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Bhanothu Shiva
Ph.D. Scholar, Department of Plant Pathology, Faculty of Agriculture, BCKV, Mohanpur, West Bengal, India

P Srinivas
Central Horticultural Experiment Station (ICAR-IIHR), Aiginia, Bhubaneswar, Odisha, India

Deepa Khulbe
Regional Research and Technology Transfer Station, OUAT, Bhubaneswar, Odisha, India

Correspondence
Bhanothu Shiva
Ph.D. Scholar, Department of Plant Pathology, Faculty of Agriculture, BCKV, Mohanpur, West Bengal, India

Screening of promising rhizobacterial agents for the management of *Fusarium oxysporum* F. sp. *melongenae* in brinjal plants

Bhanothu Shiva, P Srinivas and Deepa Khulbe

Abstract
Fusarium wilt of brinjal caused by *Fusarium oxysporum* f. sp. *melongenae* is a now serious disease in eastern coastal regions of Odisha. Due to its soil inhabiting nature, this disease is very difficult to be managed by chemical fungicides. Rhizosphere soil of healthy brinjal plants was used for isolation and screening of native bacterial antagonists for screening and their promising role of biocontrol efficacy against fusarium wilt. Among 34 bacterial strains isolated from rhizoplane and rhizosphere of brinjal roots, five isolates *viz.* isolate-01, isolate-17, isolate-23, isolate-24 and isolate-32 were found highly inhibitory against mycelial growth of *Fusarium* sp., in dual cultures. Highest inhibition of radial mycelial growth of pathogen in dual culture was induced by isolate-32 (74.1%) followed by isolate-24 (71.9%). In greenhouse experiments percent disease incidence (PDI) was lower in artificially inoculated brinjal plants treated with isolate-32 (6.3%) and isolate-24 (8.7%), with percent disease reduction over control of 86% and 80.8%, respectively. These isolates also exhibited significant difference in seed germination percentage under artificial inoculation along with pathogen, highest germination percentage was recorded by isolate-32 (93%) followed by isolate-24 (91%) as compared to pathogen inoculated control (18%). The study concluded that the two native rhizobacteria isolated from root zone of healthy brinjal plants could successfully protect the brinjal plants from the lethal infection by *Fusarium sp.* while enhancing the germination of the treated plants.

Keywords: Brinjal, biocontrol, germination percentage, *Fusarium oxysporum* f. sp. *melongenae*

Introduction
Brinjal (*Solanum melongena* L.) belongs to the family Solanaceae and is the most important and widely-consumed vegetable in India. It is grown in 691,000 hectares with production of eight to nine million tones (equivalent to one quarter of global production), which makes India the second largest producer of brinjal in the world. Brinjal is susceptible to many soilborne fungal and bacterial diseases like fusarium wilt (*Fusarium oxysporum* f. sp. *melongenae*), collar rot (*Sclerotium rolfsii*), verticillium wilt (*Verticillium dahliae*) and bacterial wilt (*Ralstonia solanacearum*) (Kalloo and Berg, 1993) [7] and (Sihachakr *et al.*, 1994) [12]. Among all the pathogens *Fusarium* spp. is the most destructive pathogen that causes wilt in brinjal. Fusarium wilt, caused is a major constraint in brinjal production in India. The disease is widely distributed in tropical, subtropical and some warm temperate regions of the world. The pathogen is difficult to control since it is soil-borne and has a wide host-range, including several hundred species representing 44 families of plants. Infection is through root-to-root transmission, movement of soil and dissemination by farm implements, and insect transmission. A combination of high temperature and poor drainage favour development of the disease which causes 75 to 81% yield loss during summer in India (Das and Chattopadhyay, 1953; Rai *et al*, 1975; Rao *et al*, 1976) [4].

This pathogen blocks the xylem transport system and causes severe wilting and death of brinjal plants (Altinok, 2005) [1]. Under optimal infection conditions, such as temperature, high soil moisture level, soil compaction and poor soil drainage, this pathogen can completely destroy the grown plants. Infected plants exhibit leaf chlorosis and slight vein clearing on outer leaflets, followed by yellowing and dropping of leaves, then xylem browning of the stem and finally death of the aboveground parts. The infection and the symptoms are observed when the temperature is about 25° C.

Use of chemical, cultural and biological measures are some common practices followed to control this disease to some extent. Being a soilborne plant pathogen, it is difficult to control

using a conventional chemical fungicide, because spores of this fungus survives for many years in the soil. Intensive use of chemical fungicides accumulates toxin in the environment and create residue problems. Rhizospheric microorganisms are the ideal control for soilborne plant pathogens. The objective of present study was to isolate native rhizobacteria from the rhizosphere and rhizoplanes of brinjal crop and screen them for their bio-control potential against the devastating fusarium wilt pathogen of brinjal crop.

Materials and Methods

The study was carried out during 2017-18 in the Department of Plant Pathology, Odisha University of Agriculture and Technology (OUAT), Bhubaneswar, Odisha, India, which Agro-climatically falls under East & South East Coastal Plain zone.

Isolation, purification and identification of pathogen

Several diseased brinjal plants were collected during the field surveys. The diseased plant part was washed in running tap water and cut into small bits (2-3 mm). These bits were surface sterilized with Sodium hypochlorite (20%) solution for 60sec and subsequently washed thrice in sterile distilled water to remove traces of Sodium hypochlorite. The bits were picked up and placed using sterilized needle and forceps in the center of the Petri plates containing water agar and sub-cultured on Potato Dextrose Agar (PDA). The inoculated plates were incubated in BOD incubator at 25 ±2ºC for seven days. The plates were observed at regular intervals for the development of the typical fungal colonies.

The fungal colonies produced white, pink, salmon or gray-coloured colonies with velvety to cottony surfaces and readily changed the colour up on spore production. Microscopically, the filaments were hyaline and septate. They typically branched at acute and at right angles. The production of both fusoid macroconidia (hyaline, multicellular clusters, macroconidia with foot cells at the base of the macroconidium) and microconidia (hyaline, unicellular) were characteristic of the genus *Fusarium* spp. was purified and identified using the microscopic charterers including the shape of conidia (Booth, 1977) [2], primary and secondary characteristics according to the Nelson *et al.,* (1983) [9].

Pathogenicity test of the *Fusarium* spp.

Pathogenicity tests were carried out to establish the ability of fungal isolates to produce typical diseases symptom(s) under artificial condition on brinjal seedling crates. The inoculum of the pathogen was grown on milled maize grain seeds, added to the coir pith @ 10g kg^{-1}, moistened with water and mixed thoroughly. Pathogenicity test of soil borne fungi was carried out in green house using coir pith as substrate in protrays by axil-puncture method (Winstead and Kelman, 1952; Rashmi *et al*, 2012) [14, 11]. Suitable check was maintained without addition of inoculum to the coir pith. The seedling crates were watered at regular interval to maintain soil moisture. The seedlings grown were observed after 15 days for symptom development. Re-isolations or both the fungal pathogens (*Fusarium* spp.) were done from infected seedlings and the cultures obtained were compared with initial cultures to confirm the identity and pathogenicity of pathogen.

Isolation and purification of native rhizobacteria

Isolation of rhizobacteria from collected soil samples was carried out by dilution plate technique as described by Islam (2009) [6]. One gram of rhizosphere soil was taken into a test tube and 9ml of sterile distilled water and stirred thoroughly for few minutes in order to obtain a uniform 1:10 dilute soil suspension. In case of multiple samples, the tubes were shaken for 10 min on a rotary shaker. This was used as stock solution resulting 10^{-1} dilution. One ml of 1:10 stock suspension was transferred with the help of sterile pipette into the 2nd test tube containing 9 ml sterile water and shaken thoroughly resulting 10^{-2} dilution. Serial dilution technique was performed up to 10^{-4} dilution and the final dilution was made up to 10^{-4} dilution. 0.1ml of an appropriately diluted culture was spread over the surface of nutrient agar (NA) plate using sterile glass spreader. The plates are then incubated until the colonies appear. The surface of the plate was kept dry so that the spread liquid is soaked. The Plates were incubated at 25°C± 2 for 2-4 days in inverted position so that vapours condensed from the lid may not hamper the growth of the isolated bacteria. After incubation bacterial colonies were counted and representative colonies were selected /marked for isolation and purification.

The bacterial colonies with distinct types observed on the basis of their morphological characteristics, were selected and isolated on NA slants. The streaked NA plates were incubated at room temperature for 2 days. Purification was done by streaking NA plates from single colony.

Screening and evaluation of selected antagonistic native rhizobacteria against *Fusarium* spp.

***In vitro* screening of rhizobacterial isolates for their antagonist properties by dual culture method**

The antagonistic potential of the rhizobacterial native isolates against soil borne fungal pathogens was investigated by dual culture method (Dennis and Webster, 1971a, Buysens and Scheffer, 1992) [5, 3]. The extent of antagonistic activity by rhizobacterial isolates against *Fusarium* spp. pathogen was recorded on fifth day by measuring the radial growth of the pathogen in dual culture plates and in control plate. The per cent inhibition of radial growth of *Fusarium* spp. over control was calculated (Vincent (1927) 13[].

$$\text{Percentage inhibition} = \frac{(\text{Control-Test})}{\text{Control}} \times 100$$

***In-vivo* screening of antagonistic rhizobacteria against *Fusarium* spp.**

To study the efficacy of rhizobacterial isolates selected through *in-vitro* screening, the surface sterilized brinjal seeds (cv. Utkal Anushree) were planted in the potrays containing standard soil media inoculated with *Fusarium* spp. After one week and one day before transplanting the brinjal seedlings, selected rhizobacterial isolates were incorporated in soil media at the rate of 5 ml per well at 10^9cfu/ml. Three weeks old seedlings were root dipped in bacterial suspension of selected antagonistic bacteria (10^9cfu/ml) for 45 min and transplanted into pathogen-rhizobacteria mixture coir pith (Lemessa and Zeller, 2007) [8]. The seedlings were maintained in green house at 24-28°C temperature and 75-90% relative humidity. The seedlings were watered with sterile water when necessary.

***In-vivo* evaluation of selected antagonistic rhizobacteria for biocontrol of *Fusarium* spp.**

Five selected rhizobacterial isolates with higher inhibition under *in vitro* tests were further tested in green house on brinjal plants to evaluate their ability to control soil borne diseases. Potrays containing standard soil mix and milled

maize grains inoculated with *Fusarium* spp. After one week and one day before transplanting the seedlings, antagonists were incorporated in the coir pith at a rate of 5 ml per well at 10^9cfu/ml. Three weeks old brinjal seedlings were root dipped in bacterial suspension of antagonistic bacteria (10^9cfu/ml) for 45 min and transplanted into pathogen-antagonist mixture coir pith (Lemessa and Zeller, 2007) [8]. Treatments were replicated four times. Appropriate positive and negative controls were maintained. The disease incidence and biocontrol efficiency were calculated as follows:

$$\text{Percent incidence} = \frac{\text{Number of diseased plants}}{\text{Total number of plants observed}} \times 100$$

Statistical analysis

The data obtained in the experiments was analyzed using appropriate analysis Programme -Statistical Methods for Agricultural Workers, ICAR, New Delhi (Panse and Sukhatme, 1989) [10].

Experimental Results

Isolation and identification of soil borne pathogen

The soilborne pathogen *Fusarium* spp. was isolated from diseased samples of brinjal plants collected from the OUAT fields, RRTTS farm, CHES farm and local farmer's fields during survey.

The fungal colony produced white, pink, salmon or gray-coloured colonies with velvety to cottony surfaces and readily changed the colour up on spore production. Microscopically, the filaments were hyaline and septate. They typically branched at acute and at right angles. The production of both fusoid macroconidia (hyaline, multicellular clusters, macroconidia with foot cells at the base of the macroconidium) and microconidia (hyaline, unicellular) were characteristic of the genus *Fusarium* spp.

Plate 1: Isolation of pure culture of *Fusarium* spp. (a) Wilt symptoms and death of above ground parts (b) Rotting below collar region (c) Discoloured stem vascular tissue (d) Pure culture *Fusarium* spp. Pathogenicity of *Fusarium* spp.

The pathogenicity of the isolate of *Fusarium oxysporum* was proved under artificial condition on brinjal seedlings. The inoculum of the pathogen was grown on milled maize grain seeds, added to the moistened coir pith @ 10g kg^{-1} and mixed thoroughly. Suitable check was maintained without addition of inoculum to the coir pith. The seedling crates were watered at regular interval to maintain soil moisture. The seedlings were observed after 15 days for symptom development. Symptoms consisted of collapse of entire seedlings and drooping of petiole, rachis and leaflets. Gradually the leaves turned yellow and light brown to straw coloured. Symptoms due to wilting of the plants in the protrays inoculated with *Fusarium* culture were similar to that of the plants wilted in the main field. Upon re-isolation of the fungus (*Fusarium* spp.) was done from infected seedlings and the cultures obtained were compared with initial cultures to confirm the identity and pathogenicity of pathogens.

Plate 2: Pathogenicity test (a) artificial inoculation of *Fusarium* spp. on healthy brinjal plant (b) complete drooping and wilting of plants

Isolation and purification of rhizospheric bacteria

A total of thirty-four different bacterial isolates were isolated from soil samples collected during the survey of solanaceous vegetable growing areas. The isolates were purified on PKV, KB, and NA media. The bacterial isolates were coded in a series from Iso-1 to Iso-34. Out of these bacterial isolates, 25 were rhizobacterial isolates and 9 isolates were endophytic in nature.

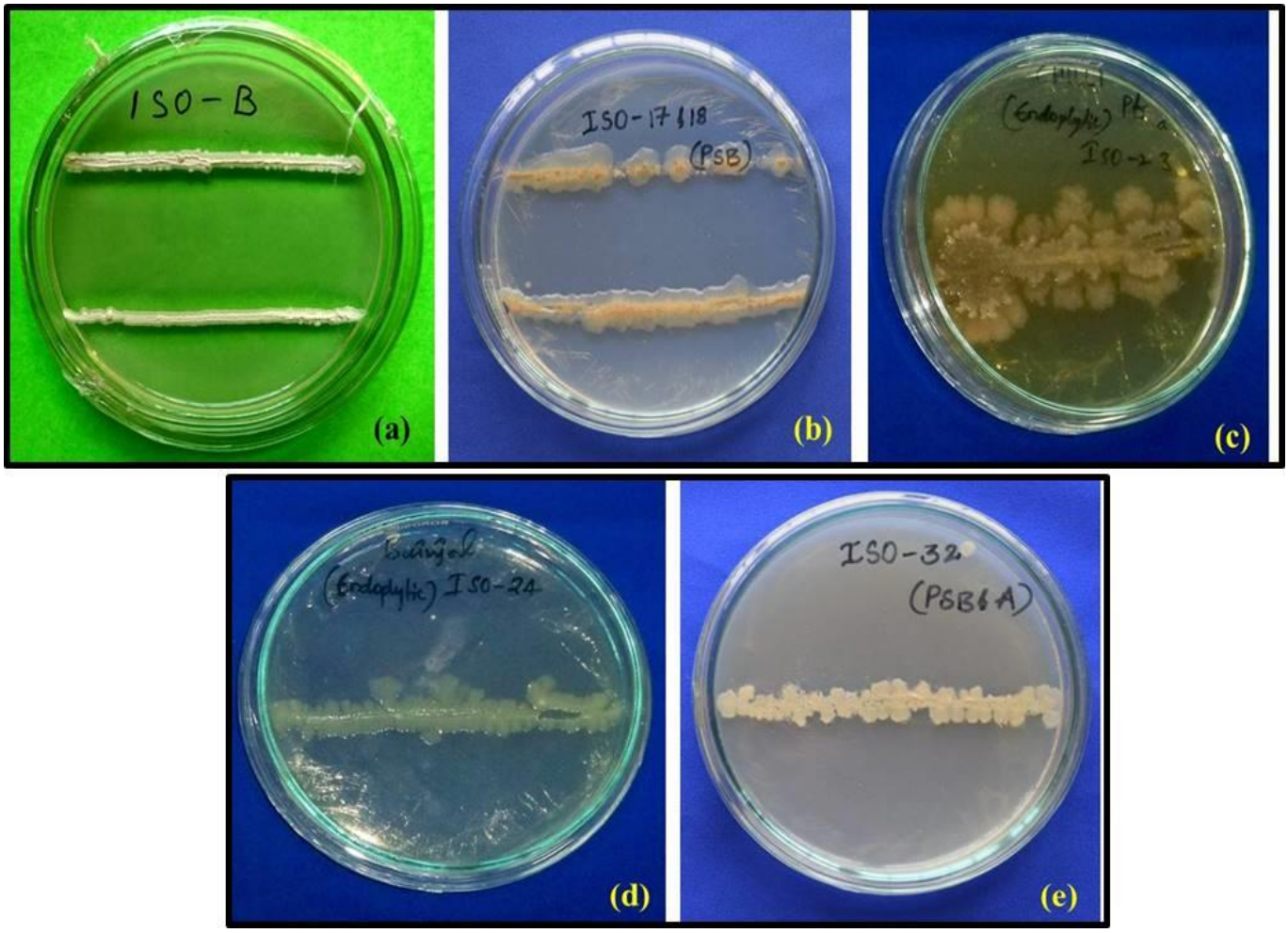

Plate 3: Pure cultures of selected rhizobacterial isolates

Screening of isolated rhizobacteria for antagonistic potential

Preliminary *in-vitro* bioassay of isolated rhizobacterial isolates was carried out against *Fusarium* spp. by the dual culture method. The intensity of the antagonism by various isolates against the pathogens was recorded as percent inhibition of mycelial growth by scoring in a scale from 0 (no inhibition) to >75% as (+++++) (data not presented). The efficiency of isolates 01, 17, 23, 24 and 32 was highest (55% inhibition or more), while other strains were either inferior or inefficient in checking the mycelial growth of the pathogens.

***In vitro* evaluation of selected rhizobacterial isolates against *Fusarium* spp.**

In vitro evaluation of selected rhizobacterial isolates (isolate-01, isolate-17, isolate-23, isolate-24 and isolate-32), against *Fusarium* spp. was carried out using dual culture method to

test their efficiency to inhibit the mycelial growth of isolated fungal plant pathogen.

Antagonistic activity of selected rhizobacteria against *Fusarium* spp. by dual culture method

The data presented in the given Table 1, have been revealed that antagonistic effect of all the selected isolates against *Fusarium* spp. showed significant reduction in mycelial growth. The per cent inhibition over control in wilt disease ranged from 74.1 to 60.4 per cent. Maximum per cent inhibition over control was shown by isolate-32 (74.1 per cent) followed by isolate-24 (60.4). The significant difference in radial growth observed by control (90mm) whereas isolate-32 (23.3mm) recorded lowest followed by isolate-24 (25.3mm).

Table 1: Antagonistic activity of rhizobacterial isolates against *Fusarium* spp. in dual plate

Treatment	Radial growth (mm) *	Per cent inhibition over control*	Inhibition zone (mm) *
Iso-01	28.0	68.9	16.7
Iso-17	34.0	62.2	13.0
Iso-23	35.7	60.4	12.7
Iso-24	25.3	71.9	18.3
Iso-32	23.3	74.1	20.0
Control	90.0	0.0	0.0
SE(m)±	0.40	0.5	0.4
C.D.≤ (0.05)	1.3	1.4	1.3

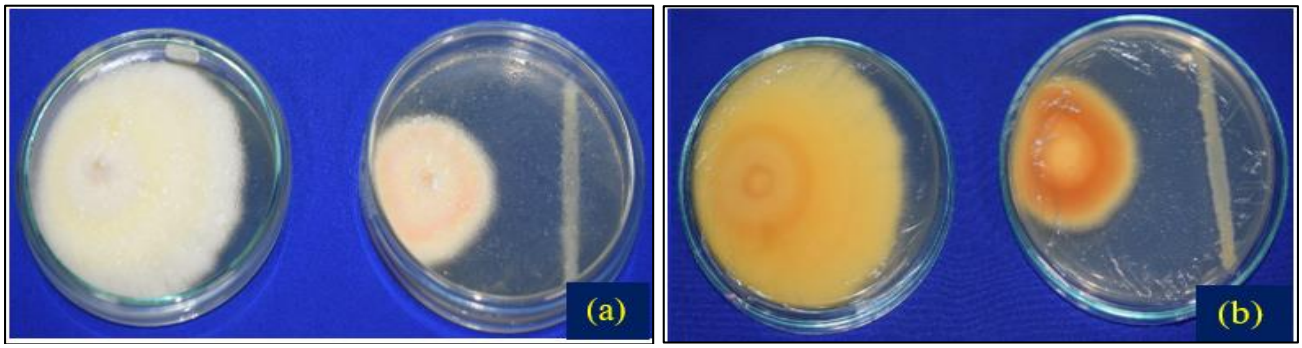

Plate 4: Dual culture of selected rhizobacterial isolate with *Fusarium* spp. (a) Normal position (b) Inverted position

***In vivo* evaluation of selected native antagonistic rhizobacteria against fusarium wilt disease**

The effect of selected antagonists was investigated for their biocontrol potential against fusarium wilt. All the isolates gave significant control of wilt diseases when compared with inoculated control. Incidence of the diseases reduced to the level of 6.3% with isolate-32 which gave 86.0% disease control over inoculated control. Effect of seed treatment with native rhizobacterial isolates on seed germination in artificially inoculated protrays under greenhouse conditions was also evaluated (Table 2).

Table 2: Effect of seed treatment with native rhizobacterial isolates on *in vivo* incidence of fusarium wilt under artificial inoculation of pathogen

Treatment	Disease Incidence (%)	Disease reduction over control (%)
Iso-01	10.4	76.9
Iso-17	9.4	79.1
Iso-23	13.2	70.7
Iso-24	8.7	80.8
Iso-32	6.3	86.0
Control	45.0	00.0
SE(m)±	1.5	
C.D. (≤0.05)	4.6	

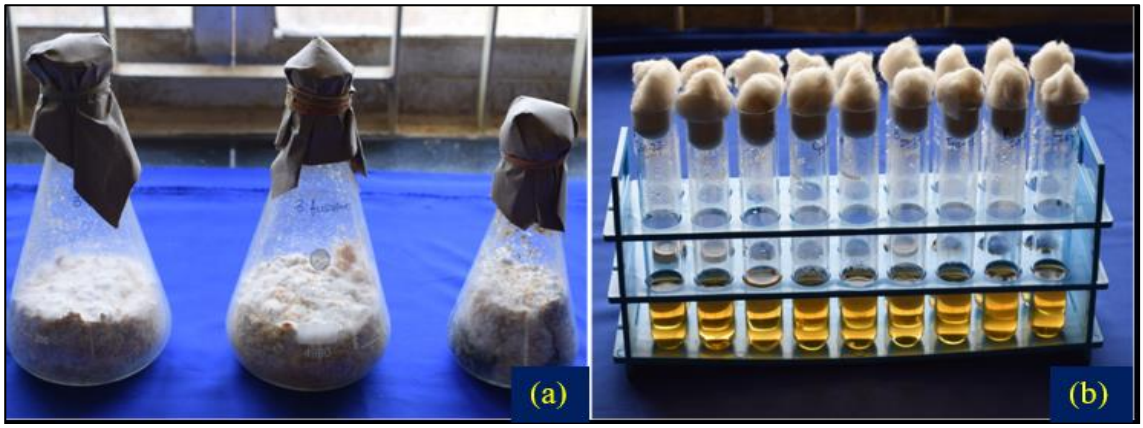

Plate 5: Mass multiplication of pathogen and selected bacterial isolates for artificial inoculation to evaluate the disease management (a) Mass multiplication of *Fusarium* spp. in milled maize grain (b) Selected rhizobacterial isolates

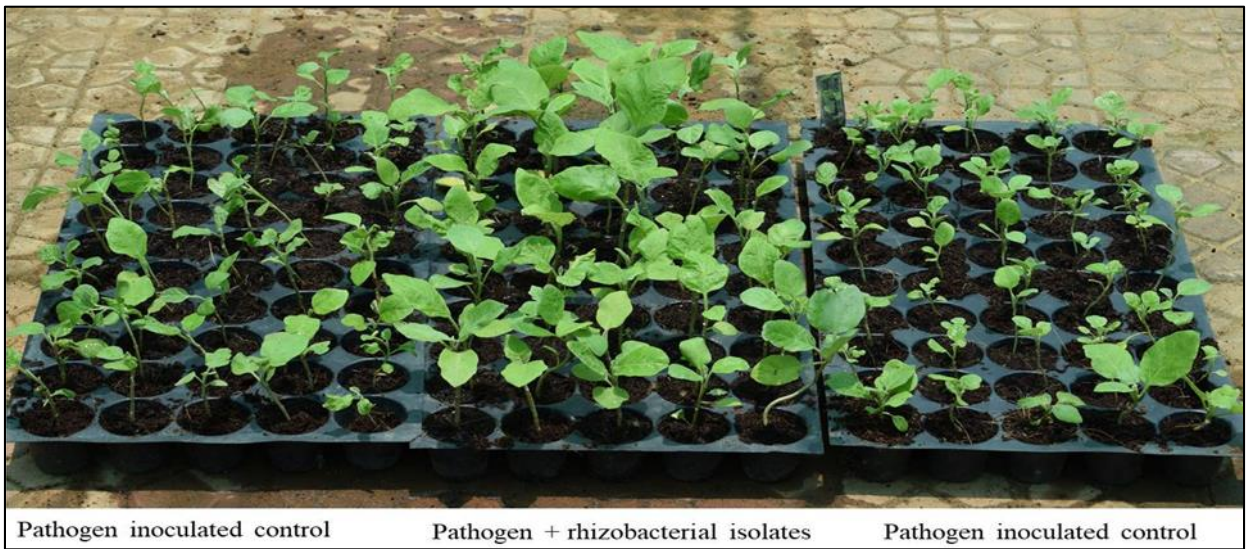

Plate 6: Effect of rhizobacteria on management of wilt disease

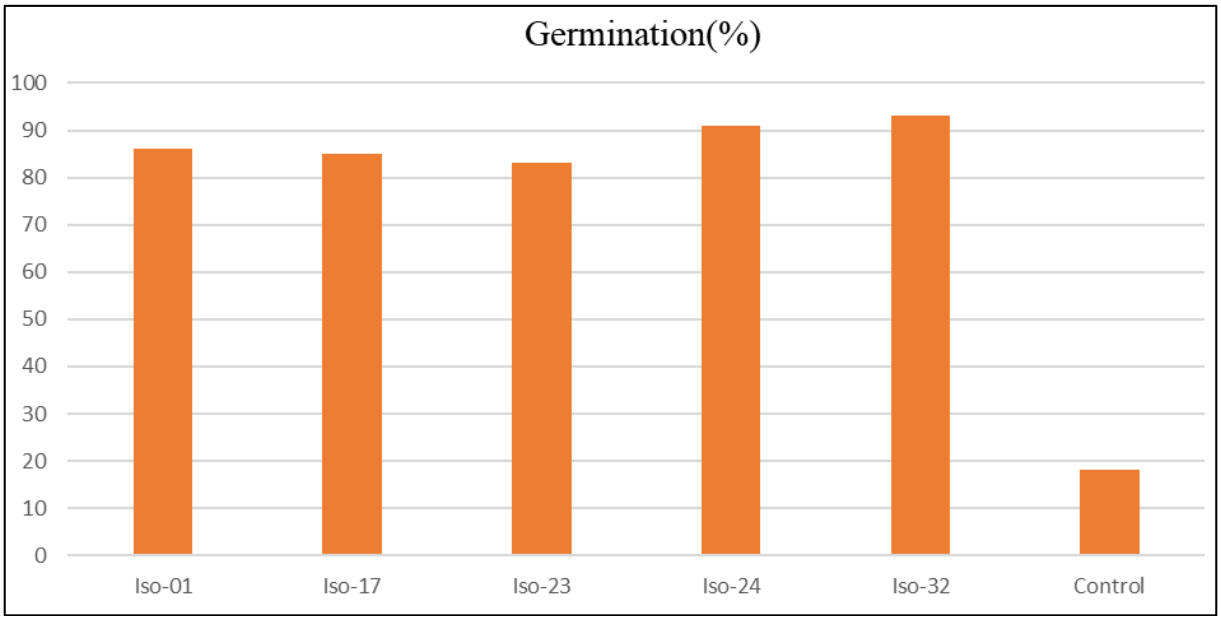

Fig 1: Effect of seed treatment with native rhizobacterial isolates on germination (%) under *in vivo* conditions

The results (fig.1) showed that the percent germination of brinjal seeds treated with five rhizobacterial isolates ranged between 83% and 93% as compared to 18% germination in control treatment plants inoculated with fusarium wilt pathogen alone. Among individual isolates isolate-32 effected highest germination (93%) followed by isolate-24 (91%). Selected native rhizobacterial isolates shown desired germination and at par to each other.

Conclusion

Native microbes are the best while bioprospecting agriculturally important microorganisms from any agro-ecological system. Vegetables being highly economically important crop receive more pesticides for management of several pest and diseases. However, as the fruits, the edible parts of the plants, come in direct contact with deadly pesticides, it is imperative to explore more native microbes which can counter pathogens more effectively. The present study concluded that native rhizobacterial strains isolated from the brinjal crop can be successfully used for managing soil borne *Fusarium* spp. affecting brinjal crop besides enhancing the growth of the treated plants. Among five rhizobacterial isolates two isolates isolate-32 and isolate-24 identified as having highly potential antagonistic properties along with plant growth promotion ability, which would pave way for eco-friendly management of fusarium wilt of brinjal.

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