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MICROBIAL BIOCONTROL OF *MELOIDOGYNE INCOGNITA* IN BANANA PHOSPHATE SOLUBILIZING BACTERIA FROM *ARACHIS HYPOGAEA* SOIL AS A NOVEL AGENT

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ABSTRACT

Banana is one of the most important food and cash crops in India and around the world. Plant-parasitic nematodes pose a significant threat to banana production and, consequently, food security. Among these, *Meloidogyne* species are the primary nematodes associated with banana plants in the Thrissur districts Kerala. In this study, we investigated the nematocidal activity of phosphate-solubilizing bacteria against *Meloidogyne* infections in banana plants. We isolated these bacteria from soil samples of *Arachis hypogaea*. The morphological and biochemical characteristics of the phosphate-solubilizing bacteria were analyzed. Additionally, molecular identification was performed using 16S rRNA sequencing. The results indicated that the phosphate-solubilizing bacteria were effective in inhibiting *Meloidogyne* infections in banana plants.

KEYWORDS: Banana, nematodes, *Meloidogyne* species, bacteria, biological control.

INTRODUCTION

Fruits and vegetables are an important component of a healthy diet. Some fruits like bananas offer great medical benefits. This is partly because bananas aid in the body's retention of calcium, nitrogen, and phosphorus, all of which work to build healthy and regenerated tissues. Bananas are an excellent source of potassium. Potassium can be found in a variety of

fruits, vegetables, and even meats, however, a single banana provides you with 23% of the potassium that you need on a daily basis. Nutritional breakdown of Bananas One medium banana (about 126 grams) is considered to be one serving. One serving of banana contains 110 calories, 30 grams of carbohydrate and 1 gram of protein. Bananas are naturally free of fat, cholesterol and sodium. Vitamin B6 - .5 mg Manganese - .3 mg Vitamin C - 9 mg Potassium - 450 mg Dietary Fiber - 3g Protein - 1 g Magnesium - 34 mg Folate - 25.0 mcg Riboflavin .1 mg Niacin - .8 mg Vitamin A - 81 IU Iron .3 mg (Sampath Kumar *et al*., 2012). In Banana production India has first rank in the world. In agriculture, the production of fruits and vegetables are of so vital importance that it provide three to four time more income than cereals per unit of land. The fruit crops hold a great promise for accelerating income of the farmers. Realizing the importance of fruit cultivation many farmers are diverting their resources towards plantation of fruit crops. Area under fruit crops is, therefore, increasing day by day (Maurya *et al.*, 1996). India is the largest producer of fruits next to China. The annual production of fruits has been estimated to be 88.97 MT from an area of 06.38 Million ha. Over the decades, increase in area and production accounts to around 30.00 percent and 54.00 percent, respectively (Anonymous, 2015). In India it is cultivated in 802.6(000) hac. Area with the total production of 29724.6 (000) MT. It is the most popular fresh fruit all over the world and its name comes from the Arabic word banan, which means finger (Ruchi Sharma and Wilson Kispotta 2017).

Banana is the second most important crop in India; it is one of the most nematode infecting plants. Banana trees contain high moisture content it is very favourable for nematodes. Nematodes enter through roots and destroy the whole plants it also spoils the surface. Root-knot nematodes *Meloidogyne* Sp. are obligate endoparasites of great economic importance, being among the major limiting factors in the production of field and plantation crops. Their vernacular name comes from the galls (root knots) induced by *Meloidogyne* on the roots of their host plant. The most important species are the tropical *M. arenaria*, *M. incognita*, and *M. javanica*, and the temperate *M. hapla*. The reaction of a plant to parasitism by root-knot nematodes depends on the plant species and cultivar. Crop rotation, field period, season, initial population density and soil type also influence the severity of damage. Typical system include stunted growth, wilting, leaf discoloration and deformation of the roots. A number of methods followed to control the nematodes such as chemical control, organic agents, and biological control have been tried. Chemical control agents are effective but it is expensive and lead to cause soil pollution. After many researches rhizospheric microorganisms has been

described and it is tested for activity as biocontrol agents against soil nematodes. These microorganisms possess some microorganisms possess some mechanisms to promote plant growth and control pathogens. *Paenibacillus polymyxa*, *B. megaterium* and *B. circulans* are common soil bacterial bio fertilizers belonging to plant growth promoting bacteria. These bacterial activities include nitrogen fixation, phosphate solubilisation, and potassium solubilisation. Moreover, additional environmentally safe and economically feasible root-knot nematode control practices needs to be available. The main focus of this work is to evaluate the dual effect of some bacterial agents against the root-knot nematode *M. incognita* infestation in sandy soil.

The root knot nematodes *M. incognita* and *M. javanica* occur on banana and plantain roots wherever this crop is grown. *M. arenaria* and occasionally some other *Meloidogyne* species may also be found associated with banana and plantain. In spite of their widespread occurrence and high abundance, root-knot nematodes are not considered important pathogens of banana and plantain. Root-knot nematodes often occur on banana roots together with the other pathogenic nematode species *Radopholus similis* and *Pratylenchus* Sp. The damage caused by these other nematode species is more visible (root necrosis) and more destructive (toppling of plants) than the symptoms (galling) and other adverse effects caused by *Meloidogyne* Sp. Moreover, *R. similis* and, to a lesser extent, *Pratylenchu* Sp, tend to outnumber and eventually replace root knot nematode populations. When they occur together, the root lesion inducing nematodes destroy the root tissues and thus provide feeding sites for *Meloidogyne* Sp, (Waele *et al*., 1998).

MATERIALS AND METHODS

Collection of soil samples

The soil samples from groundnut field was collected from Thrissur districts Kerala.

Collection of nematodes

The *Meloidogyne incognita* nematode was collected and gifted by Dr. Giribabbu, Scientist, National Research Centre for Banana Trichy. The nematodes were maintained at lab, until the experiment was conducted.

Enumeration and isolation of phosphate solubilization of microorganism's microbes

The soil sample about 1g was collected and dissolved in 100 ml of sterile distilled water. The enumeration of microbes was carried out by the serial dilution method. The 1ml of each 10^{-4},

10^{-5}, and 10^{-6} dilutions were mixed with 20 ml of Pikovskaya agar medium and poured into the petri plates. Sealed the petri plates with the parafilm and incubated the plates at 37°C in a bacteriological incubator for 48 hr.

Morphological analysis

The bacterial species form characteristic colonies on Pikovskaya's Agar Media was further screened for morphological analysis was studied using the Gram staining kit (K001-1KT, Hi Media) by the standard procedure. The stained cells were observed under a compound microscope. The Gram's reaction and cell morphology for efficient PSB strains were recorded.

Biochemical analysis (Williams & Wilkins 1981)

Indole test

About 4 ml of tryptophan broth was taken in a sterilized test tubes. Inoculated the tube aseptically by taking the growth from 18 to 24 hrs culture. Incubated the tube at 37°C for 24-28 hours. Added 0.5 ml of Kovac's reagent to the broth culture and observed for the presence or absence of ring.

Citrate Utilization Test

Streaked the slant back and forth with a light inoculum picked from the center of a well-isolated colony. Incubated aerobically at 35 to 37°C for up to 4-7 days. Observed a color change from green to blue along the slant.

Tsi test

Inoculated a well isolated bacterial colony using TSI by first stabbing through the centre of the medium to the bottom of the tube and then streaked the surface of the slant. Left the cap loosen and incubated the tube at 35 in ambient air for 18 to 24 hours. Observe the reaction.

Catalase Test (Tube Method)

Poured about 1-2 ml of hydrogen peroxide solution into a test tube. Taken several colonies of the 18 to 24 hours test organism using a sterile wooden stick or a glass rod, and immersed in the hydrogen peroxide solution and observed for immediate bubbling.

Oxidase Test

Taken a filter paper soaked with the substrate tetramethyl-p-phenylenediamine dihydrochloride, Moisten the paper with sterile distilled water. Picked the colony to be tested with wooden or platinum loop and smeared in the filter paper, Observed inoculated area of paper for a color change to deep blue or purple within 10-30 seconds.

Nitrate Test

Inoculated nitrate broth with a heavy growth of test organism using aseptic technique in a sterile test tube. Incubated the tubes at an appropriate temperature for 24 to 48 hours. Then, one dropperfull of sulfanilic acid and one dropperfull of a α-naphthylamine was added to each broth. At this point, a color change to RED indicates a positive nitrate reduction test.

Gelatin hydrolysis test

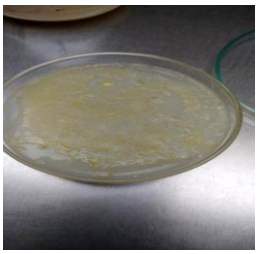
There are several methods for determining gelatinase production, all of which make use of gelatin as the substrate. The standard and most commonly employed method is the nutrient gelatin stab method. Inoculated a heavy inoculums of test bacteria (18-to24-hour-old) by stabbing 4-5 times (half inch) on the tube containing nutrient gelatin medium and incubated the inoculated tube along with an un inoculated medium at 35°C, or at the test bacterium's optimal growth temperature, for up to 2 weeks. Removed the tubes daily from the incubator and placed in ice bath or refrigerator (4°C) for 15-30 minutes (until control is gelled) every day to checked for gelatin liquefaction. (Gelatin normally liquefies at 28°C and above, so to confirm that liquefaction was due to gelatinase activity, the tubes are immersed in an ice bath or kept in refrigerator at 4°C). Tilted the tubes to observe if gelatin has been hydrolyzed.

RESULTS AND DISCUSSION

There is a serious lack of knowledge on nematode pests of staple food crops in ESA European Space Agency cropping 7 areas and consequently only minimal awareness among farmers, extension workers and 8 politicians of the damage they cause. The major plant-parasitic nematodes present in the sampling areas were .*R. similis*, *Helicotylenchus* spp., *Meloidogyne* spp. and *Pratylenchus* spp. The *Helicotylenchus* species consisted of *H. multicinctus* and a few *R. similis* with a proportion of 3:1. The perineal patterns of *Meloidogyne* spp. females indicated that *M. incognita* was common and *M. javanica* was rarely present.

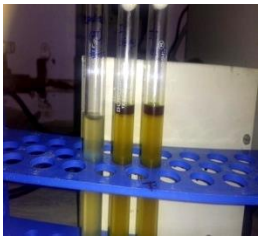
Biochemical test

Isolation of Rhizome Bacteria



We have isolated Phosphate solubilizing rhizome bacteria from groundnut. Whitish yellow colonies are observed.

Biochemical Test



Indole Test, Cherry red colour was observed. It indicates positive result.

Citrate utilization test

Deep blue colour was observed. It indicates the organism utilizes the citrate.

Triple Sugar Iron Test

Yellow colour was observed. It indicates glucose fermentation and acid production.

Catalyse Test

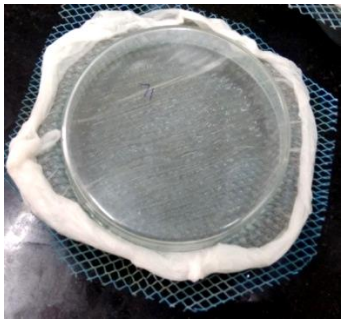
Bubbles were formed. It indicates the presence of catalyse.

Oxidase test

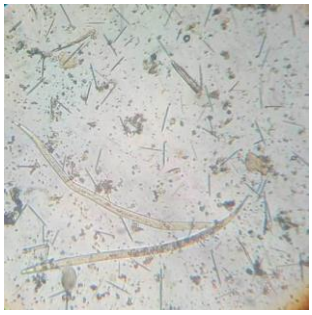
There is no colour change in the oxidase disc. It indicates the negative result.

Musa Spp., infected roots

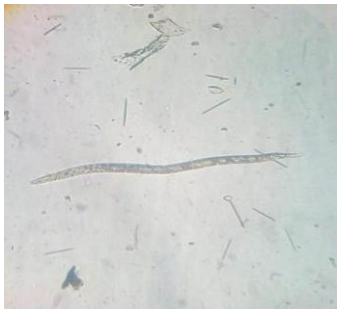
Nematodes Filtration



Collection of nematodes



Meloidogyne Spp., 10X



10X

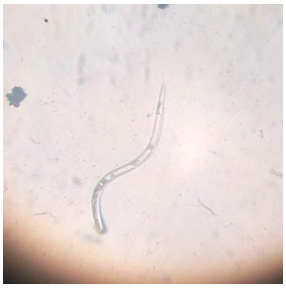
10X

Plate- 1: Nematodes collection.

10X

10X

10X



10X

Chemical composition of nutrient agar medium

S.NO	Name of the chemical	Quantity
1.	Peptone	0.5 gm
2.	Sodium chloride (Nacl)	0.5 gm
3.	Yeast extract	0.2 gm
4.	Beef extract	0.1 gm
5.	Agar powder	1.75 gm

Chemical composition of nutrient broth

S.NO	Name of the chemical	Quantity
1.	Peptone	0.5 gm
2.	Sodium chloride (Nacl)	0.5 gm
3.	Yeast extract	0.2 gm
4.	Beef extract	0.1 gm

DNA Isolation of preparation in stock solution.

S.NO	Type of the solution	Name of the chemical	Quantity
1.	0.5 M TrisHCl (pH-8.0)	Tris base	3.028 g
		Distilled water	40 mL
	0.5 M EDTA (pH-8.0)	EDTA	9.31 g
		Distilled water	40 mL
	10mM TrisHCl (pH-7.5)	Tris base	0.03 g
		Distilled water	20 mL
4.	TAE buffer (50X-1Litter)	Tris base	242 g
		Glacial acetic acid	57.1 mL
		EDTA	100 mL
5.	0.5 % Bromophenol blue	Bromophenol blue	500 mg

DNA Isolation of preparation in working solution

S.NO	Type of the solution	Name of the chemical	Quantity	P^H
1.	Solution 1	Tris-HCl	50 mM	8.0
2.		EDTA	20 mM	8.0
3.	Solution 2	Saturated NaCl solution	6 mM	-
4.		SDS	10 %	-

Preparation for Proteinase K (20 mg/mL).

S.NO	Name of the chemical	Quantity
1.	Proteinase K	10 mg
2.	Autoclaved distilled water	500 µL

Preparation for TE buffer

S.NO	Name of the chemical	Quantity	P^H
1.	Stock 0.5 M TrisHCl	2.0 mL (10 mM)	8.0
2.	Stock 0.5 M EDTA	0.2 mL (1 mM)	8.0

Preparation for 0.8% agarose gel

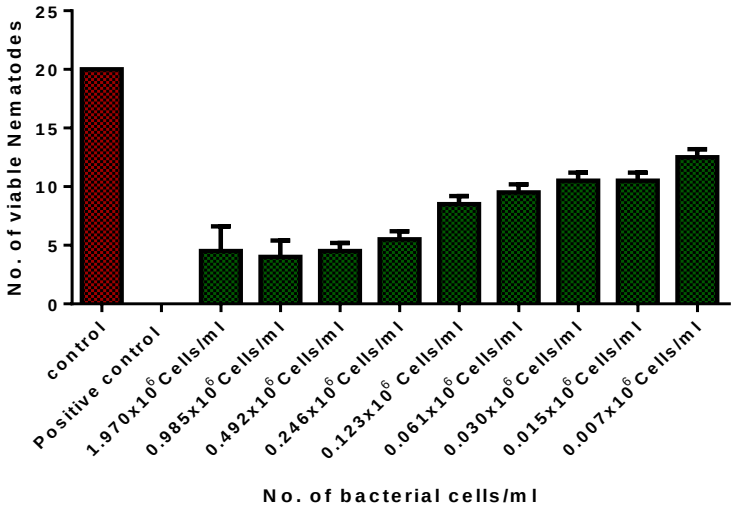
S.NO	Name of the chemical	Quantity
1.	Agarose	0.24 g
2.	1X TAE	30 mL

PCR amplification profile

S.NO	Temperature	Time
1.	95^0C	30 Sec
2.	95^0C	30 Sec
3.	56^0C	30 Sec
4.	72^0C	30 Sec
5.	72^0C	10 Sec
6.	4^0C	∞

The PCR mix consisted of the following components

S.NO	Name of the chemical	Quantity
1.	PCR Product (ExoSAP treated)	10-20 ng
2.	Primer	3.2 pM (either Forward or Reverse)
3.	Sequencing Mix	0.28 µl
4.	DMSO	0.30 µl
5.	5x Reaction buffer	1.86 µl
6.	Sterile distilled water	make up to 10µl



Graph 1. No: Bacterial cells / ml.

Various genera of banana parasitic nematodes were found in Rusitu Valley. This agrees well with Gowen and Queneherve (1990), Kashaija *et al.* (2000), Araya (2002), Pattison (2011), and Chavez and Araya (2010) reported that nematode species with different feeding habits usually exist as mixed populations in banana fields. Migratory endoparasitic nematodes that feed on the root cortex of banana plants (*R. similis* and *Pratylenchus* sp.), sedentary endoparasitic nematodes that penetrate banana roots, migrate and settle at a feeding site *Meloidogyne* sp. as well as the ectoparasitic nematode *Helicotylenchus* sp. were observed to be prevalent in Rusitu Valley from the survey. The results are consistent with those by

(Gowen *et al.* 2005) reported that nematode parasitism in the banana crop are characterized by several nematode species causing simultaneous infections.

Nematode genera detected in this survey are similar to those found in Costa Rica (Araya *et al*., 2002), Uganda (Kashaija *et al*., 1994), Ecuador (Chavez and Araya, 2011), Tamil Nadu, India (Plowright *et al.*, 2013), Sasser, 2013), and Viljoen 2010). *Meloidogyne* sp. was the most abundant nematode inhabiting banana fields in Rusitu Valley. These findings concur with those of Wang and Hooks (2009) who also found that *Meloidogyne* spp. was the most dominant plant-parasitic nematode species extracted from the soils of banana fields in Hawaii. Cropping of susceptible crops such as sugarcane, citrus, pineapple, and legumes with banana probably lead to the high abundance of *Meloidogyne* sp. in the soil around banana plants in the surveyed area.

The root-knot nematode was found to produce typical root galling symptoms in most of the root samples. Soil samples exhibited abundant J_2 of *Meloidogyne* sp. but the banana plants in the orchard did not showed any external above ground symptoms. There is no doubt that the galled roots are prone to attacks by various soil microorganisms which interact with nematode, resulting in root rotting decay of the root system. The attack of *M. incognita* caused 30.95% yield loss, deterioration of fruit quality and delayed crop duration (Jonathan and Rajendran 2000).

Biological control of nematodes, Nematologists in all over the world are working very hard to identify and lean to manipulate natural enemies of nematodes so they can be used as biological control agents. Nematodes have many natural enemies including fungi, bacteria and predacious nematodes. Certain fungi capture and kill nematodes in the soil. *Arthrobotrys* spp., *Dactylaria* spp., *Dactylella* spp., *Catenaria* spp., and *Trichothecium* spp., are the genera most commonly represented. The biological control of root knot nematode on tomato under green-house condition by using predaceous fungi has been reported Singh *et al*., 2001; Neher 2000.

Some fungi capture nematode by adhesion, but many employ specialized devices that include networks of adhesive branches, stalked adhesive knobs, non-constricting rings and constricting rings. The surface of the nematode is penetrated and the fungus hyphae grow throughout the nematode body, digesting and absorbing its contents. Under favorable conditions, large numbers of nematodes may be captured, and killed especially by those fungi

that form adhesive net-works or hyphal loops. *Trichoderma harzianum*, *Trichoderma virens*, *Aspergillus niger*, *Paecilomyces lilacinus* and *Pochonia chlamydosporia* are found promising biocontrol agents. Daneel (2015) while investigating the biocontrol potential of *P. lilacinus* against *M. incognita* on okra found the fungus to be quite effective and economically better than nematicides. According to Sikora (2005) carbofuran at the rate 1 kg/ha was less effective than *P. lilacinus* against *M. incognita*.

Now mycorrhiza is not restricted to its use only as biofertilizers, its potential role in the biological control of plant parasitic nematodes is reported by many repoters. Sikora (2005) found that prior presence of VAM fungi *Glomus mosseae* has resulted into an increase in plant resistance against *Meloidogyne* spp. A bacterial parasite of nematodes *Pasteuria penetrans*, has received much attention and research effort in recent years, *P. penetrans* is probably the most specific obligate parasite of nematodes, with a life cycle remarkably well adapted to parasitism of certain phytonematodes.

It directly parasitizes juvenile nematode, thus affects penetration and reproduction. *Pasteuria penetrans* can survive several years in air dried soil apparently without loss of viability. Seed bacterization, soil drenching and bare root dip application with *Pseudomonas fluorescens*, *Pasteuria penetrans*, *Bacillus subtilis*, *B. polymyxa* effectively controls plant parasitic nematodes was also reported by many workers. Among the predatory nematodes, monarchs may be proved efficient predators because of stronger predatory potential, high rate of predacity and high strike rate. In conclusion, *R. similis, Pratylenchus* sp., *Helicotylenchus* sp. and *Meloidogyne* are the major plant-parasitic nematodes associated with banana.

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