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Inorganic Phosphate Solubilizing Potential of *Arthrobotrys Conoides* and *Duddingtonia Flagrans*, A Nematode Trapping Fungi A Potential Biocontrol Agent

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Abstract Insufficient nutrient and pest infection are two major predicaments for low crop yield in agriculture. Nematodes, a most infectious parasite pest in plants responsible for major yield loss. Chemical phosphate fertilizer made large reserves of it in soil, but part of it is made available to the plants because of irreversible fixation of it with divalent cation. In the present study, *Arthrobotrys conoides* (JX979095) and *Duddingtonia flagrans* (JX979096) a nematode trapping fungi were isolated from the arable soil of Anand district and studied for its phosphate solubilization potential. Among the different P sources, bioleaching of Tricalcium Phosphate (TCP) was found to be more efficient compared to other P salts. Phosphate solubilizing ability was enhanced in presence of D-glucose as compared to other C sources. In case of nitrogen source ammonium sulfate at 0.5 % showed best P solubilization followed by casein, urea and sodium nitrate respectively. *Duddingtonia flagrans* was showing maximum P solubilization 12.92% in compare to *Arthrobotrys conoides* which showing 10.32% of 0.5% applied. Fungi showed citric acid and malic acid peak in HPLC profile against standard which may be responsible for the fall in pH and subsequently P solubilization. Thus this study demonstrates that the isolated nematophagous fungi can have dual role, which is as biocontrol of plant parasitic nematodes as well as biofrtilizer when applied in soil. Application of such type of fungi in to field can enhance the soil fertility crop production.

Keywords: *Arthrobotrys conoides*, biocontrol, biofrtilizer, *Duddingtonia flagrans*, nematophagous fungi.

Introduction

Phosphorus is an essential nutrient both as a part of several key plant structure compounds as well as a catalyst in the conversion of numerous key biochemical reactions. It is the major nutrient after nitrogen (N) that limits plant

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growth and unlike the later, there is no large atmospheric source for this element that can be made biologically available (Gyaneshwar *et al*., 2002; Ezawa *et al*., 2002). The availability of phosphorus in the soil is somewhat limited which explains the need for application of soluble fertilizers for adequate plant growth (Vassilev *et al*., 2001). Soluble phosphorus, either from fertilizer or natural weathering reacts with clay, iron, and aluminium compounds in the soil and is converted readily to less available forms by the process of phosphorus fixation. Phosphorus is fixed as insoluble iron and aluminium phosphates in acidic soils or as calcium phosphates in alkaline soils (Tunesi *et al*., 1991; Lopez and Garcia, 2001; Zhang *et al*., 2001). Because of the spiraling cost of phosphatic fertilizers coupled with low recovery (10- 30%) of phosphorous applied in the field, the developing tropical countries are attempting to utilize their indigenous reactive ground phosphate rock as a cheap alternative (Sabannavar and Lakshman, 2009). Many soil bacteria, *Pseudomonas, Azospirillum, Bacillus, Rhizobium, Burkholderia, Arthrobacter, Alcaligenes, Serratia, Enterobacter, Acinetobacter, Flavobacterium and Erwinia* and fungi especially *Aspergillus, Penicillium, Trichoderma* have the ability to solubilize elemental phosphate (Pi) and make it available to plants (Oberson *et al*., 2001; Engamberdiyeva *et al*., 2003, Rodriguez *et al*., 1996).

Phosphate solubilization ability of the microorganisms is considered to be one of the most significant features associated with plant phosphate nutrition. Many researchers have reported that phosphate solubilizing microorganisms are very important as soil inoculums to improve the plant growth and yield (Young, 1994; Young *et al*., 1998; Goldstein *et al*., 1999; Fasim *et al*., 2003).

Fungi are reported to solubilize phosphate by producing organic acids and are known to have a higher efficiency of solubilization than bacteria. Several mechanisms like lowering of pH by acid production, ion chelation and exchange reactions in the growth environment have been reported to play key roles in this phenomenon by the phosphate solubilizing microorganisms (Halder *et al*., 1991; Abd Alla, 1994; Whitelaw *et al*., 1991). There are several evidences showing that the solubilisation of soil phosphates in many cases is due to the excretion of organic acids (Stevenson, 1967; Goldstein, 1995; Kim *et al*., 1997).

Nematophagous fungi are predacious microbes that can capture, trap and digest nematodes (Nordbring *et al*., 2006). Nematophagous fungi have been the subject of intense research over several decades which include fundamental studies on their ecology, distribution and systematics as well as their potential as biological control agents against nematode pathogens of plants and animals (Li *et al*., 2000; Liu and Zhang, 2003; Dong *et al*., 2004; Mo *et al*., 2005; Zhao *et al*., 2005).

Many researchers have studied several nematophagous fungi have been characterized for their ability to trap and digest plant parasitic nematodes (Tunlid and Jansson, 1991; Siddiqui and Mahmood, 1996; Xiang *et al*., 2006; Yang *et al*., 2007; Sharma and Pandey, 2009). However their phosphate solubilisation potential still awaits intense exploration. Given the fact that the point of action of nematophagous fungi is on the field when used as a biological control agent, evaluation of phosphate solubilization potential of this microbe will assist in utilizing it for release of vital minerals in the soil that support plant growth thus leading to exploitation of both the economically important features, *viz*., mineral mobilization and biological control, of this versatile microbe.

In the present study, we explored the ability of our nematophagous fungi isolates *Arthrobotrys conoides* and *Duddingtonia flagrans* for solubilising inorganic phosphate and in the process identified the organic acids secreted by it through HPLC based analysis.

Materials and methods

Microorganism and its culture and preservation

Arthrobotrys conoides (JX979095) and *Duddingtonia flagrans* (JX979096) used in the present work were originally isolated from the ecological niche of Anand, Gujarat (Pandit *et al*., 2014) and minted on Corn Meal agar (CMA; Hi-Media, 1.7%W/V) by inoculation and incubation at 28 ± 1 ℃ for 7 days and preserved at 4 ℃ until use. The isolate was sub cultured every 15 days following establishing microbiological procedure.

Phosphate solubilization study

Isolates were checked for its phosphate solubilizing ability on PVK agar (Pikovskaya, 1948) (Hi-Media) pH 7. Eight mm-diameter mycelial growth from a 7 days old culture grown on CMA was placed in centre on PVK agar plate in triplicate duly amended with 0.5% Tricalcium Phosphate (TCP) and incubated at 28 ± 1 ℃ for 4 days prior to observation for detecting the characteristic halo zone.

Phosphate solubilization study in different liquid media

Phosphorus solubilizing ability of isolates was tested in four different liquid media *viz*., PVK, AYG (Young, 1994), NBRIP and NBRIY medium (Nautiyal, 1999) to identify the one that is most suitable for phosphate

solubilization (Srividya *et al*., 2009). The pH of all media was adjusted to 6.5 with 1N HCl. Conidio and chlamydospores from 15 days old CMA plates were harvested by adding 5 ml sterile distilled water. Two fifty ml flasks in triplicate, each containing 70 ml PVK broth and amended 0.5% TCP were inoculated with 2 ml of the spore suspension and incubated on a rotary shaker at128 rpm at 28_ ± 1 ℃ for 7 days. Uninoculated medium containing the same amount of TCP served as control.

Solubilization of different phosphate sources

Tricalcium phosphate in the PVK broth was replaced by different phosphate salts which included Rock phosphate (RP), Zink phosphate (ZNP) and Aluminium phosphate (ALP) in order to study solubilization efficacy of different P sources by isolates.

Effect of different carbon and nitrogen sources on phosphate solubilization

One percent glucose as carbon source in the PVK broth was replaced with same amount of sucrose, lactose, maltose and galactose for studying the effect of different carbon sources on phosphate solubilization by the isolates. Stock solutions of each sugar was prepared in distilled water, sterilized separately and added to the medium to get required final concentration. Similarly, for determining effect of different nitrogen sources on phosphate solubilization, 0.5% of different nitrogenous compounds which included casein, peptone, urea and sodium nitrate respectively were added to the PVK medium *in lieu* of 0.5% ammonium sulphate.

Estimation of solubilized phosphorus

Solubilized P was estimated by Vanado-molybdate method as described in APHA 1995, from the culture supernatant centrifuged at 10000rpm for 15 min. Change in pH was estimated using pH meter equipped with glass electrode. Uninoculated flasks were used as control. This procedure was followed from day 3 to 7. All the experiments were performed in triplicate and mean values were expressed in terms of percent (%) solubilization of phosphate for an application of 0.5% of the source (w/v) at pre inoculation stage.

Identification of organic acids secreted by the fungal isolate

Organic acid secreted by the isolates were identified through HPLC based analysis from the 7 days old culture supernatant filtered through a 0.45 µm pore

size using a 4 mm diameter micro filter syringe (Millipore Inc., USA). 100 µl of this filtrate was injected into a C18 EXIL and Wakosil II 5C18 columns (PERKIN ELMER series-200). The mobile phase comprised of a mixture of water: methanol: TFA at the ratio of 15:85:0.1, working at a constant flow rate of 0.5 ml/min at 28 ℃. The unknown organic acids were determined by comparing the retention times on chromatograms with oxalic, citric, acetic, malic and succinic acid as standards.

Results

In the present study we, in vitro demonstrated phosphate solubilization potential of two Indian isolates of nematophagous fungi which can trap and digest plant parasitic nematodes (Pandit *et al*., 2014). When grown on PVK agar supplemented with 0.5% TCP, characteristic halo zone was observed after 4 days of incubation confirming the property of phosphate solubilization. This halo zone was formed may be due to secretion of organic acids by the fungi which lower the pH of the media, subsequently solubilizing tricalcium phosphate.

Maximum phosphate solubilization was observed in PVK medium 12.85+/- 0.104, 10.25 % +/- 0.154, followed by NBRIY 11.56 +/- 0.141, 9.98% +/- 0.14,) and NBRIP 10.96 +/- 0.23, 0.121, 9.86% +/- 0.216 and AYG medium 11.36 +/- 0.113, 9.45% +/- 0.148 for *D. flagrans* and *A. conoides* respectively (Figure 1). Phosphate solubilization is executed as a result of organic acid secretion and reduction of pH of the medium. Figure 2 shows that there was a fall in pH from 6.5 to 3.0 for *D. flagrans* and 3.2 for *A. conoides* after 7 days of growth in TCP-supplemented PVK medium. Positive correlation was clearly found between phosphate solubilization and corresponding fall in pH of the medium. This observation is in line with results of several researchers who observed that fungi solubilizing phosphate through secretion of organic acids those results in sharp fall in pH value of the medium (Rashid *et al*., 2004).

Four different phosphate-salt, *viz*., tricalcium phosphate (TCP), zinc phosphate (ZNP), aluminium phosphate (ALP) and rock phosphate (RP) were evaluated in this study. It was found that TCP was best solubilized by the isolates that are 10.32% +/- 0.191 by *A. conoides* and 12.76 +/- 0.134 by *D. flagrans* followed by ALP and ZNP. RP was found to get marginally solubilised amongst the four phosphate salts 3.25 +/- 0.168 % by *A. conoides* and 5.75+/- 0.195 by *D. flagrans* (Figure 3).

The capability of different carbon and nitrogen sources to compliment phosphate solubilization by isolates was also evaluated. Among different carbon sources that were used, glucose as carbon source was found to solubilize maximum amount of tricalcium phosphate (Figure 4). Among the different

nitrogen sources surprisingly, urea was found to be a poor source of nitrogen for the isolates with the lowest degree of phosphate solubilization. A non synthetic organic source of nitrogen, *viz*., casein and peptone were used and the later was found to solubilize more TCP by all the isolates as compared to casein. Ammonium sulphate as nitrogen source supported maximum phosphate solubilization (Figure 5).

In the present study, we analyzed the spent culture broth using HPLC to identify organic acids that were secreted by the fungi during the process of phosphate solubilization. Secreted organic acids were identified through comparison of retention time of known organic acids which were used as standards. HPLC results indicated that the organism predominantly secreted citric acid and malic acid as two major organic acid components into the media (Figure 6).

Discussion

Nematophagous fungi have been the subject of intense research in India. However, species of *Arthrobotrys* and *Duddingtonia flagrans* received special attention for their potential as agent for controlling plant and animal gastrointestinal nematodes respectively.

Antagonistic potential of the plant biological control agent *Trichoderma sp.* against *A. oligospora* was estimated to be a high of > 80 % indicating possible, decreased efficacy of the later in presence of the former in the field (Mukhopadhyaya *et al*., 2001a). The influence of various physical and chemical agents on growth of different *A. oligospora* isolates were also studied (Mukhopadhyaya *et al*., 2001b), Nagee and co workers reported successful isolation of several nematophagous fungi from the ecological niches of the state of Gujarat (Nagee *et al*., 2001). Chauhan and co workers reported the isolation of *A. musiformis*, a chlamydospore-bearing *Arthrobotrys sp.* and demonstrated its ability to trap live nematodes (Chauhan *et al*., 2002). In the year 2001, Sanyal and Mukhopadhyaya studied predatory activity of *Duddingtonia Flagrans* against Caprine parasitic gastroenteristitis. The same group, in the year 2003 demonstrated the environmental impact of use of *Duddingtonia flagrans* in the field playing the role of a potential animal biological control agent as well as influence of faecal dispersal time on larval translation of ovine *Haemonchus contortus*. Effective research was conducted to assess the potential of these fungal spores for use as commercial product for biological control of animal parasitic nematodes (Sanyal and Mukhopadhyaya, 2003) and implications of fungicidal effects of Benzimidazole compounds on *Duddingtonia flagrans* were also evaluated (Sanyal *et al*., 2004).

In an interesting study, Nagee and Mukhopadhyaya demonstrated a method for use of soft-walled conidia as effective tool for controlling animal gastrointestinal nematodes (Nagee *et al*., 2008). The same group undertook molecular study to characterize an expressed sequence Tag (EST) corresponding to the cuticle degrading gene serine protease (PII) that was identified from worm-induced *A. oligospora* using the differential display technology.

However, very little attention was paid to explore the potential of phosphate solubilization of these economically important, nematophagous fungal group. Given the fact that point of action of these organisms as animal biological control agent is in the field, evaluating its merits for other field-related positive features is therefore a logical line of research. This prompted us to undertake a preliminary study to understand the phosphate solubilizing capability of these microbes.

PVK medium solubilized TCP greater than other media possibly due to its more balanced composition of glucose, ammonium sulfate, $MgSO_4.H_2O$, $FeSO_4.7H_2O$, yeast extract, NaCl and KCl. Nautiyal in his study found that amount of glucose as a carbon source played an important role in phosphate solubilization (Vassilev *et al*., 2001). Although AYG medium contained greater amount of glucose, it remained deprived of NaCl, KCl and $MnSO_4$. Hence it appears that NaCl, KCl, $FeSO_4.7H_2O$ and $MnSO_4.H_2O$ also played vital role in growth and metabolism of isolates and subsequently in phosphate solubilization. Yeast extract as a media component bears a correlation with phosphate solubilization capability of the isolates. Media which were deprived of yeast extract such as NBRIP and NBRIY solubilized less amount of phosphate compared to PVK and AYG. Further, the same observation was found to be true when solubilization of phosphate in AYG medium was analyzed which contained lesser amount of yeast extract as compared to PVK media. Possible reason for this observation is the non-synthetic composition of yeast extract which is rich in nitrogen, vitamins and other growth stimulating compounds. These are the reasons for identifying PVK as the media of choice for studying *in vitro* phosphate solubilization of these fungi in this study which is in line with studies across the world on microbial phosphate solubilization. It was also seen that NaCl and KCl played important role in phosphate solubilization by this fungal isolate. Since AYG medium containing 1g/L of ammonium sulphate but not NaCl or KCl and demonstrated less solubilization of phosphate compared to NBRIY and PVK media respectively, where both contained NaCl and KCl as two prominent media component.

Variation was also observed in the pH value of PVK media when glucose and sucrose were used as carbon source as compare to lactose, galactose and

maltose (data not shown). This may be due to secretion of the type as well as amount of different organic acids when one or another sugar is utilized by the fungus as the carbon source. In order to study the effect of different nitrogen sources on phosphate solubilization, both synthetic as well as non synthetic form of nitrogen sources were used. Our study on phosphate solubilization in various growth media was in line with this observation when their individual nitrogen source and amount was correlated with this phenomenon.

There are several mechanisms which are involved in phosphate solubilization but secretion of organic acids followed by lowering of pH was found to be the most widely accepted mechanism (Nahas, 1996). Many researchers have identified organic acids such as gluconic (Stephen and Jisha, 2011), citric, gluconic, lactic, succinic and propionic acid (Chen *et al*., 2004) individually or in combination to be responsible for phosphate solubilization (James and Cathy, 1992; Nahas *et al*., 1996). Others have also detected low molecular weight organic acids like acetic, propionic, isobutyric, isovaleric, valeric, isocaproic, lactic, fumaric and succinic acid when characterized through gas chromatographic techniques (Vazquez *et al*., 2000). These two acids may be responsible for lowering the pH of medium and phosphate solubilization by *Arthrobotrys conoides.* (isolate RPAN-12) studied in our program.

Industrialization and green revolution has enhanced productivity of the nation but at the same time has significantly contributed to massive abuse of nature. While extensive use of fertilizer has resulted in better crop production, it has also lead to imbalance of the sensitive ecosystem. This indicates therefore to the need for alternative methods to improve soil health without causing long term damage to soil and environment. This is the primary reason for the recent focus on biofertilizers. *Cyanobacteria, Rhizobium*, endophytic diazotrophs and phosphate solubilizing microorganisms are therefore being used as microbial inoculants and seen as a non toxic, ecologically friendly and harmless ways of improving soil health (Kannaiyan *et al*., 2004). Phosphorus is a major growth-limiting nutrient, and unlike nitrogen, there is no large atmospheric source that can be made biologically available (Ezawa *et al*., 2002). Attempt to look for phosphate solubilizing trait in economically important fungi which are biological control agents with potential for controlling gastrointestinal nematodes with and foci of action being the soil, is therefore natural and logical. Duponnois and co workers in the year 2006 first demonstrated phosphate solubilizing trait of *A. oligospora*. The present study further strengthens this finding and is the first report from India to demonstrate phosphate solubilization feature of an Indian isolate of nematophagous fungus. In this era when cross country transaction of biological material faces

increasing import restrictions, it becomes necessary and important to profile isolates such as ours belonging to genus, that is isolated from the same ecological niche where it can be used on larger scale for dual purpose of biological control as well as phosphate solubilization Biofertilizer.

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