Molecular Identification of Rhizospheric Thermo-halotolerant *Aspergillus terreus* and its Correlation to Sustainable Agriculture

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High phosphate solubility is one of the most important factors for increasing plant growth. This study focused on the zinc phosphate (ZP) solubilizing capacity of thermo-halotolerant *Aspergillus terreus*, where the growth showed halo zones on Pikovskaya agar medium that appeared at high NaCl concentrations (up to 10%) and a wide range of temperatures (up to 45 °C). Acidification of the broth was assumed to be the major mechanism for ZP solubilization by *A. terreus*, where the growth was related to the pH decrease in the medium containing ZP. Under pot conditions, *A. terreus* increased the biomass and phosphorus content of *Hordeum vulgare* plants. *A. terreus* showed a phosphorus solubilization ability with a NaCl concentration of up to 10%; therefore, *A. terreus* can be of great benefit in maintaining the available phosphate levels for crops in saline soils. Finally, it was found that *A. terreus* with ZP can substitute chemical fertilizer and help improve crop production.

Keywords: Aspergillus terreus; Zinc phosphate; Solubilization; Biofertilizers

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INTRODUCTION

Phosphorus (P) is one of the most important macronutrients, after nitrogen, for the growth and development of plants (Hameeda *et al*. 2008; Sharma *et al*. 2013). The majority of soil P, approximately 95% to 99%, is in an insoluble form linked with metals, such as iron, zinc, aluminum, and calcium; therefore, it cannot be used by plants (Son *et al*. 2006) and must be converted into a soluble form. Phosphorus participates remarkably in plant metabolism, photosynthesis, energy and sugar production, and synthesis of nucleic acids, and it enhances nitrogen fixation (Saber *et al*. 2005).

Many microorganisms in agricultural soil plays an important role in sustainable agriculture (Abdel-Ghany *et al.* 2015, 2018a,b). Phosphate-solubilizing microorganisms (PSMs) can play a great role in dissolving both fertilizer P and bound P in the soil, which is environmentally friendly and sustainable (Khan *et al*. 2007).

These PSMs can solubilize and mineralize P from the inorganic and organic pools of the total soil P and may be utilized as inoculants to increase the availability of P to plants (Richardson 2001; Oliveira *et al*. 2009; Abdel-Ghany *et al.* 2013). Bhattacharyya and Jha (2012) stated that in the natural environment numerous microorganisms in the soil and rhizosphere are effective at releasing P from the total soil P through solubilization and mineralization. Numerous researchers have conducted investigations on PSMs in soils (Gupta *et al*. 2012), mangrove (Holguin *et al*. 2001), and the rhizosphere (Oliveira *et al*. 2009). Nahas (1996) reported that fungi possess a better ability to solubilize insoluble

phosphate than bacteria. A broad range of soil fungi are reported to solubilize insoluble P, including *Aspergillus niger* and *Penicillium* species, which are the most common fungi capable of phosphate solubilization (Whitelaw *et al*. 1999; Nelofer *et al*. 2016).

Conflicting results have been reported for the effect of temperature on phosphorus solubilization by fungi and bacteria. Varsha *et al.* (2002) recorded 28 °C as the best temperature for P solubilization by *Aspergillus aculeatus*. *A. awamori* is capable of solubilizing insoluble phosphate over a wide range of high temperature (Walpola *et al*. 2012). On the other hand, Johri *et al.* (1999) reported solubilization at a low temperature of 10 °C.

Several mechanisms, including lowering the pH by acid production, ion chelation, and exchange reactions in the growth environment, have been reported to play a role in phosphate solubilization by soil fungi and bacteria (Abd-Alla 1994; Whitelaw 1999; Han *et al*. 2006). *Penicillium* and *Aspergillus* are able to change insoluble soil phosphates into soluble forms by secreting acids, such as formic, acetic, propionic, lactic, glycolic, fumaric, and succinic acids. These acids reduce the soil pH and bring about the dissolution of phosphate bonds (Bolan *et al*. 1997). Most of the reports in the literature suggested that tri-calcium phosphate is dissolved by acidification. Therefore, any microorganisms that acidify the external medium will show some level of phosphate-solubilizing activity (Chai *et al*. 2011; Sanjotha *et al*. 2011).

Inoculation with phosphate-solubilizing fungi (*A. terreus* and *P. pinophilum*) and the application of reactive phosphate rock improves the P uptake of plants, which results in an increased dry matter yield of sorghum under controlled conditions (Steiner *et al*. 2016). These PSMs have considerable synergistic effects on the growth and development of crops (Tallapragada and Gudimi 2011). Salt-tolerant or halophilic soil microorganisms that also exhibit the ability to solubilize insoluble P facilitate the development of saline-alkali soil-based agriculture (Zhu *et al*. 2011). The objective of this study was to select promising phosphate-solubilizing fungi under stressful salt and temperature conditions for the development of sustainable agriculture.

EXPERIMENTAL

Rhizosphere Sampling for Phosphate-solubilizing Fungi

Wild halophilic plants (*Suaeda monoica*) in Jizan, Saudi Arabia were uprooted from salt marsh soil (Fig. 1A) and gently shaken to remove the superfluous soil, and the rhizosphere soils were placed in clean sterilized polyethylene bags. A known weight of roots with soil was immersed in a flask containing sterilized distilled H_2O. After being shaken, suitable dilutions were prepared. One milliliter of the suitable rhizosphere soil suspension was transferred to each sterilized petri plate and covered with melted, but cooled medium. The plates were incubated at 28 °C for 7 d to 10 d, and the developing fungi were counted and isolated.

Salt and Temperature Tolerance of the Fungal Isolates

The salt tolerance of the isolates was determined by inoculating the cultures in triplicate on Czapek medium amended with NaCl concentrations of up to 15% w/v. Rosebengal (1/15000, w/v) and chloramphenicol (50 ppm) were added to the growth medium to suppress bacterial growth. For temperature tolerance, the fungal isolates were cultivated on Czapek agar medium and incubated at different temperatures up to 50 °C.

The fungal growth in terms of the colony diameter was recorded after 7 d of incubation. The fungus that was highly resistant to NaCl (halophilic/halotolerance) and temperature was purified.

Morphological and Molecular Identification of Fungal Isolate

The fungus that was highly resistant to NaCl (halophilic/halotolerance) and temperature was purified, identified morphologically and microscopically as *A. terreus* (Fig. 1B) according to established literature protocols (Raper and Fennell 1973; Pitt and Hocking 1997), and confirmed by molecular identification.

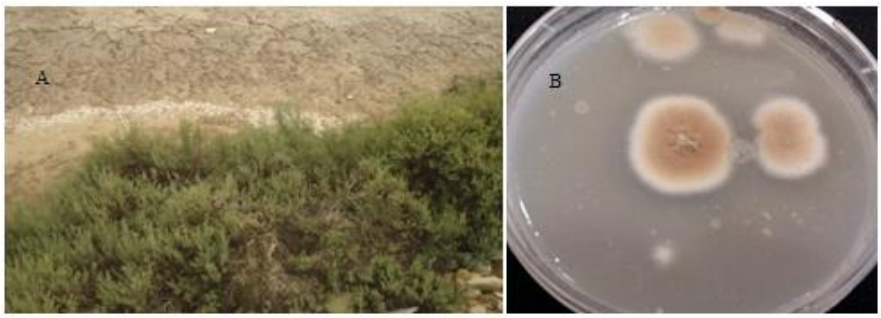


Fig. 1.(A) Hypersaline soil with *Suaeda monoica* plant for fungal isolation and (B) *A. terreus* colony growth

DNA Extraction and PCR Amplification

Fungal mycelia (0.2 g) of fungus grown on potato dextrose medium were harvested and placed in liquid nitrogen for 10 min, then vigorously homogenized to extract its genomic DNA. A DNA extraction buffer (500 µL) containing of 200 mM Tris-HCl, 240 mM NaCl, 25 mM EDTA, and 1% SDS at pH 8.0 has been mixed with the DNA extract, then shaken for 5 min and then centrifuged for 5 min at 10,000 rpm. Next, the supernatant has been mixed with an equal volume of phenol:chloroform (1:1 v/v) for 30 min and again centrifuged for 5 min at 12,000 rpm. The upper part has been gently outgoing and mixed with an equal volume of 3 M sodium acetate (pH 5.2) and 2 volumes of 96% ethanol for 60 min at -20 °C. After centrifugation, the collected DNA was washed with ethanol (70%), dried until removal of the ethanol, and re-suspended in 100 µL of distilled water.

Molecular identification of *A. terreus* was based on Internal transcribed spacer (ITS) rDNA sequence (18S- 28S rRNA), according to White *et al.* (1990). The sequences of the ITS1 and ITS4 primers were 5‘-TCCGTAGGTGAACCTGCGG-3‘and 5′-TCCTCCGCTTATTGATATGC-3′, respectively. Molecular evolutionary genetic analysis software MEGA6 (Germany) was used for phylogenetic analyses. The sequence was annotated and submitted to the NCBI Gen Bank database. A basic local alignment search tool (BLAST) homology search was performed to find the closest homology, and a sequence analysis was performed by comparison with ITS rDNA sequences of ten other fungal species obtained from the Gen Bank database. The evolutionary tree was reconstructed using the neighbor joining method.

Phosphate-solubilizing Ability Test

Aspergillus terreus was checked for a phosphate-solubilizing ability on Pikovskaya (PKV) agar medium that contained 0.5 g/L $(NH_4)_2SO_4$, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.02 g/L NaCl, 0.02 g/LKCl, 0.003 g/L $FeSO_4 \cdot 7H_2O$, 0.003 g/L $MnSO_4 \cdot H_2O$, 5 g/L $Ca_3(PO_4)_2$,

10.0 g/L glucose, 0.5 g/L yeast extract, 15.0 g/L agar, and 1000 mL of distilled water (Pikovskaya 1948) containing zinc phosphate (ZP). The formation of a clear halo zone around the fungal growth after 5 d of incubation indicated a phosphate-solubilizing ability.

Zinc Phosphate Solubilization Efficiency

The PKV agar medium containing ZP was prepared and poured into sterilized petri plates. *A. terreus* (5-mm disc of fungal colony) was spotted on these plates and incubated for 7 days at different temperature ranged from 10 to 45 °C. Those that showed halo zones around the colonies were capable of phosphate solubilization. The solubilization index was calculated according to Eq. 1,

$$SI = \left(\frac{C + H}{C}\right) \quad (1)$$

where *SI* is the solubilization index, *C* is the colony diameter (cm), and *H* is the halo zone diameter (cm).

Effect of the Salinity on Zinc Phosphate Solubilization

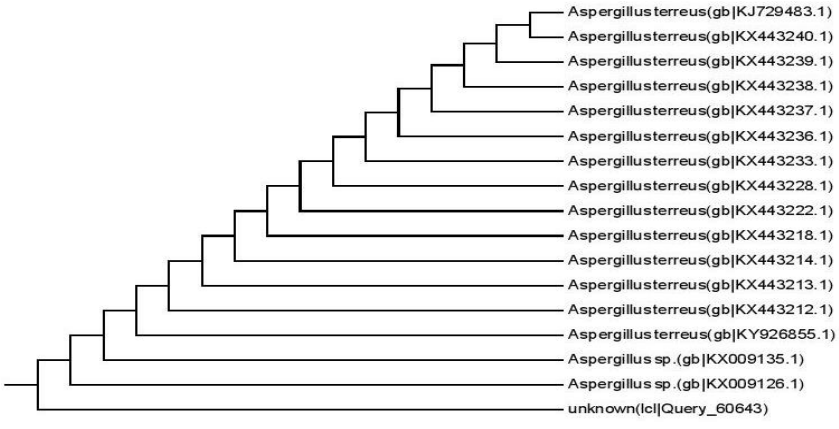
The effect of the salinity on the phosphate solubilization ability of *A. terreus* was determined in a PKV medium amended with NaCl (0% to 10%) over 5 d. The medium was inoculated with *A. terreus* and incubated at 30 °C. The ZP solubilization efficiency was determined using the method mentioned above.

Pot Experiment

Grains of *Hordeum vulgare* were surface sterilized, rinsed six times with sterile water, and dried. Spore suspensions of *A. terreus* at different levels (2 mL and 4 mL, each containing 2×10^6 spores) were added to autoclaved soils 48 h before sowing. The uninoculated seeds served as a control treatment for comparison. Zinc phosphate was added (0.5 g/kg) to the soil before seeding, except to the control. The plants were irrigated with sterilized water when required. The pots with different treatments were arranged in a randomized complete block design with eight replicates for each treatment. The treatments were as follows: control (soil without ZP and *A. terreus*), soil treated with ZP, soil treated with ZP and 2 mL of spore suspension, and soil treated with ZP and 4 mL of spore suspension. After 20 d, a random sample (six plants of *H. vulgare*) was taken from each treatment to determine some growth parameters, including the plant height (cm), dry weight/plant (g), nitrogen (N) (extracted from the plants with sulfuric acid and detected using the semimicro Kjeldahl method), and P (extracted by nitric perchloric acid digestion and detected using the vanadono-molybdo-phosphoric colorimetric method).

RESULTS AND DISCUSSION

The identification of the *A. terreus* fungus was confirmed using molecular characterization, which is based on ITS rDNA (Figs. 2Aand 2B). This method of molecular identification of fungi at the species level is primarily based on the variable nature of the ITS regions of DNA (Romanelli *et al*. 2010; Delgado-Serrano *et al*. 2016). The 18S rRNA sequence of the *A. terreus* isolate was searched for on a database (BLAST) using multiple sequence alignment (Fig. 1B).



(A)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Aspergillus terreus strain PKKS1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene,	957	957	99%	0.0	100%	KJ729483.1
Aspergillus terreus strain NN071355 18S ribosomal RNA gene, partial sequence; internal transcribed spacer	953	953	100%	0.0	99%	KX443240.1
Aspergillus terreus strain NN071354 18S ribosomal RNA gene, partial sequence; internal transcribed spacer	953	953	100%	0.0	99%	KX443239.1
Aspergillus terreus strain NN071332 18S ribosomal RNA gene, partial sequence; internal transcribed spacer	953	953	100%	0.0	99%	KX443238.1
Aspergillus terreus strain NN071330 18S ribosomal RNA gene, partial sequence; internal transcribed spacer	953	953	100%	0.0	99%	KX443237.1
Aspergillus terreus strain NN071249 18S ribosomal RNA gene, partial sequence; internal transcribed spacer	953	953	100%	0.0	99%	KX443236.1
Aspergillus terreus strain NN058790 18S ribosomal RNA gene, partial sequence; internal transcribed spacer	953	953	100%	0.0	99%	KX443233.1
Aspergillus terreus strain NN058534 18S ribosomal RNA gene, partial sequence; internal transcribed spacer	953	953	100%	0.0	99%	KX443228.1
Aspergillus terreus strain NN058355 18S ribosomal RNA gene, partial sequence; internal transcribed spacer	953	953	100%	0.0	99%	KX443222.1
Aspergillus terreus strain NN053548 18S ribosomal RNA gene, partial sequence; internal transcribed spacer	953	953	100%	0.0	99%	KX443218.1
Aspergillus terreus strain NN045440 18S ribosomal RNA gene, partial sequence; internal transcribed spacer	953	953	100%	0.0	99%	KX443214.1
Aspergillus terreus strain NN045427 18S ribosomal RNA gene, partial sequence; internal transcribed spacer	953	953	100%	0.0	99%	KX443213.1
Aspergillus terreus strain NN044279 18S ribosomal RNA gene, partial sequence; internal transcribed spacer	953	953	100%	0.0	99%	KX443212.1
Aspergillus terreus small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.	953	953	100%	0.0	99%	KY926855.1
Aspergillus sp. isolate SR44 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and i	953	953	100%	0.0	99%	KX009135.1
Aspergillus sp. isolate SR23 small subunit ribosomal RNA gene, partial sequence; internal transcribed space	953	953	100%	0.0	99%	KX009126.1

(B)

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Query  1    ATAAATGCGTCGGCGGGCGCCGGCCGGGCCTACGGAGCGGAAGACGAAGCCCCATACGCT  60
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Sbjct  518  ATAAATGCGTCGGCGGGCGCCGGCCGGGCCTACGGAGCGGAAGACGAAGCCCCATACGCT  459
Query  61   CGAGGACCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCGTCCCCCGGGAGCCGGGGGAC  120
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Sbjct  458  CGAGGACCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCGTCCCCCGGGAGCCGGGGGAC  399
Query  121  GAGGGCCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCCCC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  398  GAGGGCCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCCCC  339
Query  181  CCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGCAA  240
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Sbjct  158  GTTCATGTTGGGGTCTCCGGCGGGCACGGGCCCGGGGGCGAGTCGCCCCCGGCGGCCAG  99
Query  421  CAACGCTGGCGGGCCCGCCGAAGCAACAAGGTACAATAGTCACGGGTGGGGAGGTTGGGC  480
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Sbjct  98   CAACGCTGGCGGGCCCGCCGAAGCAACAAGGTACAATAGTCACGGGTGGGGAGGTTGGGC  39
Query  481  CATAAAGACCCGCACTCGGTAATGATCCTTCCGCAGGT  518
            ||||||||||||||||||||||||||||||||||||||
Sbjct  38   CATAAAGACCCGCACTCGGTAATGATCCTTCCGCAGGT  1
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Fig. 2.(A) Phylogenetic relationships between the *A. terreus* strain and the ITS sequences of closely related fungal strains retrieved from the NCBI GenBank database; and (B)cluster analysis of *A. terreus* (B)

From the alignment profile results, it was concluded that the *A. terreus* strain PKKS1 18S rRNA amplicon closely matched other *A. terreus* isolates (> 99%). The

constructed phylogenetic relatedness (Fig.2B) of the whole sequence of *A. terreus* strain PKKS1 18S rRNA was compared with closely related strains from the BLAST database. This procedure revealed the molecular identity of the isolated fungus strain of this study. The highest growth, irrespective of the NaCl concentration, was recorded with the isolate *A. terreus*, which grew with up to 10% NaCl (Table 1).

The growth of *A. terreus* in general decreased with an increasing NaCl concentration, but the maximum growth was noted at 2% NaCl. This concentration appeared to be optimal for growth. Similar data were obtained by Nasmetova *et al*. (2010) with halophilic *A. terreus* that was isolated from hypersaline environments. Nasmetova *et al*. (2010) and Nazareth *et al*. (2012) reported that *A. fumigatus* and *A. terreus* can be isolated with a high frequency from salty soil and hypersaline water samples, with salinities ranging from 3% to 15% NaCl. Kanse *et al*. (2015) found that increasing the salinity had no effect on the phosphate solubilization ability of *Talaromyces funiculosus.* In the present study, *A. terreus* was able to grow at temperatures of up to 45 °C (Table 2), but the growth decreased at temperatures above 35 °C. This result confirmed the thermotolerance of *A. terreus* and agreed with the results of Ahirwar *et al*. (2017).

Table 1. Growth of *A. terreus* under Different NaCl Concentrations

Salt Concentration (%)	Growth (cm) ±SD*
0.0	5.47±0.06
2	5.53±0.15
4	4.43±0.12
6	3.07±0.12
8	1.43±0.06
10	0.93±0.06
12	0.00±0.00

SD*, ± standard deviation

Table 2. Growth of *A. terreus* at Different Temperatures

Temperature (°C)	Growth(cm) ±SD*
10	0.00±0.00
15	1.33±0.15
20	3.30±0.17
25	4.07±0.12
30	5.43±0.12
35	5.70±0.10
40	4.30±0.26
45	2.23±0.06

SD*, ± standard deviation

These results indicated that *A. terreus* may be a potential candidate for ZP solubilization at environmentally stressed temperatures (Fig. 3), as the phosphate solubilization efficiency of *A. terreus* at different temperatures up to 45 °C was clearly high from the appearance of clear halo zones around the fungal colonies. Similarly, other researchers (Gharieb *et al*. 1998; Sayer and Gadd 1998) have reported phosphate solubilization at different temperatures by *Aspergillus* sp. Halos were produced because of the solubilization of insoluble phosphates, which in turn were mediated *via* the production of organic acid in the surrounding medium (Gaur1990).

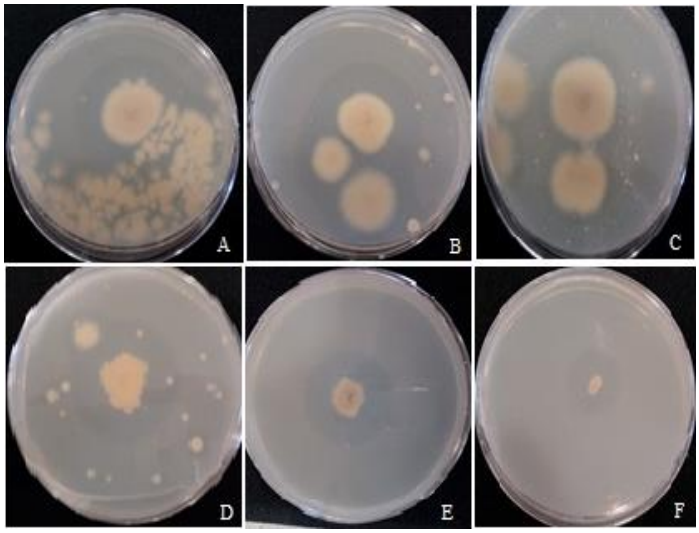

Fig. 3. Phosphate solubilizing ability (halo zone) of *A. terreus* at different temperatures: (A) 30 °C; (B) 25 °C; (C) 20 °C; (D) 35 °C; (E) 40 °C; and (F) 45 °C

The greatest decrease in the medium pH was observed with the ZP-amended medium and unamended medium during *A. terreus* growth after up to 10 d of incubation, particularly at a low concentration (500 ppm) (Table 3). The presence of insoluble ZP was not the factor necessary to stimulate acid production. Solubilization might have occurred because of the production of acids by *A. terreus,* as the pH of the culture broth shifted from neutral to highly acidic. Previous studies on the effects of the pH on the availability of phosphate have had contrasting results (Whitelaw 1999). However, the effects of the pH on the solubility and speciation of metals have been well documented (Gadd and Sayer 2000; Gadd 2001). Fankem *et al*. (2006) stated that phosphate solubilization is the result of the combined effects of pH decrease and organic acid production by microorganisms. Another explanation for phosphate solubilization was reported by Kim *et al*. (2005), who stated that organic acids may form soluble complexes with metal ions associated with insoluble P, and thus P is released.

Table 3. pH Detection at Different Incubation Periods and Different ZP Concentrations

Incubation Period (d)	Medium Supplemented with ZP			Control Medium±SD*
	500ppm±SD*	1000ppm±SD*	2000ppm±SD*	
2	6.63±0.15	6.63±0.12	6.83±0.06	6.57±0.12
4	6.23±0.06	6.37±0.06	6.43±0.12	6.40±0.10
6	4.07±0.15	4.17±0.15	5.73±0.06	4.23±0.55
8	3.30±0.17	3.43±0.12	4.70±0.17	3.77±0.06
10	2.37±0.15	3.10±0.17	3.87±0.31	2.47±0.12

SD*, ± standard deviation

A. terreus showed P solubilization with up to 10% NaCl present (Table 4). Similarly, Nautiyal (1999) reported solubilization in the presence of 10% NaCl, but there was a general decrease in solubilization with an increasing NaCl concentration. According to Srividya *et al*. (2009), the phosphate-solubilizing ability of *A. niger* was enhanced by the presence of NaCl. In addition, previous studies reported that solubilization of inorganic phosphates was improved in the presence of 1% NaCl (Kim *et al*. 1997; Kang *et al.* 2002).

The strain *A. terreus* can thus be of great benefit in maintaining the available phosphate levels for crops in saline soils.

Table 4. Solubilizing Efficiency of the ZP at Different NaCl Concentrations

NaCl Concentration (%)	Colony Diameter (cm) ±SD*	Halo Zone (cm) ±SD*	SI±SD*
0.0	3.43±0.12	6.43±0.06	2.9
2.5	3.07±0.12	5.10±0.17	2.7
5.0	3.03±0.15	5.13±0.12	2.7
7.5	2.53±0.15	4.47±0.06	2.8
10.0	2.13±0.12	3.30±0.10	2.5

SD*, ± standard deviation

This is not the first study to use *Aspergillus* sp. for phosphate solubilization or as a biofertilizer (Jain *et al*. 2012; Xiao *et al*. 2013). The best growth of *Hordeum vulgare* was recorded for the plant treated with *A. terreus* at low and high spore suspensions with ZP, followed by the control with ZP and no fungal inoculation, when compared with that of the control without ZP and fungal inoculation (Fig. 4).

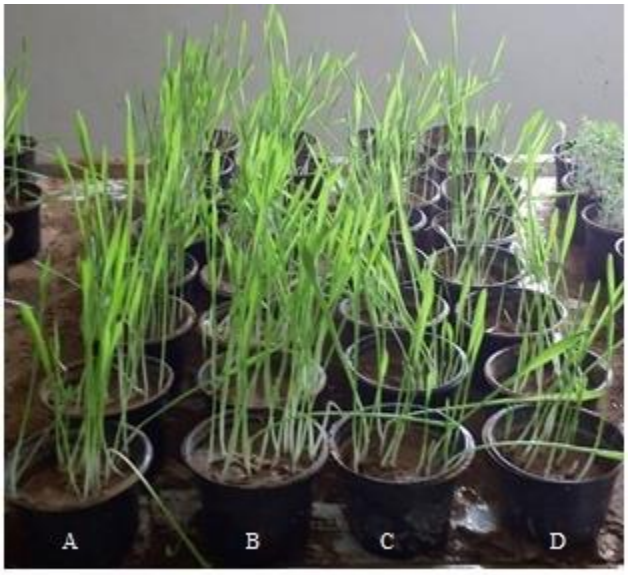

Fig. 4. *Hordeum vulgare* treated with phosphate solubilizing *A. terreus*: (A) control (soil without ZP and *A. terreus*); (B) soil treated with ZP; (C) soil treated with ZP and 2 mL of spore (2×10^6) suspension; and (D) soil treated with ZP and 4 mL of spores (2×10^6)

The control plant revealed that a deficiency of available phosphate retarded plant growth for various parameters, such as the shoot length and fresh weight, while increases in these parameters may have been because of the P released from ZP by fungal inoculation (Table 5).

Table 5. Effects of Phosphate Solubilizing *A. terreus* on Some Growth Parameters, P, and N Percentages in *Hordeum vulgare* Plants

Treatment	Height (cm) ± SD*	Fresh Weight (mg) ± SD*	Protein (%)	P (%)	N (%)
Control (Soil)	24.17 ± 1.26	368 ± 10.41	22.56	0.33	4.2
Soil +ZP	26.67 ± 0.29	382 ± 2.00	23.87	0.41	4.7
Soil+ZP+ Fungus**	34.33±0.29	425±27.30	27.50	0.65	7.5
Soil+ZP+ Fungus***	34.83±0.58	424±25.58	27.52	0.65	7.7

SD*, ± standard deviation
Two mL** and 4 mL*** of distilled water, each containing 2×10^6 *A. terreus* spores

CONCLUSIONS

1. Rhizosphere *A. terreus* appeared to have a promising effect on the solubilization of zinc phosphate (ZP) under stressful temperature and salinity conditions.
2. The pot experiment studies revealed that *A. terreus* increased the P and nitrogen concentrations in *H. vulgare* plants.

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