments The authors are greatly indebted to Gisselly Mendoza for their technical assistance. This work was funded by own resources from the Laboratory of Xenobiotics of CINVESTAV-IPN, Mexico. Also the first author wish to thank the Mexican National Council of Science and Technology (CONACyT) by his doctoral scholarship.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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