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Research Article
Characterization of Phosphate Solubilising Bacteria Isolated from Rhizosphere Soils of *Piper nigrum* L.

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Abstract

Background and Objective: Phosphorus is an important plant nutrient next to Nitrogen. The amount of phosphorus available for plant growth in the soil is very less due to its insoluble nature. In this study, isolation and evaluation of phosphate solubilising bacteria from the rhizosphere soils of *Piper nigrum* L. grown in two pepper growing locations of Karnataka state of India was carried out. **Materials and Methods:** Rhizosphere soil samples collected from two locations were subjected to serial dilution and plated on Sperber's medium. These isolates were subjected to morphological and biochemical tests evaluation for plant growth promoting property and release of water soluble phosphorus. All experiments were performed in triplicates and data are expressed as Mean±standard deviation. **Results:** A total of eighteen phosphate solubilising bacterial isolates were isolated from the pepper rhizosphere soils samples from two different locations of Karnataka state of India. Isolate T6 released maximum water soluble phosphorus of 82 µg mL^{-1} and compared to un inoculated control, all the treatments involving inoculation of Phosphate solubilising bacteria showed higher plant height. Among all the isolates, six isolates showed positive and twelve isolates showed negative results for gram reaction. All the isolates were positive for catalase and oxidase test and all were negative for urease production and lipase test. All the isolates were rod shaped. **Conclusion:** Phosphate solubilising bacterial isolates isolated from rhizosphere of *P. nigrum* L. exhibited solubilisation of insoluble phosphorus and also plant growth promotional property. They can be used to improve the phosphorus nutrition and growth of black pepper.

Key words: Phosphate solubilising bacteria, rhizosphere, *P. nigrum* L. water soluble phosphorus, bioassay

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The availability of nutrients is one of the factors influencing productivity of a land. Among the major plant nutrients, Phosphorus occupies principal position next to nitrogen with respect to fertilizer amendment. Therefore, phosphatic fertilizers are world's second largest bulk agricultural chemicals and hence second most widely applied chemical on the earth[1]. Phosphorus makes up about 0.05-0.5% of the total plant dry weight[2] which plays key role in the metabolism, growth and development of the plant. As a result, its concentration and availability influences crop yield. Out of large portion of phosphatic fertilizer applied to the soil only small fraction is utilized by the plant and the reaming is transformed into insoluble form by P fixation[3]. The process of P fixation depends on pH and soil type. In acid soils, P fixation is mediated by free oxides and hydroxides of iron and aluminium, while in case of alkaline soil, it is fixed by calcium[4]. Some microorganisms have the ability to dissolve the insoluble form of phosphorus and release soluble forms (such as HPO_4^- and $H_2PO_4^-$). The process of conversion of insoluble inorganic phosphate into soluble form is called mineral phosphate solubilisation and the organisms are called phosphate solubilising microorganisms[5]. These organisms are also reported to secrete plant growth hormones like auxins and gibberellins which causes growth promotion, flower induction and fruit setting[6,7]. The use of PSM as a biofertilizer is becoming popular because, they reduce the requirement of chemical phosphatic fertilizer thereby reducing cultivation cost and also decreasing environmental pollution.

Black pepper (*Piper nigrum* L.) is also known as "king of spices" is a widely used spice commodity[8]. This crop grows successfully 1500 m above MSL and between 20° North and 20° South of equator. It requires 2000-3000 mm rain fall. Fruits of this plant which has not attained full ripening are harvested, subsequently dried and used as spice. It has characteristic pungent aroma and taste which makes it suitable agent as a food flavouring agent. Also, it is an essential ingredient in Indian system of medicine. An oleoresin called 'piperine' is a principal ingredient responsible for the characteristic pungency which also enhances bioavailability[8]. Therefore, it is used in pharmaceutical preparations. There is a huge demand for black pepper and to meet this demand fertilizer application and wide scale use of broad-spectrum organophosphate pesticides are practiced. This leads to degradation of environment and shortening of lifespan of pepper vines[9]. In addition, there is also growing demand for organic cultivation of agricultural produce which is sustainable as well as safe from environmental and human health perspective. One of the important practices in organic agriculture is use of beneficial soil microbes to improve soil physical, chemical and biological properties. As phosphate solubilising microbes enhances availability of phosphorus to plant, it can be one of the alternatives to chemical farming practices. Though there are many phosphate solubilising microorganisms developed, they have not been very consistent in their performances everywhere due to their poor adoptability to changing agro-climatic conditions[10]. The present investigation was conducted with an intention to isolate phosphate solubilisers from areas where the pepper is an important crop cultivated and observation of *Piper nigrum* L. characteristics. The isolated isolates may be of use in this location.

MATERIALS AND METHODS

Study area: This study was carried out during August, 2018 to April, 2019. It was carried out at the PG Department of Biotechnology, Alva's College, Moodubidire, D.K. (District), Karnataka State, India. The black pepper rhizosphere soil samples were brought from two locations viz., 1. Mudradi village of Hebri taluk, Udupi District and 2. Hodala Village of Thirthahalli Taluk, Shimoga District.

Collection of soil rhizosphere sample: Rhizosphere soil samples surrounding the roots of three year old black pepper seedlings at one plot each from two locations (Hodla village, Thirthahalli taluk, Shimogga District and Mudradi Village, Hebri Taluk, Udupi District) were collected. Soil surrounding the pepper plants was loosened using a small spade. The soil adhering to the root was aseptically collected in a sterile polythene bag and brought to the laboratory. In a plot, 10 rhizosphere soil samples from 10 different plants were collected and pooled. In laboratory, clods and stones from the pooled soil samples were removed. From this sample, 10 g representative sample was prepared and used for isolation of P-solubilisers.

Isolation of P-solubilizing bacteria: Ten gram of representative soil sample was serially diluted till 10^{-4} dilution. One mL of aliquot each from 10^{-3} and 10^{-4} were transferred into separate sterile petri plates. Molten lukewarm Sperber's medium[11] was poured aseptically. This medium consisted of glucose 6.0 g L^{-1}; Yeast extract 0.5 g L^{-1}; $MgSO_4.7H_2O$, 0.25 g L^{-1}; $CaCl_2$ g L^{-1}; Agar, 15 g L^{-1}; pH, 7.0-7.2. After cooling, 3 mL/100 mL of 10% $CaCl_2$ and 2 mL/100 mL of 10% K_2HPO_4 was added. The plates were rotated in clockwise and anticlockwise directions to ensure proper mixing. These plates were incubated upside-down position at 30±2°C for 48 hrs. Colonies forming clear zone was carefully selected, purified and maintained on agar slants.

Morphological and biochemical characterization: Gram staining, catalase test, starch hydrolysis, urease production and Lipase test were carried out as per the standard procedures[12].

Estimation of water soluble phosphorus: The isolates were grown in sterilized liquid Sperber's liquid medium (20 mL) at 30°C for 2 days with continuous shaking at 150 rpm. Aliquots (1 mL) of each culture (10^8 CFU mL^{-1} of each inoculant) were then transferred to a 100 mL flask (n = 3 per isolate) containing sterilized liquid medium (50 mL) and incubated for 3 days with continuous shaking at 30°C. Sterilized uninoculated medium served as a control. A sample (10 mL) of each culture and control were taken and centrifuged at 8000 rpm for 10 min. The clear supernatant was employed to determine the amount of P released into the medium. The soluble phosphate released into the medium was analyzed by following phosphomolybdic blue colour method[13].

Bioassay studies: To test plant growth stimulating effect of Phosphates solubiliser, green gram seedling bioassay was conducted. These seeds were surface sterilized using 70% ethanol for 3 min. and 3% sodium hypochlorite for 3 min[14]. These surface sterilized seeds were rinsed six times in sterile water and dried. These surface sterilized seeds were soaked in different culture broth for an hour. They were sown aseptically in sterile sand medium and sterile water was added twice a day. The seedlings were maintained for 2 weeks. Simultaneously, sterilized seeds soaked in sterile un-inoculated Sperber's medium served as control. Treatment and control were maintained in triplicates. The plant heights of all the plants were recorded.

Statistical method: All experiments were performed in triplicates and data are expressed as mean±standard deviation.

RESULTS

Eighteen phosphate solubilising isolates were isolated from rhizosphere soils of *P. nigrum* L. two locations. All the isolates produced clear zones on Sperber's medium.

A sum total of 18 phosphate solubilizing bacterial isolates were isolated from *Piper nigrum* L. Among 18 isolates, 8 isolates were from Mudradi location and 10 from Thirthahalli

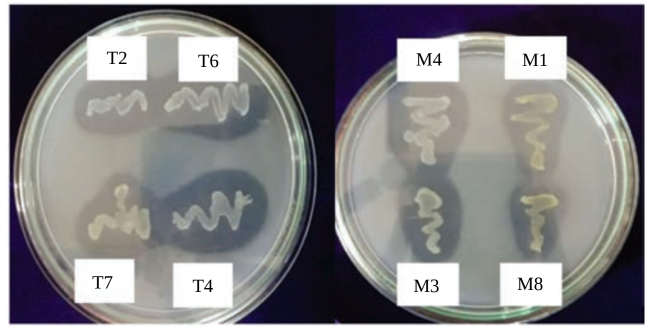

Fig. 1: Clear zones formed in Sperber's medium by selected phosphate solubilising isolates isolated from black pepper rhizosphere soils of Hodla (T2, T4, T6 and T7) and Mudradi villages (isolates M1, M3, M4 and M8)

Table 1: Morphological and biochemical properties of phosphate solubilising bacteria isolated from rhizosphere soils of *P. nigrum* L.

Isolate numbers	Gram reaction	Catalase test	Oxidase test	Urease test	Lipase test
T1	-	+	+	-	-
T2	-	+	+	-	-
T3	-	+	+	-	-
T4	-	+	+	-	-
T5	-	+	+	-	-
T6	-	+	+	-	-
T7	+	+	+	-	-
T8	+	+	+	-	-
T9	+	+	+	-	-
T10	+	+	+	-	-
M1	-	+	+	-	-
M2	-	+	+	-	-
M3	-	+	+	-	-
M4	-	+	+	-	-
M5	-	+	+	-	-
M6	-	+	+	-	-
M7	+	+	+	-	-
M8	+	+	+	-	-

location. Isolates were selected based on their ability to form clear zone around their colonies (Fig. 1). These isolates subjected to morphological and biochemical characterization. The results are presented in Table 1. From this it is clear that six isolates were gram positive and twelve were gram negative. All the isolates showed positive result for catalase and oxidase test and all were showing negative result for urease and lipase test.

Table 2 represents the water soluble phosphorus released by the phosphate solubilising bacterial isolates isolated from rhizosphere soils of Hodla. The amount of water soluble phosphorus released from different isolates isolated from Hodla soil ranges from 32.0±1.00-82.46±1.06 µg mL^{-1}. Isolate T6 released maximum and T1 released minimum amount of water soluble phosphorus.

Table 2: Water soluble phosphorus (µg mL^{-1}) released by phosphate solubilising bacteria isolated from hodla soil

Isolate no.	Water soluble phosphorus released (µg mL^{-1}) (Mean±SD)
T1	32.00±1.00
T2	60.30±0.57
T3	40.50±0.76
T4	55.40±0.58
T5	54.30±0.76
T6	82.46±1.06
T7	58.40±1.00
T8	45.67±0.59
T9	43.01±0.65
T10	42.66±0.56

Table 3: Water soluble phosphorus (µg mL^{-1}) released by phosphate solubilising bacteria isolated from Mudradi soil

Isolate no.	Water soluble phosphorus released (µg mL^{-1}) (Mean±SD)
M1	38±0.87
M2	33±0.76
M3	31±0.44
M4	70±1.01
M5	26±0.98
M6	25±0.66
M7	30±0.56
M8	31±0.75

Table 4: Effect of inoculation of phosphate solubilising bacterial isolates from hodla rhizosphere soil on plant height

Isolate no.	Plant height (cm) (Mean±SD)
Control	9.8±0.62
T1	16.2±0.88
T2	23.3±0.92
T3	14.2±0.65
T4	13.7±0.87
T5	13.6±1.06
T6	19.5±0.91
T7	15.1±1.00
T8	15.3±0.95
T9	14.8±0.98
T10	12.6±1.06

Table 5: Effect of phosphate solubilising bacterial isolates from Mudradi soil on growth of green gram plant height

Isolate no.	Plant height (cm) (Mean±SD)
Control	9.8±0.62
M1	16.3±1.08
M2	14.7±0.93
M3	14.2±0.95
M4	20.3±0.87
M5	12.6±0.76
M6	11.5±0.97
M7	12.2±0.92
M8	13.6±0.99

Table 3 represents the water soluble phosphorus released by the phosphate solubilising bacterial isolates isolated from rhizosphere soils of Mudradi. Amount of water soluble phosphorus released from different isolates isolated from Mudradi soil ranges from 26±0.98-70±1.01 µg mL^{-1}. Isolate M4 released highest water soluble phosphate and M6 released minimum water soluble phosphate.

The effect of bacterial inoculation on green gram plant height 2 weeks after sowing are represented in Table 4 and 5. All the treatments involving inoculation by phosphate solubilising bacteria showed increased plant height compared to control. Least plant height was recorded in un-inoculated control.

Inoculation with isolate T2 showed maximum plant height 23.3 cm and that with isolate M6 exhibited least plant height among bacterial inoculation treatments.

DISCUSSION

There is a growing trend in use of beneficial soil microorganisms in crop production because many people are being aware of hazards associated with food products produced using chemical inputs. Therefore, use of biological inputs that can enhance crop growth is an attractive cost effective, environmentally friendly and sustainable approach[15]. Rhizosphere is the source of many microbes which competitively colonize plant roots and stimulate plant growth[16]. Phosphate solubilising microorganisms are one such beneficial microbes which has ability to transform insoluble form of phosphorus into soluble available form. The performance of these isolates is not consistent because of their poor adoptability to changing agro-climatic conditions[17]. This necessitates the use of bioinoculants adapted to that particular location for harnessing their maximum potential. Phosphate solubilising microbes can be routinely screened by a plate assay method using Sperber's or Pikovskaya medium[18,19]. The phosphate solubilising microbes grows on these media and form clear zone around the colony[20]. The clear zone formation results from conversion of insoluble forms of phosphorus in the media to soluble form[21]. Eighteen phosphate solubilising bacterial isolates were isolated from two locations. All the isolates produced clear zone on Sperber's medium confirming their ability of phosphate solubilisation. When these isolates subjected to gram staining and other biochemical properties, six isolates were found to be gram positive and twelve were gram negative. All the isolates showed positive result for catalase and oxidase test and all were showing negative result for urease and lipase test. Several authors reported Phosphate solubilising bacteria showing both positive and negative results for gram staining[22,23]. Even the other biochemical studies on phosphate solubiliser were also in consistent with earlier studies[24].

All the isolates released water soluble phosphorus and isolate T6 isolated from the Hodla village released maximum water soluble phosphorus. It has been observed that isolates which shows clear zone around their colonies when grown on Sperber's medium are capable of solubilising insoluble phosphate[25]. The studies have shown that the microbial solubilisation in the liquid medium has often been due to synthesis and excretion of organic acids[26]. Phosphate solubilising microbes are capable of producing variety of organic acids from simple carbohydrates and organic acid production is reported to be the one of the mechanisms of phosphate solubilisation[27]. Acidification of periplasmic space by production of gluconic acid from glucose is also reported to be a mechanism underlying phosphate solubilisation in some organisms[28].

Phosphate solubilising microbes and other beneficial soil inhabiting microbes found to produce plant growth promoting substances like IAA. The GA and cytokinins and have been related to enhancement of plant growth[29-31]. It was found by several researchers that phosphate solubilising microbes produce IAA, GA and cytokinins[32,33]. The IAA, GA and cytokine are known to increase the plant height[34]. In the present investigation, there was increased plant height observed in all the treatments containing phosphate solubilising bacteria compared to un-inoculated control. Isolate T2 produced maximum increment in plant height compared to all the other isolates. There is a large variation in the amount of IAA and GA produced by different strains of phosphate solubilising rhizobacteria and is attributed to variability in the metabolism of different phosphate solubilising bacteria[15].

The positive effects exerted by beneficial microbes such as stimulation of plant growth promotion, tolerance to abiotic and biotic stresses based on their ability to produce auxins, gibberellins, cytokines salicylic acids and abscisic acid. Therefore, microbes which are associated with plants have ability to modulate hormone levels and metabolism in plant tissue[35]. In the light of these observations increased plant height in treatments with inoculated treatments may be due to release of water soluble phosphates and production of plant growth hormones by the bacteria. The growth factor producing efficiency and field experiments needs to be conducted which will further improve the knowledge on this aspect.

CONCLUSION

Even though, soil contain surplus amount of phosphorus, they may not be available to plant growth. Every plant has preference for particular group of its microbial partner; a study on the phosphate solubiliser associated with pepper was conducted. All the phosphate solubiliser isolated promoted the plant growth and released water soluble form of phosphorus from the insoluble source.

SIGNIFICANCE STATEMENT

This study identified that *P. nigrum* L. is a good source of phosphate solubilising microorganisms which along with improving P nutrition also improves plant growth. These bacterial agents along with improving crop nutrition helps in improving soil health and decreasing cost of cultivation. As the research in black pepper is scanty and there is a huge demand for organically grown pepper, many beneficial isolates from the black pepper rhizosphere can be isolated and evaluated at field level.

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