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Plant Growth Promotion by an Extracellular HAP-Phytase of a Thermophilic Mold *Sporotrichum thermophile*

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Abstract Phytase of the thermophilic mold *Sporotrichum thermophile* Apinis hydrolyzed and liberated inorganic phosphate from Ca^{+2}, Mg^{+2}, and Co^{+2} phytates more efficiently than those of Al^{3+}, Fe^{2+}, Fe^{3+}, and Zn^{2+}. The hydrolysis rate was higher at 60 °C as compared to 26 °C. Among all the organic acids tested, citrate was more effective in enhancing solubilization of insoluble phytate salts by phytase than others. The dry weight and inorganic phosphate contents of the wheat plants were high when supplemented with phytase or fungal spores. The plants provided with 5 mg phytate per plant exhibited enhanced growth and inorganic phosphate. With increase in the dosage of phytase, there was increase in growth and inorganic phosphate of plants, the highest being at 20 U per plant. The compost made employing the combined native microflora of the wheat straw and *S. thermophile* promoted growth of the plants. The plant-growth-promoting effect was also higher with the compost made using *S. thermophile* than that from only the native microflora.

Keywords Phytase · Insoluble phytates · *Sporotrichum thermophile* · Plant growth promotion · Compost

Introduction

Phytases [*myo*-inositol hexakisphosphate phosphohydrolase; EC 3.1.3.8 and EC 3.1.3.26] belong to a subclass of phosphatases, which catalyze the hydrolysis of phytic acid, the principal storage form of phosphorus in cereals, legumes, oil seeds, nuts, and others [1]. Large proportion of soil phosphorus exists in the organic form, of which phytic acid is the predominant form [2]. Phytate is well known to be an anti-nutrient and a major source of phosphorus pollution in animal manure [3–5]. In soil, it is either adsorbed to clays or precipitated as insoluble salts of iron and aluminum in acidic soils or as insoluble calcium

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salts in alkaline soils [2]. Therefore, a phytase with high activity on phytates has a demand for soil amendment [2].

Phosphorus deficiency in soils is a major constraint for agricultural production worldwide. Plants fail to utilize the insoluble phytates directly, and, therefore, these phytates should be dephosphorylated by phytases/acid phosphatases before assimilation. There are a few reports explaining the role of phytase in improving the growth of the plants and reducing the phosphorus pollution. A β-propeller phytase from *Bacillus subtilis* was constitutively expressed through roots in tobacco and *Arabidopsis* [6]. In tobacco, phytase activity in transgenic leaf and root extracts was seven to nine times higher than those in wild-type extracts. However, a purple acid phosphatase exhibiting phytase activity was reported from root exudates of tobacco [7]. Yip et al. [8] showed that the tobacco line transformed with a neutral *Bacillus* phytase exhibited phenotypic changes in flowering, seed development, and response to phosphate deficiency. The transgenic line showed an increase in flower and fruit numbers, small seed syndrome, lower seed IP6 to IP5 ratio, and enhanced growth under phosphate starvation conditions compared with the wild type.

Sporotrichum thermophile occurs widely in soils and plays an important role in decomposing plant materials by producing an array of cell-wall-degrading enzymes [9–12]. *S. thermophile* produces phytase in submerged as well as solid-state fermentations [13–16]. Phytase of *S. thermophile* is a glycoprotein histidine acid phosphatase phytase with broad substrate specificity [17]. In this investigation, we have tested the applicability of extracellular phytase of *S. thermophile* in hydrolyzing insoluble phytates and plant growth promotion. To the best of our knowledge, this is the first report on insoluble phytate hydrolysis and plant growth promotion by the phytase of a thermophilic mold.

Materials and Methods

Source of the Strain and Culture Conditions

The thermophilic mold *S. thermophile* Apinis BJTLR50 was isolated from a soil sample collected from Rohtak, Haryana state (India), and routinely grown on Emerson's YpSs [18] agar medium. The conidiospores from 6-day-old sporulated solid media slopes were harvested by washing using normal saline containing 0.1% (*v*/*v*) Tween-80 and the spore suspension was adjusted to $\sim 1 \times 10^7$ CFU/ml for inoculation of the phytase production medium. *S. thermophile* was cultivated in the phytase production medium [14] at 45 °C. The culture filtrate containing phytase activity was subjected to acetone precipitation and the concentrated enzyme was dialyzed and used in the plant growth experiments.

Phytase Assay

Phytase activity was assayed by quantitating the inorganic phosphate liberated from sodium phytate according to Fiske and Subbarow [19] as described earlier [13–17]. One unit of phytase is defined as the amount of enzyme that liberates 1 nmol of inorganic phosphate per second under the assay conditions.

Plant biomass and Inorganic Phosphate Estimation

The fresh root and shoot were dried in a hot oven at 60 °C for dry weight measurements. The dried parts were ashed in an incinerator at 400 °C for 4 h and dissolved in 0.9 M H_2SO_4

[20]. The inorganic phosphate content was estimated according to Fiske and Subbarow [19].

Preparation and Hydrolysis of Insoluble Phytate Salts

In order to study the hydrolysis of metal phytates, 100-mM stock solutions of Ca^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, and Al^{2+} ions were prepared by dissolving $CaCl_2{\cdot}2H_2O$, $CoCl_2{\cdot}2H_2O$, $FeSO_4{\cdot}7H_2O$, $MgCl_2{\cdot}6H_2O$, $MnCl_2{\cdot}4H_2O$, $ZnCl_2{\cdot}7H_2O$, $FeCl_3{\cdot}6H_2O$, and $AlCl_3{\cdot}6H_2O$ in distilled water [2]. Equal volumes (0.5 mL each) of 100-mM salt solutions (Ca^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, and Al^{2+}) and 10 mM sodium phytate were mixed and incubated overnight at 4 °C. The precipitated salts were centrifuged at 10,000×*g* at 4 °C for 5 min. The supernatant was decanted off and the precipitated salts were washed thrice with 50 mM Na-acetate buffer (pH5.0) and finally resuspended in 0.5 mL of the same buffer. Phytase assays were initiated by incubating 0.5 mL of phytate salts with 0.5 mL of enzyme (10 U mL^{-1}) at 26 and 60 °C. Aliquots of the mixture were taken at desired intervals. The salts were pelleted by centrifugation and the amount of inorganic phosphate in the supernatant was determined. The substrate and enzyme controls were also run simultaneously and values were deducted from the test run.

For assessing the effects of organic acids on the hydrolysis of insoluble phytate salts, the precipitated phytate salts were suspended in 0.5 mL phytase (5 U mL^{-1}) and 0.5 mL of organic acid (4 mM of citrate, malate, and oxalate) was then added to each tube. The mixtures were incubated at 60 °C for 12 h with gentle shaking [2], and the amounts of inorganic phosphate liberated were determined. The controls were also run simultaneously and values were deducted from the test run.

Effect of the Mold and Its Phytase on Plant Growth

The sterilized wheat (*Triticum aestivum* L.) seeds were germinated on one tenth MS agar medium [21] and the seedlings (3–4 cm) were transferred to one tenth MS broth (30 mL) containing sodium phytate (10 mg) as phosphorus source in 100-mL culture tubes. Phytase (10 U) and fungal spores were added to the tubes along with the control (with inorganic phosphate replacing sodium phytate). The tubes were incubated at 26±1 °C and a 16-h photoperiod in cool white fluorescent light (3.2 klx or 40 μmol $m^{-2}s^{-1}$) for 20 days. The effect of different concentrations of sodium phytate on plant growth was evaluated in liquid cultures. After 20 days of incubation, the seedlings were monitored for growth and inorganic phosphate content of the plants. The effect of different enzyme doses on plant growth was also assessed in a similar manner.

Wheat Straw Compost and Its Utilization for Plant Growth

Wheat straw was ground in a grinder and moistened with distilled water, placed in enamel-coated metallic trays (45×30×7.5 cm) and autoclaved at 15 lb psi for 30 min, inoculated with conidiospores of *S. thermophile*, and incubated at 45 °C for 10 days. In other sets, native microflora of wheat straw alone or along with *S. thermophile* was used for composting. The decomposed wheat straw was mixed with soil vermiculite (1:1 ratio) and inoculated with surface-sterilized seeds of wheat and incubated for 30 days. A set of plants was harvested from each set of experiments and analyzed for plant growth parameters. The soil vermiculite supplemented with one tenth MS medium was used as control for plant growth.

Each experiment was conducted in six replicates and the average values are presented.

Results

Hydrolysis of Insoluble Phytates

Phytase of *S. thermophile* was able to hydrolyze various insoluble phytates to a varied extent. The hydrolysis rate of insoluble phytates was higher at 60 °C (Fig. 1a) than that at 26 °C (Fig. 1b). It released inorganic phosphate from Ca^{+2}, Mg^{+2}, and Co^{+2} phytates more efficiently than those of Al^{3+}, Fe^{2+}, Fe^{3+}, and Zn^{2+}. There was a gradual increase in inorganic phosphate with incubation time. The effect of organic acids on the hydrolysis of these insoluble phytates was also investigated. Among all the organic acids tested, citrate was more effective than others in solubilizing insoluble phytate salts with phytase (Fig. 2).

Effect of Fungus and Phytase on Plant Growth Promotion

When the surface-sterilized seeds were germinated on one tenth MS agar and the seedlings (3–4 cm) were transferred to one tenth MS broth (30 mL) containing sodium phytate as phosphorus source, both the fungus as well as the enzyme promoted the growth of the wheat seedlings (Fig. 3a, b). The growth and inorganic phosphate content of the plants were better than the control.

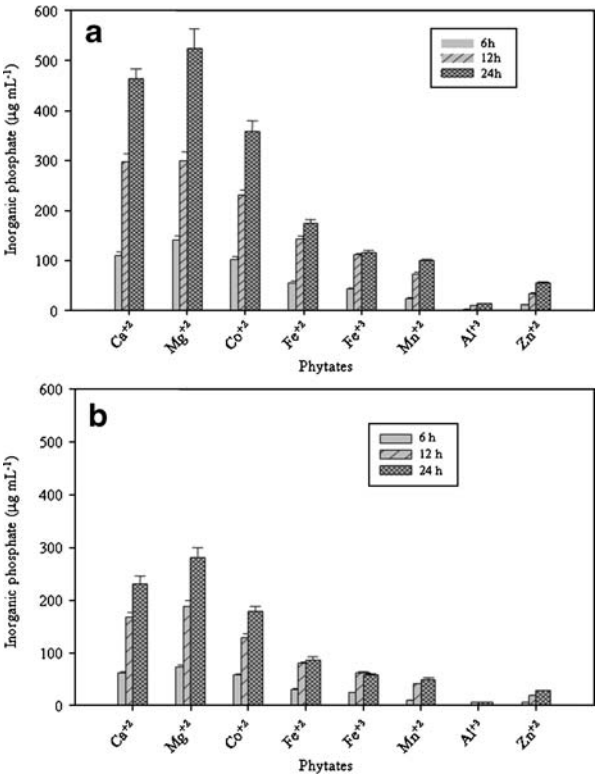

Fig. 1 **a** Effect of phytase (5 U) on the hydrolysis of insoluble phytates at 60 °C over a period of time. **b** Effect of phytase (5 U) on the hydrolysis of insoluble phytates at 26 °C over a period of time

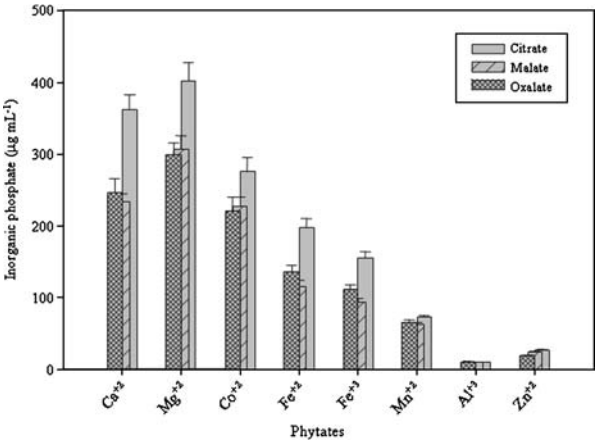

Fig. 2 Effect of organic acids (2 mM) on the hydrolysis of insoluble phytates by the phytase (5 U) of *S. thermophile* at 60 °C after 12 h of incubation

The effect of different concentrations of sodium phytate was assessed in liquid cultures. Sodium phytate (5 mg per plant) was adequate for liberating enough phosphorus for the growth of the seedlings (Fig. 4a, b). The plant growth, root/shoot length, and inorganic phosphate content of test plants were better than the control plants.

An enzyme dose of 20 U per plant was found adequate to liberate enough amount of inorganic phosphate required for supporting plant growth (Fig. 5a–c, Fig. 6). The plant

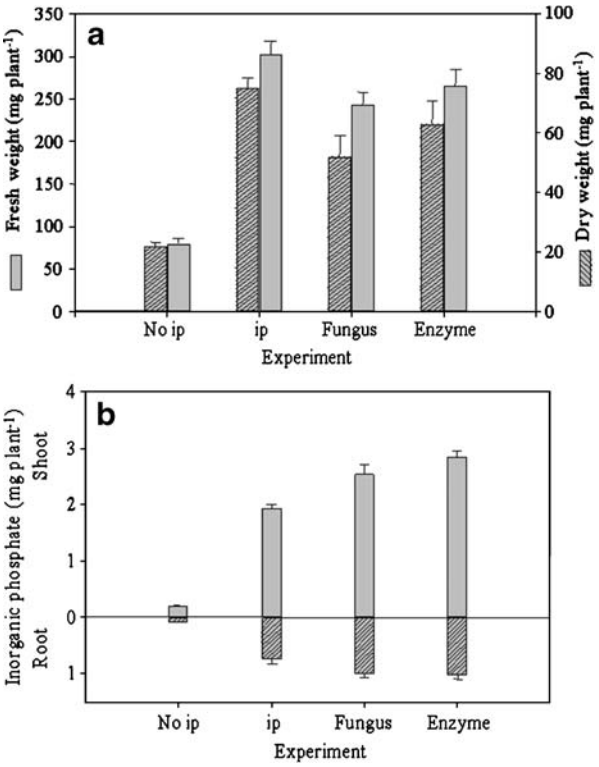

Fig. 3 Effect of the fungus and its phytase on plant growth (**a**) fresh/dry weight and (b) inorganic phosphate content of wheat seedling (*ip*=inorganic phosphate, *No ip*=without inorganic phosphate)

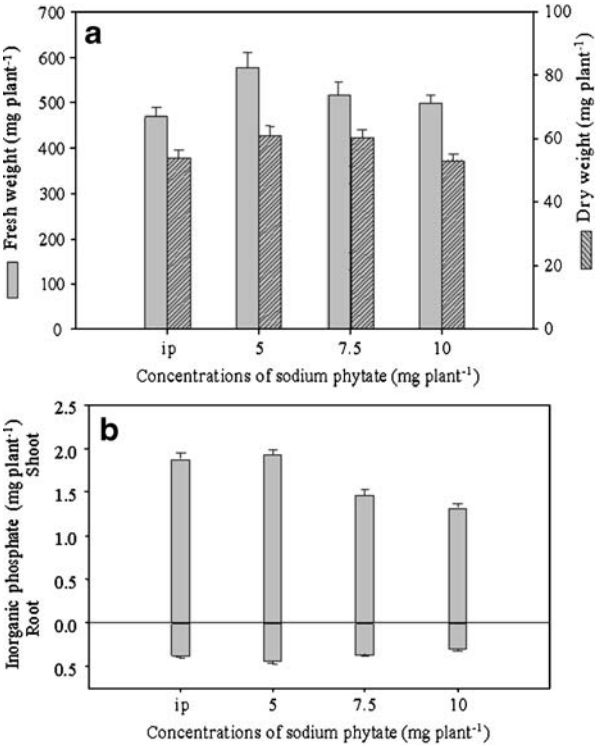

Fig. 4 Effect of different concentrations of sodium phytate on plant growth (**a**) fresh/dry weight and (**b**) inorganic phosphate content of wheat plant (*ip*=inorganic phosphate)

growth, root/shoot length, and inorganic phosphate content of test plants were higher than the control.

Wheat Straw Composting and Its Utilization for Plant Growth

The compost prepared by the combined action of native microflora of wheat straw and *S. thermophile* promoted the growth of plants. The inorganic phosphate content of the wheat plants was also high as compared to those cultivated on the compost prepared either with only native microflora or *S. thermophile* (Table 1). The difference in the plant-growth-promoting effect was clear after 10 days, and it further became prominent after 30 days.

Discussion

Phytase of *S. thermophile* was able to hydrolyze various insoluble phytates efficiently. The hydrolysis rate of these salts was higher at 60 °C than that at 26 °C; this is due to the fact that the mold phytase is optimally active at 60 °C [17]. Tang et al. [2] observed similar trend for the hydrolysis of insoluble phytate salts by fungal, bacterial, and wheat phytases. The phosphate liberation reported by Tang et al. [2] was, however, lower than that with *S. thermophile* phytase.

Among all the organic acids tested, citrate was more effective in enhancing enzymatic solubilization of phytate salts, as reported by Tang et al. [2]. Organic acids achieve this by either a direct exchange with inorganic phosphate adsorbed to soil constituents or by a

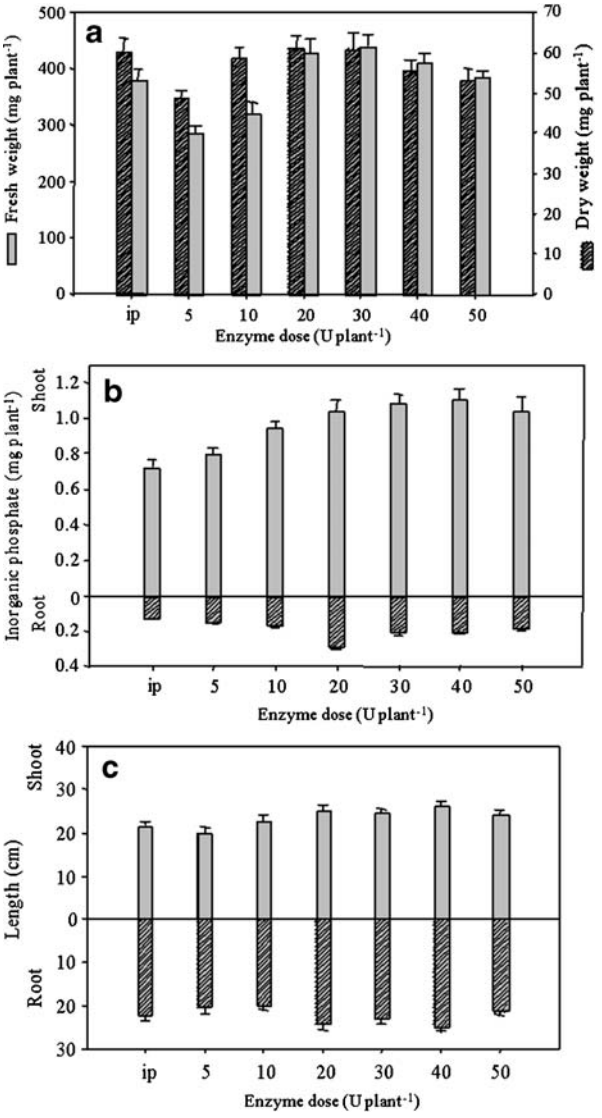

Fig. 5 Effect of different doses of enzyme on plant growth, **a** fresh/dry weight of plants, (**b**) inorganic phosphate content of plants, and **c** root/shoot length of the wheat plants (*ip*=inorganic phosphate)

chelation of metal ions in cation–Pi complexes [2]. The main mechanism for desorbing free phytate could be a metal complexation by these acids because, as compared to malate and oxalate, which carry two carboxyl groups, citrate carries three carboxyl groups and, hence, has a higher capacity to complex cations [2]. For example, at pH5.0, oxalate and malate mainly carry two negative charges, while the ratio of citrate^{2-}–citrate^{3-} is approximately 1:1.

The fresh and dry weight of the plants was higher in the test tubes supplemented with phytase and fungal spores. The inorganic phosphate content of the root and shoot was also improved by the phytase. The compost prepared by the combined action of native microflora of the wheat straw and *S. thermophile* enhanced growth and inorganic phosphate content of the plants as compared to those with the compost made using them separately. The effect was also higher when *S. thermophile* was used alone in comparison with that of

Fig. 6 Photograph showing the treated and untreated wheat plants. **a** Plant grown in normal MS broth without inorganic phosphate, **b** plant grown in normal MS broth with inorganic phosphate, **c** plant grown in normal MS broth with sodium phytate as phosphorus source supplemented with enzyme, **d** Plant grown in normal MS broth with sodium phytate as phosphorus source inoculated with mold

native microflora of wheat straw and control. *S. thermophile* is a good decomposing organism secreting an array of different enzymes [10–13, 22]. There are a few reports on the plant-growth-promoting effect of phytases and phytase-producing microbes, but this is the first report on the effect of phytase of a thermophilic mold on plant growth. The ability of soil microorganisms to solubilize various forms of organic phosphorus is well documented [20, 23, 24], as the plants are not able to utilize phosphorus directly from the organic phosphorus sources particularly *myo*-inositol phosphates. The importance of soil microorganisms for increasing the availability of phosphorus from phytates to plant roots has been suggested by Tarafdar and Marschner [20], who showed that the phosphorus

Table 1 Effect of wheat straw compost on the growth of wheat seedlings.

Time	Composting	Plant weight (mg±SD)		Plant	
(day)		Fresh weight	Dry weight	Length (cm±SD)	Inorganic phosphate (μg/plant±SD)
10	Control[a]	152.4±8.4	24.6±1.0	26.3±1.4	561.2±30.4
	WS+NM	140.0±4.8	20.2±1.4	21.4±1.1	656.5±40.8
	WS+NM+ST	146.0±6.0	29.6±1.8	26.9±2.0	763.8±52.0
	WS+ST	139.0±5.9	23.1±1.1	27.6±1.7	721.4±39.8
20	Control	332.4±18.2	46.8±2.7	46.1±2.8	1,163.8±110.4
	WS+NM	286.6±20.5	39.2±1.9	48.8±3.2	997.6±69.9
	WS+NM+ST	386.3±22.0	48.6±2.4	65.1±5.6	1,516.8±138.0
	WS+ST	329.0±17.9	43.6±3.3	62.1±4.3	1,382.1±121.0
30	Control	405.4±30.8	62.6±4.0	57.4±2.9	1,499.5±109.8
	WS+NM	384.0±21.7	47.3±2.9	62.6±5.0	1,367.2±120.0
	WS+NM+ST	592.8±32.1	98.2±7.6	89.4±6.9	2,116.0±201.6
	WS+ST	569.0±20.7	83.1±4.4	81.2±4.7	1,888.6±165.9

WS wheat straw, *NM* native microflora of wheat straw, *ST S. thermophile*

[a] One tenth MS medium

nutrition of wheat grown in soil supplied with phytate increased when the plant was co-inoculated with a mycorrhizal fungus, *Glomus mosseae* and *Aspergillus fumigatus* (a phytase producer). Phytase- and phosphatase-producing fungi were used as seed inoculants to attain a high P nutrition of plants in the soils containing high phytate phosphorus [24]. Transgenic *Arabidopsis* plant expressing MtPHY1, a full-length cDNA encoding an extracellular phytase from a legume *Medicago truncatula*, led to significant improvement in organic phosphorus utilization and plant growth [25]. When phytate was supplied as the sole source of phosphorus, dry weight of the transgenic *Arabidopsis* lines were 3.1-fold to fourfold higher than the control plants and total phosphorus contents were 4.1- to 5.5-fold higher than the control, suggesting the great potential of phytase for improving plant phosphorus acquisition and for phytoremediation. The growth and phosphorus nutrition of *Arabidopsis thaliana* plants supplied with phytate was improved significantly after the introduction of phytase gene from *Aspergillus niger* [23]. Growth and phosphorus nutrition of the transformed plants was ameliorated, and it was equivalent to control plants supplied with inorganic phosphate, suggesting the phytase activity of plant roots as a significant factor in the utilization of phosphorus from phytate and opportunity for using gene technology to improve the ability of plants to utilize accumulated forms of soil organic phosphorus. Similarly, Richardson et al. [23] had also highlighted the potential role of soil microorganisms for increasing the availability of phosphorus from phytates through phytase action. Idriss et al. [26] investigated the effect of a phytase-producing *Bacillus amyloliquefaciens* on the growth of maize seedlings. Both phytase and the bacterium promoted the growth of maize seedlings; however, in contrast, no such plant-growth-promoting effect was seen with the culture filtrate of the phytase-less mutant. Greenhouse study and field experiments have demonstrated that both wild-type *Bacillus mucilaginosus* and its transgenic strain (containing phytase gene) promoted the tobacco plant growth [27]. Furthermore, the transgenic strain promoted plant growth better than the wild-type and control plants. These approaches can be applied for boosting the productivity in agriculture and horticulture.

Conclusions

The mold and its phytase and the compost made using the mold promoted the growth of wheat seedlings with concomitant increase in their phosphorus content. The mold can be added to soils using compost as a carrier. This investigation has thus confirmed that the phytase of thermophilic molds such as *S. thermophile* can be potential candidates for making phosphorus available from insoluble phytates for plant growth promotion.

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