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Phosphate dissolving fungi: Mechanism and application in alleviation of salt stress in wheat

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ABSTRACT

The present investigation reveals the solubilization efficiency of tri-calcium phosphate (TCP), Udaipur rock phosphate (URP), aluminium phosphate (AP) and ferric phosphate (FP) by *Aspergillus niger* (ITCC 6719) and *Trichoderma harzianum* (ITCC 6721) as function of carbon concentrations. Increasing glucose concentration from 1 to 7% in the growth medium, though improved the phosphorus (P) solubilization significantly but each fungal strain preferred different optimum carbon concentrations for mediating solubilization of different P sources. The two fungi employed different mechanisms to reduce medium pH for release of P from TCP, AP and FP. However, URP was solubilized solely through fungal production of citric, succinic, propionic, malic and acetic acid. A linear increase in citric acid production with increasing carbon concentration was recorded during FP solubilization by *T. harzianum*. The cell free culture filtrate of *A. niger* detected high phytase and low acid phosphatase activity titre whereas results were vice versa for *T. harzianum*. Both the fungal strains possessed plant growth promoting attributes such as auxin and sidreophore production and could solubilize Zn. In hydroponic system (with 60 mM of sodium chloride concentration), supplementation with culture filtrate from each fungal strain increased the shoot growth of wheat seedlings significantly compared to non culture filtrate control. Use of *A.niger* as bio-inoculant could be a sustainable approach to improve soil P availability, promote plant growth and alleviate adverse effect of salt stress.

1. Introduction

Phosphorus (P) in soil exists in various organic and inorganic fractions and their content varies with the soil type (Satyavir et al., 2014). The concentration of organic P in organic matter rich soils may be as high as 40–80% of total soil P (Tsai and Rosseto, 1992) with phytate P as the most predominant and recalcitrant form of organic P (Turner et al., 2003). Direct utilization of phytate P by plant roots is very poor (Unno et al., 2005) and adsorption of phytate ions to soil particles as well as their precipitation as calcium, iron and aluminium phytate, makes them resilient to enzymatic hydrolysis (Tang et al., 2006). On the other hand, the high fixation/sorption capacity of Indian soils reduces the fertilization effectiveness of added chemical P. The poor availability of phytate-P/mineral-P in soil highlights the need for recycling of both organic and inorganic P pool of soil. Considering the imminent world phosphate rock scarcity scenario, a rational management of P and a global shift towards a sustainable agricultural production system with low reliance on chemical fertilizers need to be adopted.

Microbial exploitation of soil P is one of the promising, environment-friendly strategies that can improve the P nutrition in a sustainable manner (Gaind, 2013). Phosphorus availability from inorganic P sources can be enhanced by phosphate solubilizing microorganisms (PSM) and from organic P by phosphate mineralizing microorganisms. Till date, the research has mainly been focused on screening and selection of PSM based on their ability to solubilize tricalcium phosphate, a chemical form of inorganic P found in alkaline soils. The use of microorganism for organic P recycling is the less explored area. Ideally, for developing microphos inoculants, phosphate dissolving microorganisms should be screened for solubilization of different inorganic P sources (Bashan et al., 2013) and mineralization of organic P sources. To harness the maximum benefit of inoculation under different ecological zones, there is need to develop the bio-inoculant using a microbial strain with multifunctional attributes such as phosphate solubilization and mineralization, zinc solubilization, phytohormones productions, bio-control properties etc. in a single strain (Jorquera et al., 2008).

Fungi have been reported to be more efficient in release of P from insoluble inorganic compounds than bacteria (Khan et al., 2010). Moreover, their ability to withstand biotic and abiotic stress under soil niche makes them the potential candidate for developing

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microphos inoculant. Although, secretion of organic acid is considered as the main mechanisms of inorganic phosphate solubilization but this may not imply to all the phosphate solubilizing fungi. Fungi show selectivity on the transformation of different P forms and the organic acids may or may not be secreted under different conditions. The nature of organic acid produced is a specific characteristic of each fungal isolate and may vary under the same cultivation conditions. Thus, considerable differences may exist among different fungal strains for solubilizing a particular P source (Toro et al., 1997). A complete insight into the fungal P solubilization mechanisms of commonly occurring insoluble P sources in soil is essential to establish new possibilities for fungal application in processes aimed at enhancing P availability and facilitate plant growth in soil. Moreover, the exogenous application of plant growth hormone such as gibberline, auxin and cytokinin has been found beneficial for alleviating the adverse effect of abiotic stress such as salinity in vegetable crops (Khan et al., 2004). The positive effect of auxin on wheat seed germination has been studied by Egamberdieva (2009). Whether the phosphate solubilization and endogenous production of auxin by a fungal strain contribute in alleviating the deleterious effects of salt stress needs to be investigated. Thus, the objectives of present study included (i) evaluation of two fungal strains for their potential to solubilize insoluble inorganic phosphates such as tri calcium phosphate (TCP), Udaipur rock phosphate (URP), ferric phosphate (FP) and aluminium phosphate (AP) and mineralize organic P (ii) studying the effect of carbon concentration on fungal release of P from different inorganic P sources (iii) to determine the mechanisms involved in solubilization of organic and inorganic P salts (iv) to evaluate the fungal strains for assay of phytase, phosphatase, cellulase and amylase activities, their plant growth promoting attributes such as indole acetic acid, ammonia, HCN and siderophore production and solubilization of potassium and zinc and finally (v) to evaluate the role of fungal solubilization of phosphate and endogenous production of indole acetic acid (IAA) in improving wheat plant growth under salt stress.

2. Materials and methods

2.1. Fungal strains

Aspergillus niger (ITCC 6719) and *Trichoderma harzianum* (ITCC 6721) used in the study were isolated from the rhizosphrere of soybean (*Glycine max.*) and compost respectively (Gaind and Nain, 2015). The fungal cultures were maintained on potato dextrose agar slants at 4°C with periodic transfer.

2.2. Growth conditions for inorganic phosphate solubilization

40 ml Pikovskaya's (1948) broth with TCP, URP, FP and AP each added separately at 5 g l^{-1} (w/v) was prepared and dispensed in 100 ml Erlenmeyer's flasks. The pH of the medium was adjusted to 7.0, before the addition of P source. The flasks were autoclaved and after cooling, the contents were inoculated separately with 0.1 ml spore suspension of *Aspergillus niger* (F1) and *Trichoderma harzianum* (F4). All the experiments were conducted in triplicate. The flasks were incubated for 15 d at 30°C under stationary conditions. Un-inoculated flasks with each P source served as their respective control. The sample (250 μl) drawn from inoculated flask of each treatment (under aseptic conditions), at periodic interval of 4,7,11 and 15 d was centrifuged at 8000 rpm for 2 min and used for estimation of soluble P by ascorbic acid method (Murphy and Riley, 1962). The fungal P solubilization efficiency (μg P ml^{-1}) was calculated as the difference for soluble P between treatment and its control. Similar experiment was conducted by varying the glucose concentration from 1 to 7% (w/v) in Pikovskaya's broth with each P source added separately (in triplicate) and inoculated with individual fungal strain to optimize the carbon concentration for maximum solubilization of different P sources. After 15 d incubation, the contents of inoculated flasks were filtered through pre-weighed Whatman No. 42 filter paper. The fungal biomass was estimated gravimetrically by drying the same at 70°C for 48 h. The filtrate was used for estimation of soluble P by the method of Murphy and Riley (1962). The changes in filtrate pH were determined by digital pH meter and organic acids were detected by high performance liquid chromatography (HPLC).

2.3. Organic acids detection by HPLC

To detect the fungal production of organic acids in the filtrates with different carbon concentrations and P sources, 2 ml of respective culture filtrate was passed through 0.22 μm pore size syringe filter (Hi-Media). The organic acids in the filtrate were analyzed by HPLC using Aminex column HPX-87- H and a UV detector set at 210 nm at 50°C. The solvent as mobile phase consisted of 5 mM H_2SO_4 run at a flow rate of 0.6 ml min^{-1}. Peaks were identified against a set of standards from known organic acids which were citric, succinic, formic, propionic, fumaric, oxalic, lactic and acetic acids and quantitative estimation of the produced acids carried out.

2.4. Solubilization of different inorganic phosphates by pure organic acids

Weighed quantities of various phosphates such as TCP, URP, FP and AP equivalent to 25 mg P_2O_5 were taken in 100 ml Erlenmeyer flask. To each flask 50 mg of succinic, formic, citric, malic, acetic and propionic acid was added separately along with 25 ml distilled water. All the treatments were duplicated, keeping control (without acid). The flasks were incubated at 30°C. The contents were filtered after 24 h and amount of P solublized by individual acid was estimated in the filtrate by taking a known amount of aliquot (Murphy and Riley, 1962). The results were expressed as % P solubilized.

2.5. Activity assay of phytase and acid phosphatase

Mineralization of P was evaluated in 100 ml Erlenmeyer's flasks containing 40 ml Czapek's Dox broth per fungal culture in triplicate. The flask contents were autoclaved, cooled and inoculated with 0.1 ml spore suspension of 5 days old broth culture of respective fungal isolate (F1 and F4) separately. The autoclaved, un-inoculated controls were also included for comparison. All the flasks were incubated at 30°C. After 3, 7, 10 and 14 d incubation, the flask contents were filtered through a previously weighed Whatman No.42 filter paper. The fungal biomass collected on the pre-weighed filter paper of respective flasks was measured gravimetrically after drying the samples at 70°C for 48 h. The filtrates were used for assay of extra cellular phytase (Engelen et al., 2001) and acid phosphatase. The assay of phytase, activity was performed at 37 °C for 65 min, using 50 μl of culture filtrate incubated with 100 μl phytate solution (prepared in 0.4 M sodium acetate buffer of pH 5.6 containing 2 mM $CaCl_2$). Light absorbance was measured at 415 nm with UV–vis Thermo Fischer Scientific Spectro-photometer. One enzyme unit (EU) of phytase was expressed as 1 μ mol inorganic P (Pi) liberated min^{-1} under the assay conditions.

The quantitative assay of extracellular acid phosphatase in cell free culture filtrate was carried out using acetate buffer (pH 5.4), substrate, *p*-nitrophenyl phosphate (*p*NPP) and 0.5 ml culture filtrate. The reaction mixture was incubated at 37°C for one h and the enzyme activity measured at 400 nm with UV–vis Thermo Fischer Scientific Spectro-photometer (Tabatabai and Bremner, 1969). The