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Phosphate Solubilizing Abilities of Two Fungi and their Potential for Promoting *Zenia insignis* Growth in Heavy Metal Contaminated Soil

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In this work, we identified and characterized phosphate solubilizing fungi (PSF) isolated from the soil of a zinc-lead mine in China. The ability of the fungi to promote growth in the legume *Zenia insignis* was evaluated in the first time. Two of the isolates exhibited especially high phosphate solubilizing abilities and heavy metal resistance. Internal transcribed spacer sequencing identified these isolates as the *Penicillium oxalicum* (P-1) and *Aspergillus japonicus* (A-1). After one week incubation, A-1 and P-1 had solubilized over 2000 mg/l of P in broth medium at spore densities of 10^8/ml and 10^7/ml, respectively. Both strains were tolerant to $Zn^{2+}$and Pb^{2+}, and solubilized phosphate in the presence of these ions. In addition, both fungi maintained high phosphate solubilizing abilities in most of the carbon, nitrogen and phosphorous treatments. When phosphorous was supplied as $Zn_3(PO_4)_2$, however, the soluble phosphate efficiency of P-1 and A-1 decreased to 14.5 and 6.8%. *Zenia insignis* inoculated with both fungi produced higher yield in soil contaminated with heavy metals than control, and soil properties were improved. These results highlight the potential role of P-1 and A-1 in increasing the amount of soluble P and plant yield as bio-fertilizers in soils contaminated with heavy-metals.

Key words: phosphate solubilizing fungi; heavy metal resistance; phosphate solubilizing amounts; plant growth promotion.

Although mining activities extract essential mineral resources, they generate numerous waste rocks and tailings, which are deposited on surface soil. Persistent mining has severely interfered with large land areas[22]. Mine tailings lack aggregate structure and organic material, and are typically low in nutrients and rich in heavy metals[32]. Thus, reversing the environmental effects of mine tailings has become an urgent research problem.

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Negating these effects is limited by two main factors; nutrient availability and heavy metal presence. Phosphorus is an essential macronutrient for plants, second only to nitrogen[18]. Most of the phosphorous in soil exists in insoluble forms, such as CaP, $Ca_3(PO_4)_2$ and $FePO_4$ [23]. In agricultural fields, although chemical fertilizers supply additional phosphate, their overuse is not only uneconomical, but damages the natural environment. Furthermore, the soluble P in chemical fertilizer is easily precipitated into insoluble forms by reacting with Ca^{2+}, Fe^{3+} and Al^{3+} before being absorbed by the plants[9]. Therefore, phosphorous depletion in soils cannot be corrected by increased

application of chemical phosphate fertilizers. Regarding the second limitation, the biological system requires trace amounts of heavy metals that are toxic at high concentrations, such as Fe, Cu and Zn. Thus, excess or deficiency of these metals creates environmental problems. Other heavy metals, such as Pb, Cr and Cd, cannot be metabolized by biological tissues. As such, they are toxic to plants and animals at quite low concentrations, on account of their non-degradable and persistent nature[2]. These metal ions also impair enzymatic activity by binding with proteins[36].

Most of the phosphate in soils is solubilized by phosphate solubilizing fungi (PSF). PSF play an important role in biogeochemical phosphorus cycling in ecosystems [19]. They transform insoluble tricalcium phosphorus into soluble forms by acidification, chelation, exchange reactions, and polymeric substance formation[9]. The products of acidification, such as citric acid and oxalic acid, are primarily responsible for decreasing the pH of the medium, and also determine the amount of soluble P[11].

Among the PSF, the fungal species *Penicillum* sp. and *Aspergillus* sp. display extremely high phosphate solubilizing abilities[5, 27], and are highly resistant to heavy metal contaminants. The *Penicillium* sp. isolated from an alum mine by Chai[7] demonstrates a phosphate solubilizing efficiency of 98% in optimized broth medium and tolerates the heavy metal irons Cd^{2+} and Co^{2+}. Sayer and Gadd[31] successfully cultivated the fungus *Aspergillus niger* on solid medium containing $Zn_3(PO_4)_2$ and $Co_3(PO_4)_2$ and demonstrated that this strain solubilized these compounds by producing citric and oxalic acid. Microorganism growth is typically restricted in solid media infused with heavy metals, as discussed in previous reports[21]. However, the phosphate solubilizing ability of PSF is more important than its growth under heavy metal conditions. Only strains exhibiting phosphate solubilizing abilities in heavy metal media are potential biofertilizers in mining restoration, but have been evaluated in few studies.

Zenia insignis, a legume species endemic to China, is a strong candidate for phytoextraction of the heavy metals Pb, Zn, Cd and Cu. Lauded for its rapid growth and metal-accumulating characteristics, *Z. insignis* has been widely planted as a tall arbor in soils contaminated with heavy metals[38]. However, whether the growth of *Zenia insignis* in contaminated soils is promoted by PSF has yet to be investigated.

The main objectives of this study are: (1) to isolate PSF with enhanced phosphate solubilizing abilities; (2) to quantify the optimal inoculation dose of PSF; (3) to evaluate the resistance of PSFs to Pb and Zn, and calculate their phosphate solubilizing efficiencies in liquid medium containing either metal; and (4) to examine whether PSF promotes the growth of *Zenia insignis*.

MATERIALS AND METHODS

Soils

Two separate soil samples were used in this research. The sample used to isolate the PSF was named the HY sample. The plants were cultivated in the soil called HS sample. Both soils were collected from different lead-zinc mines in the Hunan Province, located in the south-central region of China. The soil samples were air dried and screened through a 20 mesh, and their total Zn and Pb concentrations were determined by the Chinese Academy of Forestry. The Zn and Pb concentrations were 0.74 and 1.10 g/kg respectively in the HY soil samples, and 1.04 and 0.45 g/kg in the HS soil samples.

Screening of PSF and fungal identification

$Ca_3(PO_4)_2$-solubilizing fungi were screened from the PSF in HY using the procedure introduced by[8]. The isolation agar contained the following ingredients (g/L): sucrose (10), $Ca_3(PO_4)_2$ (1%, v/w) (10), sodium chloride (0.3), potassium chloride (0.3), magnesium sulfate (0.3), manganese sulfate (0.03), ferrisulphas (0.03), ammonium sulfate (0.5), yeast extract (0.5), sucrose (20). Following 7 days inoculation at 28 °C, colonies with clear halos were isolated and purified for further investigations. Among the isolated strains, two fungi demonstrated especially high solubilizing efficiency; these strains, denoted P-1 and A-1, were selected for identification. The DNA extraction and internal transcribed tracer (ITS) rDNA amplification methods are described in Löffler[20] and Bruns and Gardes[6], respectively. The amplification results were sequenced by the SinoGenoMax Company

(Beijing, China) and analyzed with NCBI BLAST.

Phosphate solubilizing conditions

Spore densities

To investigate the solubilizing efficiencies of P-1 and A-1, spore suspensions of both species were prepared at three densities, namely, 1×10^8/ml, 1×10^7/ml and 1×10^6/ml. All treatments were inoculated in 50 ml Erlenmeyer flasks containing 20 ml sterile broth medium. As a control, medium was also cultured with sterile water. Each experiment was performed in triplicate. The pH and the amount of soluble phosphate were assayed every day over a 14-day period.

Heavy metals

Microbial growth and biomass is curtailed in media containing heavy metals [13]. However, few studies have investigated the impacts of various heavy metals at different concentrations on the phosphate solubilizing abilities of PSF. This study investigated the effects of Zn and Pb supplied at 0, 100, 500, 1000, and 2000 mg/L. Both fungi were cultured in agar medium containing a specified concentration of either metal at 28 °C for 7 days. The results were recorded and compared. Simultaneously, 1 ml of each fungal spore suspension (1×10^7/ml of P-1 and 1×10^8/ml of A-1) were separately inoculated in sterile Pikovskaya broth medium containing heavy metals at the same concentrations. Control suspensions were also prepared. The soluble phosphate content in all treatments was measured on days 1, 5, 10, and 15.

Carbon, nitrogen and insoluble phosphorus sources

To reveal the soluble P amounts and the efficiencies with which the fungi solubilized carbon, nitrogen and insoluble phosphorus sources in the Pikovskaya broth medium, the medium was supplemented with 10 g/l glucose, sucrose, maltose, fructose or lactose as carbon source. The sole nitrogen source was 0.5 g/l ammonium sulfate, ammonium nitrate, sodium nitrate, potassium nitrate or urea. Meanwhile, five insoluble phosphate compounds, $Ca_3(PO_4)_2$, $MgHPO_4$, $FePO_4$, $AlPO_4$ and $Zn_3(PO_4)_2$, were supplied at 10 g/l. All experiments were conducted in triplicate, and the pH and soluble phosphate values were assayed after 7 days' inoculation.

Plant growth

Seeds of *Z. insignis* were soaked in 10% urea for 30 min, then surface sterilized by 0.1% $KMnO_4$ for 5 min, washed several times with sterile water, dipped in sterile water for 24 h, and finally germinated in sterile plates covered by sterilized gauzes. Once the buds had sprouted to an approximate height and weight, the sterilized HS soils were separated into three groups. Each of the five pots in each group was simultaneously cultivated with three *Z. insignis* buds and 20 ml fungal spore suspensions (ca. 1×10^7/ml). The control group was cultivated with three buds and watered with 20 ml sterile distilled water. All treatments were incubated in a greenhouse at 28 °C and 40% humidity. The height of the shoot was measured at 15-day intervals. After two months, all plants were uprooted and dried [1], and the height and dry biomass of their roots and shoots was measured. The pH, amount of soluble phosphate and content of the enzymes catalase[35], urease[26] and phosphatase[25] in the soils was also determined.

Statistical analysis

The phosphate solubilizing efficiency was calculated by the formula presented in Chai [7]. All experiments were treated as three or five duplicates, and all data statistically analyzed by one-way analysis of variance using SPSS 19.0 software. Differences among treatments were determined at the 5% confidence level by Duncan's multiple range test.

RESULTS

Screening and identification of the PSM

After 7 days' inoculation, two soil fungi demonstrated superior solubilizing abilities, and were therefore isolated and purified. The ITS sequence length of the two fungi, denoted P-1 and A-1, was 561 and 544 bp respectively. The strains were more than 99% homologous to the GenBank organisms *Penicillium oxalicum* and *Aspergillus japonicus*, respectively, and have been deposited in GenBank as Accession Nos. HQ680452.1 (P-1) and EU440776.1 (A-1).

Impacts of different spore densities

During the first day, the soluble P amounts in *P. oxalicum* cultures at three spore densities, 1×10^6/ml, 1×10^7/ml and 1×10^8/ml, were below 50 mg/l (Fig. 1). After 48 h inoculation the soluble P content rose rapidly, accompanied by

declining pH of the medium. At both higher densities (10^7/ml and 10^8cfu/ml), the soluble P concentration was maximized at 2000 mg/l during the first week (Fig 1b), while at the lowest density (10^6/ml), it was maximized at 1797 mg/l on day 8. Throughout the following week of incubation, the soluble P amounts gradually declined at 10^6/ml and 10^8/ml spore density, as the pH climbed to 8.0. Conversely, the pH of the 10^7/ml spore culture remained acidic (around 3.0; Fig. 1), and the soluble phosphate was maintained at approximately 1500 mg/l in equilibrium throughout the second week. The soluble P content and pH of the medium inoculated with *A. japonicus* is presented in Fig. 2. This species attained 100% solubilizing efficiency only after 6 days at a spore density of 10^8/ml. However, high solubilized P content (~1600 mg/l) was maintained throughout the second week, and the pH of the medium remained low at 4.0. At lower spore densities (10^7/ml and 10^6/ml), the soluble P

Table 1. Concentrations of carbon, nitrogen and phosphorus sources of soluble phosphate, and phosphate solubilizing efficiencies of fungi *P. oxalicum* and *A. japonicus*

Sources	*P. oxalicum*		*A. japonicus*	
	soluble P amount (mg/l)	efficiencies	soluble P amount	efficiencies (mg/l)
Carbon				
Sucrose	2083.5[a]	100%[a]	2089.6[a]	100%[a]
Glucose	2053.8[a]	100%[a]	1042.9[c]	50.5%[c]
Fructose	2055.1[a]	100%[a]	2057.8[a]	100%[a]
Lactose	2088.1[a]	100%[a]	172.9[d]	8.38%[d]
Maltose	2059.9[a]	100%[a]	1391.5[b]	67.4%[b]
Nitrogen				
$(NH_4)_2SO_4$	2095.1[a]	100%[a]	2053.9[a]	100%[a]
NH_4NO_3	1451.5[b]	70.8%[b]	998.1[b]	48.7%[b]
KNO_3	2091.5[a]	100%[a]	2054.6[a]	100%[a]
$NaNO_3$	2059.5[a]	100%[a]	2057.9[a]	100%[a]
Urea	1281.1[b]	62.5%[c]	2015.2[a]	98.3%[a]
Phosphate				
$Ca_3(PO_4)_2$	2059.6[a]	100%[a]	2065.5[b]	100%[a]
$MgHPO_4$	2055.2[a]	100%[a]	2049.8[b]	100%[a]
$Zn_3(PO_4)_2$	239.8[c]	14.5%[c]	112.3[d]	6.8%[c]
$AlPO_4$	2484.5[a]	95.5%[a]	2612.3[a]	100%[a]
$FePO_4$	751.3[b]	35.7%[b]	512.1[c]	24.3%[b]

Values are the averages of triplicate experiments. Superscripted values are statistically significant between treatments at $p < 0.05$ by Duncan's multiple range test

Table 2. Physicochemical and biochemical properties of plants and soils under three cultivation treatments

	Plants				Soils				
	Weight (s)[4] (g/plant)	Weight (r)[5]	Length (s) (mm/plant)	Length (r)	pH	Soluble-P (mg/kg)	Catalase[1]	Phosphatase[2]	Urase[3]
CK	0.1019[a]	0.1076[a]	130.7[a]	99.2[a]	8.02[a]	2.01[a]	11.76[a]	0.077[a]	0.379[a]
P-1	0.1401[b]	0.1496[b]	145.4[ab]	126.0[b]	8.09[b]	2.97[b]	11.92[b]	0.4600[c]	0.373[a]
A-1	0.1522[b]	0.1601[b]	146.4[b]	128.5[b]	8.17[c]	2.48[ab]	11.90[b]	0.3048[b]	0.386[a]

Results are the average of five replicates. Superscripted values are statistically significant at $p < 0.05$ by Duncan's multiple range test; Superscripts letters (s) [4] and (r) [5] denote shoot and root, respectively; [1, 2, 3] the measurement units of the three enzymes are [³%A / (g soil min)], [mg Pho / (g soil 24 h)] and [mg NH_3-N / (g soil 24 h)]

content varied along with the pH. The soluble P concentration in the 10^7/ml culture was maximized at 1601 mg/l on day 6, and at 1332 mg/l in the 10^6/ml culture on day 8.

Impacts of heavy metals

Figure 3 shows the growth of P-1 and A-1 on agar medium containing different heavy metals.

Both strains grew healthily on plates containing up to 2000 mg/l Pb^{2+}. Growth of A-1 colonies was reduced at high lead concentrations (1000 and 2000 mg/l) relative to the control, while growth of P-1 was not significantly affected.

The soluble P content in the broth medium was measured up to day 15 at 5 day intervals (Fig. 4). The soluble phosphate concentrations in suspensions of both fungi exposed to Pb^{+2} decreased at higher lead concentrations throughout the fifteen days. The soluble P amounts in the P-1 suspension were maximized at 1000.7 mg/l and 775.7 mg/l at lead concentrations of 1000 mg/l and 2000 mg/l, respectively, on day 5 (Fig. 4a), and then gradually deceased. On day 15, the respective soluble P amounts at these lead concentrations had declined to 583.4 mg/l and 327.2 mg/l, and the phosphate solubilizing efficiencies were 29.2 and 16.4%. The soluble P content of A-1 in the Pb^{2+} medium was higher than that of P-1 in the presence of 100 mg/l lead, throughout the entire 15 days (Fig 5a). The soluble P content was maximized at 2007.9 mg/l on day 10. At higher concentrations (500, 1000 and 2000 mg/l), the

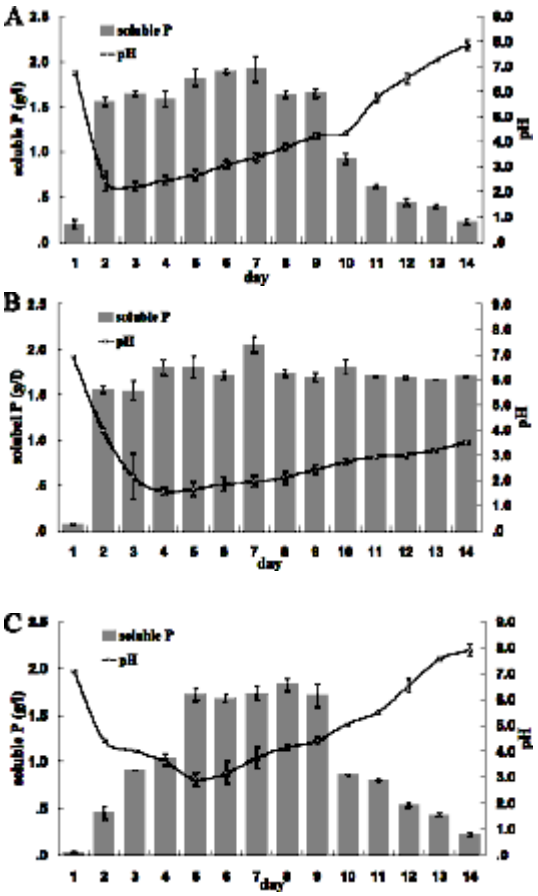

Fig. 1. Soluble phosphate concentration and pH of P-1 incubated for 2 weeks in broth medium at three different spore densities. Panels (A), (B) and (C) plot the results for the three spore suspensions, 1×10^8/ml, 1×10^7/ml and 1×10^6 /ml, respectively

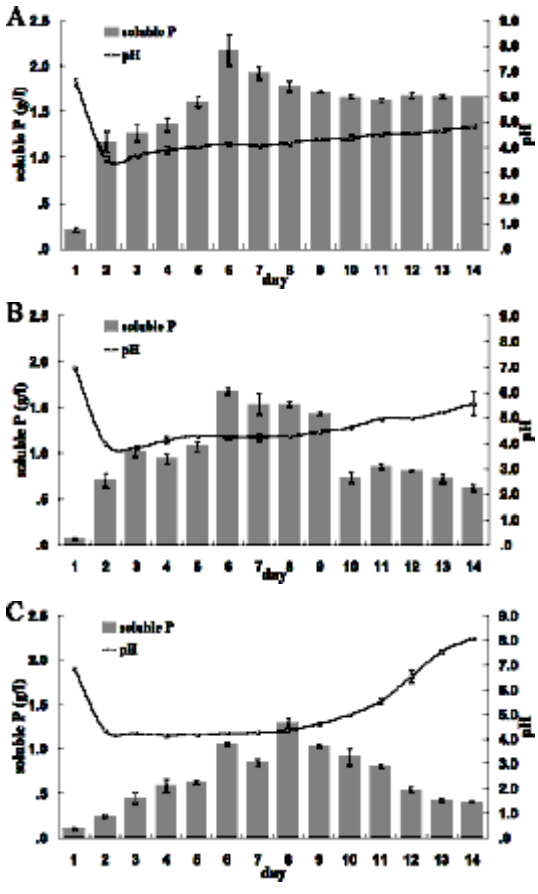

Fig. 2. Soluble phosphate concentration and pH in of A-1 culture, incubated at different spore densities in broth medium for 2 weeks. Panels (A), (B) and (C) plot the results at spore densities of 1×10^8/ml, 1×10^7/ml and 1×10^6 /ml, respectively

soluble P content was maximized at 1240.4, 658.2 and 506.4 mg/l, respectively. However, the soluble P content in A-1 exposed to lead at these concentrations was dramatically curtailed after one week incubation, reducing to below 50 mg/l on day 15.

In the presence of Zn^{2+}, the fungal growth was visibly restricted (Fig. 3). By contrast, both P-1 and A-1 grew healthily in Zn^{2+} broth medium, even at the highest concentration (2000 mg/l; photographs not shown). On day 5, the soluble phosphate content in the A-1treatment was 2090 and 182 mg/ml in the presence of 100 and 200 mg/ml Zn^{+2}, respectively. On day 5, the soluble P content in the P-1 suspension reduced as the Zn^{+2}concentration was increased to 2000 mg/l, but altered dramatically on days 10 and 15 (Fig. 4b). The soluble phosphate content had reached almost 2000 mg/l, regardless of zinc concentration, by day 15. The solubilizing efficiencies had reached 100, 97.6 and 96.6% at 500, 1000 and 2000 mg/l Zn^{+2}, respectively. The soluble P content in the A-1 culture was maximized on day 5 at all Zn^{+2} concentrations (Fig 5b), but from day 5 onward, the levels were much lower than in the P-1 culture at high concentrations (1000 and 2000 mg/l).

Impacts of three main sources

The phosphate solubilizing efficiencies of the three sources are listed in Table 1. The fungal strain P-1 attained 100% solubilizing efficiency in the presence of all five carbon sources. The A-1 strain also achieved this efficiency in media containing sucrose and fructose. In the glucose and maltose medium, the solubilizing efficiency of

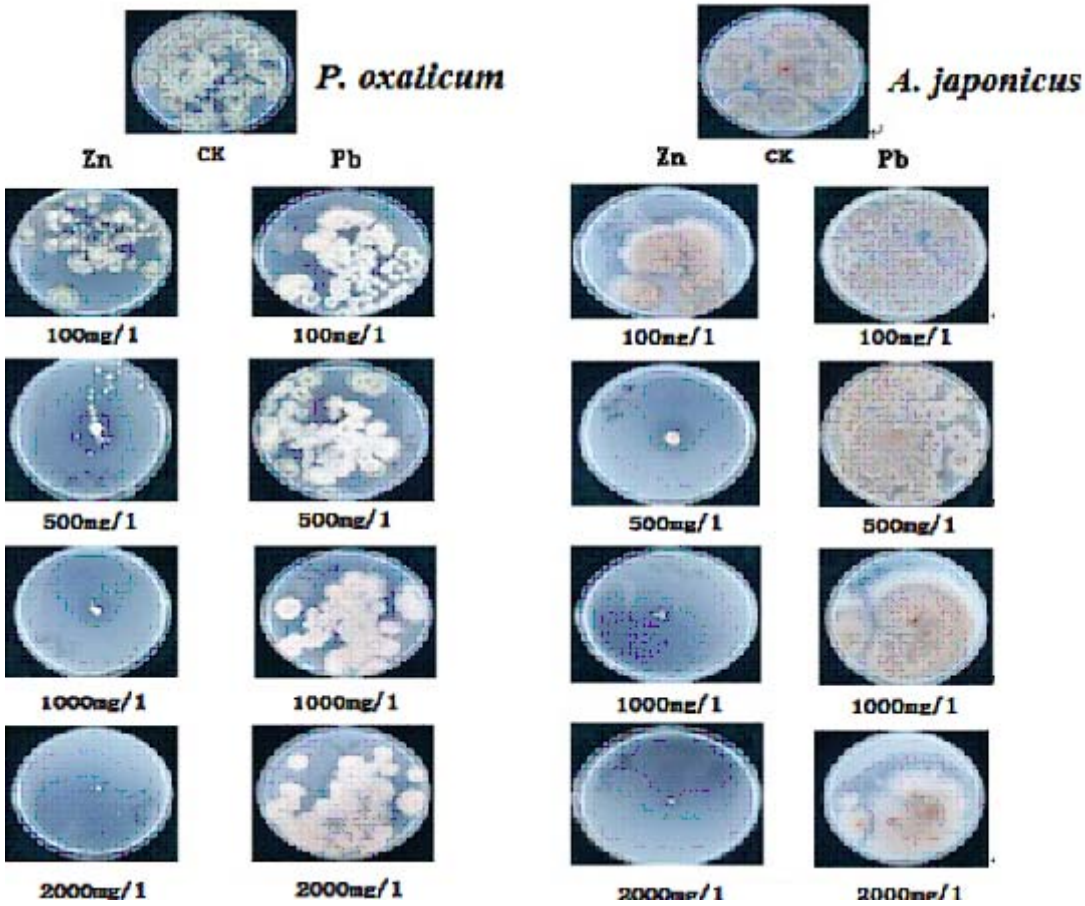

Fig. 3. Growth of P-1(left panels) and A-1 (right panels) on Pikovskaya agar plates containing three heavy metals, (Zn^{2+}, Pb^{2+}; left to right) at four concentrations (100, 500, 1000, 2000 mg/l; top to bottom). All plates were replicated in triplicate, and were randomly photographed during the experiments.

A-1 declined to 50.5 and 67.4% respectively, diminishing to 8.38% in the lactose medium. In nitrogen source experiments, both fungi achieved 100% solubilizing efficiency in media containing $(NH_4)_2SO_4$, KNO_3 or $NaNO_3$. In urea-containing medium, A-1 and P-1 demonstrated efficiencies of

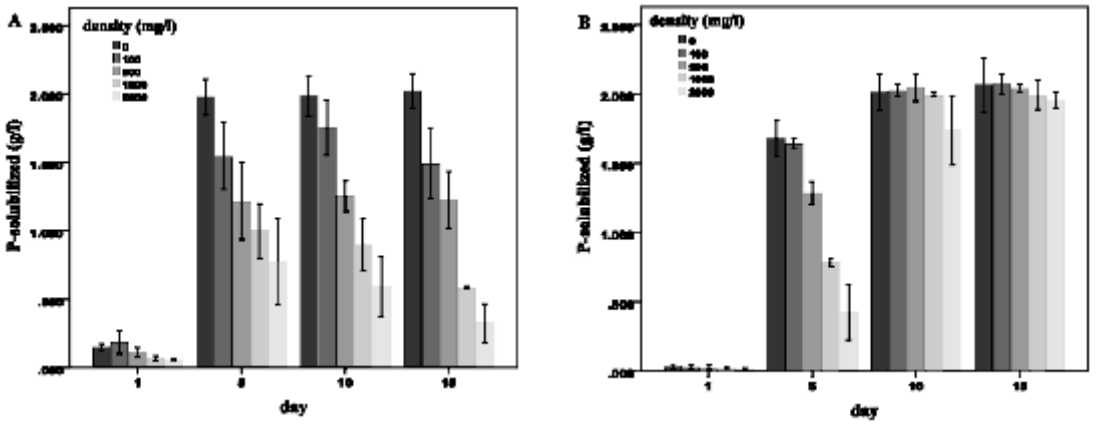

Fig. 4. Soluble phosphate concentrations in P-1 incubated in broth medium containing heavy metals Pb (A) and Zn (B) at five concentrations (0, 10, 500, 1000 and 2000 g/l, where lighter shade indicates higher density)

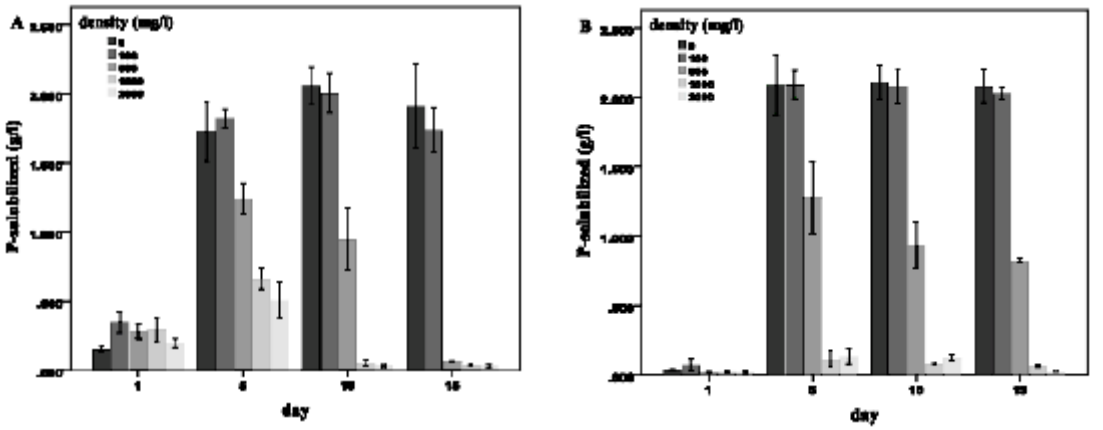

Fig. 5. Soluble phosphate concentrations in A-1 incubated in broth medium containing heavy metals Pb (A) and Zn (B) at five concentrations (0, 10, 500, 1000 and 2000 g/l, where lighter shade indicates higher density)

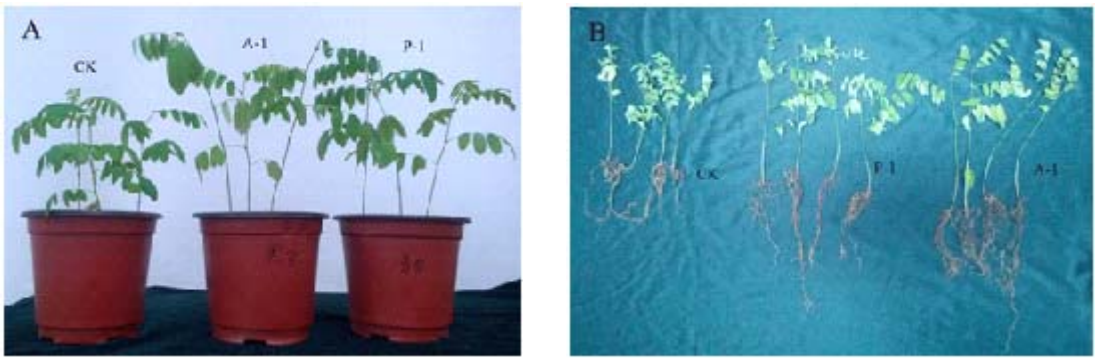

Fig. 6. Shoot heights and root lengths in the three plant treatments. (A) *Zenia insignis* seeded in soil containing heavy metal without fungal spores (left), seeded with A-1 (middle) and seeded with P-1 (right). (B) Shoot height and root depth of the *Z. insignis* treated with Ck (control), P-1 and A-1

98.3 and 62.5%, respectively. According to Duncan's multiple range test, $Zn_3(PO_4)_2$ was the most difficult compound to degrade among the five selected phosphorus sources, being solubilized with an efficiency of only 14.5% by P-1 and 6.8% by A-1. The other four insoluble phosphorus sources were solubilized up to 100%, except for $Ca_3(PO_4)_2$ as discussed earlier in this paper.

Promotion of plant growth by P-1 and A-1

After cultivating in the greenhouse for 60 days, all seedlings were harvested. The shoot heights and root lengths of seedlings inoculated with P-1 and A-1 were significantly higher than those of control plants (Fig 6). The calculated soluble phosphate content, pH and enzyme activities are listed in Table 2.

The above-ground growth of *Z. insignis* increased gradually during each 15-day interval (data not shown). On day 30, plants inoculated with P-1 and A-1 extended to 78.1 and 81.9 mm respectively, approximately 16.7 and 18.4% higher than the control (64.5 mm). On day 60, the height had increased by 27.2 and 29.5% above the control, respectively. Meanwhile, the respective root length of P-1 and A-1 had reached 128.5 and 126.0 mm on day 60, while that of the control plants was 99.2 mm. After harvesting on day 60, the A-1-inoculated plants manifested the largest root and shoot biomass of 0.1601 and 0.1522 g/plant respectively; the respective biomasses of P-1-inoculated plants were 0.1496 and 0.1401 g/plant. Inoculations with both PSF yielded much higher overall growth than that attained by the control plants.

Urease activity was not significantly different among the three treatments. The pH and catalase activity was slightly increased in the inoculated soils. The soluble P contents in the P-1- and A-1-inoculated soils were 2.97 and 2.48 mg/kg, respectively, versus 2.01 mg/kg in the control group. These increases are related to the elevated quantities of phosphatase in soils inoculated with P-1 and A-1 (Table 2).

DISCUSSION

The soluble phosphate content in the medium throughout the fortnight incubation period reflects the various solubilizing ability of the spore suspensions inoculated at different densities. The fungal strain A-1 achieved 100% efficiency at 1×10^8/ml spore suspension, but P-1 performed optimally at 1×10^7/ml rather than at the highest density of 1×10^8/ml. Both fungi attained maximum phosphate solubilizing efficiency and sustained this efficiency over 1 week at their optimum inoculation densities. Some of the P solubilized by the PSF could combine with organic acids to form organo-P compounds; this process may have reduced the soluble phosphate levels in the medium. However, as the carbon sources were consumed, the organo-P compounds could provide a subsequent energy or nutrient source, with the phosphate being re-released into the medium [15]. Thus, when the uptake rate is more-or-less balanced by the release rate, the P content in the medium would remain relatively stable. The results of this study suggest that higher solubilizing ability is not necessarily achieved by inoculating the medium with high fungal spore densities. The results also indicated the inoculation spore densities of P-1 and A-1 that maximized and sustained P solubilization, which may significantly assist further research.

The heavy-metal concentration in broth medium critically affects the quantity and stability of soluble phosphate. In this study, lead at all concentrations exerted little effect on the growth of P-1 and A-1, but the soluble P content and phosphate solubilizing efficiency was decreased at high Pb^{2+} concentrations (2000 mg/l). Heavy metals are known to persist in the environment, since they are not readily reduced by degradation[16, 21]. However, PSF can immobilize heavy metal ions by combining them with self-solubilized phosphate compounds, thereby converting them into insoluble forms that cannot be absorbed by plants[29]. Hence, the soluble P content reduces with increasing concentration of heavy metals.

Both fungal strains P-1 and A-1 grew and exhibited phosphate solubilizing abilities in broth infused with Zn^{2+} at all concentrations. By contrast, neither species thrived on Zn^{2+}-infused agar plates. These two fungi have been identified as biosorbents in aqueous solutions contaminated with heavy metals[2]. Thus, when inoculated in broth medium containing heavy metals, the fungi absorbed and diluted the ions, reducing their toxicity. However, on solid medium, the heavy metal irons were immobilized in the agar, and few would have been absorbed by the fungi. Consequently, the solid media were rendered more toxic than the

broth medium and severely restricted fungal growth.

The soluble P content and the phosphate solubilizing efficiencies of P-1 and A-1 declined at higher Pb^{2+} concentrations. However, in broth containing Zn^{2+} at the highest concentration (2000 mg/l), the phosphate solubilizing efficiency of P-1 reached almost 97%, much higher than that of A-1. This result may be attributable to different compounds excreted by the two fungi. The dominant organic acids produced by *P. oxalicum* and *A. japonicus* are oxalic acid[11] and citric acid [28], respectively. Once inoculated in the medium, the fungi secreted organic acids and then solubilized P by converting insoluble tricalcium phosphate to soluble phosphate. By day 5, the soluble P content in broth medium containing Zn^{2+} had decreased as insoluble zinc-phosphate compounds were formed by the soluble phosphate and Zn^{2+} (Fig. 4a). The insoluble zinc-phosphate compounds were then solubilized by oxalic acid, recognized as the main mineral transforming agent. Consequently, soluble P was released from the zinc-phosphate compound as the zinc oxalic precipitated out[12], increasing the amount of soluble phosphate in the medium. Since citric acid cannot solubilize zinc-phosphate compounds, the soluble phosphate content of A-1 dramatically declined at the highest Zn^{2+} concentration (2000 mg/l).

Compared with chemical phosphate fertilizers, bio-fertilizers provide a cheap phosphate fertilizer source[14], boost plant yields[3], increase the growth of shoots and roots[33], and reduce environmental damage[34]. These advantages have inspired years of PSF study, as researchers seek to exploit the organisms as bio-fertilizers. Chuang[10] reported a soluble P concentration of 322 µg/ml yielded by isolated *Aspergillus* sp. In addition, the fungus increased the dry weight and N content of *Brassica chinensis* after four weeks cultivation in pot experiments. In another study, the PSF *A. niger* promoted the growth of *Cajanus cajan*, significantly improving the dried biomass of the plant[24]. In the present study, the height and dry biomass of *Z. insignis* seeded in heavy metal contaminated soil was much improved in the presence of PSF, and the soil phosphatase enzyme activities were enhanced. In soil, P is easily immobilized into insoluble phosphate compounds, which are inaccessible to plants. PSF inoculation can increase the soluble P content in the soils, chiefly by acidification, although some phosphate solubilizing bacteria produce siderophores, plant hormones and enzymes[30] that also promote plant growth. In soils contaminated with heavy metals, the enhanced soluble P content could precipitate and immobilize the metals[29], thereby increasing the plants' resistance to heavy metals in the soils and facilitating their growth in the contaminated environment.

Catalase and urease activities are considered as indicators of microbial number and fertility in soils[26, 35]. Zhang[37] showed that PSF inoculation increases the enzyme activities in rhizosphere soil under salt stress. In the present research, catalase and urease activities in soils inoculated with the fungi P-1 and A-1 were slightly improved over the control soil, but the improvements were not significant. This result is likely attributable to minimal carbon sources in soil, which restrict the growth of PSF colonies. Phosphatase plays a crucial role in solubilizing the organic phosphate compounds in soil[17]. A positive correlation has been reported between phosphatase activity and phosphate content[4]. As detected in this study, both phosphatase activity and soluble P content were markedly increased in the soils inoculated with P-1 and A-1. The enhanced phosphatase activity increased the hydrolysis of organic phosphate compounds. Consequently, the amount of soluble phosphate available to the plants was dramatically increased, improving the yield of *Z. insignis* after 2 months cultivation.

In conclusion, the fungal strains P-1 and A-1 demonstrate high phosphate solubilizing ability under heavy metal conditions. The subsequent improvement in plant height and biomass highlights the potential of *P. oxalicum* and *A. japonicus* as bio-fertilizers in heavy metal contaminated soil.

CONCLUSIONS

Plant growth can be promoted by inoculating with phosphate solubilizing bacteria and fungi. The two strains *Penicillium oxalicum* and *Aspergillus japonicus* described in this report show great phosphate solubilizing abilities Pb and Zn tolerance, and growth promotion to *Zenia insignis*. Hence, these two strains can be treated

as the bio-fertilizers in agriculture applications and will be useful in heavy-metal remediation. Furthermore, the method to test the soluble phosphate amounts in heavy metal contaminated mediums in this report can be used to isolate phosphate solubilizing microorganisms with heavy-metal remediation potentials.

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