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Isolation and *In vitro* Studies of Native Isolates of *Bacillus subtilison* Maize Stalk Rot Incited by *Fusarium verticillioides*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Fusarium wilt of maize is a widely distributed and the most destructive disease caused by *Fusarium verticillioides*. The main objective of this experiment is to identify the effective *Bacillus* isolates against *Fusarium verticillioides* under *in vitro* condition. A total of 10 *Bacillus* spp. isolates were isolated from rhizhosphere region of maize plants in different locations of Telangana state and tested for antagonistic activity at department of plant pathology, Maize Research Centre, Agricultural Research Institute, Rajendranagar. All the isolates of *Bacillus* spp. were used for determining theirbio efficacy against Fusarium *verticillioides*. All the isolates not shown similar bio efficacy and differerd in their antagonistic activity against *F. verticillioides* mycelial growth. Among them the isolates B-ISO-3 and B-ISO-2 were found to record significantly higher percent reduction of mycelial growth 63.3 and 62.8 % respectively, followed by B-ISO-9 which recorded 61.3%t reduction of mycelial growth over control. The lowest percent reduction of mycelial growth was recorded with the isolate B-IS0-8 (34.2 %) over control.

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1. INTRODUCTION

"Maize (*Zea mays* L.) is one of the most versatile emerging crops having wider adaptability under varied agro-climatic conditions. It is cultivated in tropics, sub tropics and temperate regions under irrigated and rainfedconditions. Globally, maize is known as queen of cereals, because it has the highest genetic yield potential among the cereals" [1]. "In most of the developing countries maize is consumed directly as food. Maize occupies an important place as a source of human food (26%), animal feed (13%), poultry feed (47%), industrial products (14%) and seed (3%)" [2]. "In India, Maize is cultivated in an area of 9380.07thousand hectares with an annual production of 28752.8 thousand tons in India. In Telangana State, the crop is grown in almost all districts in an area of 630 thousand hectares with a production of 2555.64 thousand tonns and productivity of 4057Kgss/hectare" [3]. The other important maize growing states in India are Karnataka, Bihar, Rajastan, Maharashtra, Madhya Pradesh, Utter Pradesh, Andhra Pradesh, and Himachal Pradesh.

"Maize production is affected by various biotic and abiotic stresses. Among the biotic stresses, fungal diseases are one of the major constraints in realizing the potential yields of this crop. Of the fungal diseases, post flowering stalk rots poses a major threat to the productivity of maize crop. Post flowering stalk rot is complex disease which occurs at post flowering stage of the crop in both *kharif* and *Rabi* season. In India, eight fungi and three bacteria were reported to cause stalk rots [4]. Among all, Fusarium stalk rot (*Fusarium verticillioides),* Charcoal rot *(Macrophomina phaseolina),* Late wilt *(Cephalosporium maydis)* are more prevalent and destructive in India" [5]. "Among the stalk rots, Fusarium stalk rot caused by *F. verticillioides* was first reported from USA by Pammel [6] as a serious root and stalk disease". Later, Valleau [7] reported that "*F. moniliforme* was a primary cause of root and stalk rot of maize. In India, the disease was first reported from Mount Abu, Rajasthan [8] and prevalent in most of the maize growing areas of country where water stress occurs at the flowering stage of the crop". "The disease becomes apparent when crop enters senescence phase and severity increases during grain filling stage. The rotting extends from the infected roots to the stalk and causes premature drying, stalk breakage and ear dropping and thus resulting in reduction of maize yields" [9]. "The disease causes internal decay and discoloration of stalk tissues, directly reducing yield by blocking translocation of water and nutrients, thus resulting in death and lodging of the plant" [10]. The fungus survives on crop residues in the soil.

"Use of chemicals is expensive and the heavy usage of chemicals is hazardous to the environment. Among alternatives being studied, use of *Bacillus* strain has shown significant potential" [11]. "It is generally recognized that *Bacillus* species show antagonistic potential against fungal phytopathogens by antibiosis, competition or exploitation. Successful control of Fusarium species has been achieved by various *Bacillus subtilis* isolates" [12]. "Some isolates were found less effective against Fusarium species in comparison with others *Bacillus* species due to mode of action exerted or the type of antifungal metabolite produced. Therefore, many studies have been conducted to find the best *Bacillus* strain or by inducing secondary metabolites production" [13,14]. "Therefore, isolation and screening of native strain is suggested" [15]. Foreign strain or commercial inoculants has been shown less effective in other countries due to different edaphic or climatic conditions. Ji et al. [16] isolated bacterial isolate CNU114001 which was identified as *Bacillus amyloliquefaciens*exhibited 70% mycelial growth reduction against *C. orbiculare, F. oxysporum, P. digitatum*and*P. grisea.* Figueroa-Lopez et al*.* [17] reported "11,520 bacterial isolates, exhibited 95 percent survival efficiency out of which 622 isolates showing 53–99 percent *F. verticilliodes* growth inhibition". "An analysis of the plant-growth promoting (PGP) properties and biocontrol attributes of four bacilli (*Bacillus simplex* 30N-5, *B. simplex* 11*, B. simplex* 237 and *B. subtilis*30 VD-1)" was studied by Khan et al*.* [18]. Among these *B. subtilis* 30VD-1 (30VD-1) showed most effective antagonism against *Fusarium* spp. under *in vitro* conditions. The aims of this work were to determine the ability of *Bacillus* species to inhibit *Fusarium verticillioides* and to evaluate the ability of the best strain bacterium *in vitro*.

2. MATERIALS AND METHODS

2.1 Isolation of Biocontrol Agents from Rhizosphere

2.1.1 Serial dilution method

Antagonistic bacteria were isolated fromthe rhizosphere soil collected from different crops

grow in various places of Telangana. The plants were pulled out gently with intact roots and the excess soil adhering on roots was removed gently. Ten grams of rhizosphere soil collected from different crops was transferred to 250 mi Erlenmeyer flask containing 100 ml of sterile distilled water separately. After thorough shaking, the antagonist present in the suspension was isolated by serial dilution plate method. From the final dilutions of 10^{-3} ,10^{-4}, 10^{-5} and 10^{-6}. One ml of each aliquot was pipetted out poured into sterilized petri dishes containing Nutrient agar medium and they were gently rotated clockwise and anticlockwise for uniform distribution and incubated at room temperature (28 ± 2ºC) for 24 hours. Colonies with characteristics of *Bacillus* spp. were isolated individually and purified by streak plate method [19] on Nutrient agar medium. The pure cultures of the antagonists were maintained on respective agar slants of 4ºC respectively

2.1.2 Identification of bacterial colonies

"Pure cultures of bacteria were streaked on nutrient agar plates separately and incubated at room temperature until single colony developed. Individual colony was examined for Gram staining and endospore staining" [20].

2.1.3 Gram staining

"A drop of sterile distilled water was placed in the center of glass slide. A loopful of inoculum from young culture was taken, mixed with water and placed in the center of the slide. The suspension was spread out on slide using the tip of inoculation loop to make a thin smear. The smear was dried in air and fixed through mild heating by passing the slide 3 to 4 times over the flame. The smear was then flooded with crystal violet solution for 1 min and washed gently with flow of tap water. Then the slide was flooded with iodine solution. After incubation at room temperature for 1 min, iodine solution was drained out followed by washing with 95% decolorizer. After that, it was washed with water within 15 to 30 seconds and blotted carefully. The smear was incubated with safranin solution for 1 minute. The slide was washed gently in flow of tap water and air dried. The slide was examined under microscope at 100X power with oil immersion and data was recorded for different isolates" [20].

2.1.4 Endospore staining

"A bacterial smear was taken on a clean slide, air dried and gently heat fixed. Then the slides were flooded with malachite green, for 3-5min using the flame of burner. The slides were washed gently in flow of tap water to remove dye. After cooling the slides, safranin was drained on to the slide. The slide was washed gently in flow of tap water and air dried. The slides were observed at 100X with oil immersion and data was recorded for different isolates" [20].

2.1.5 Screening of bacterial antagonists

The bacterial isolates of *Bacillus subtilis* were tested for their inhibitory effect on growth of *Fusarium verticillioides* by following the dual culture technique [21]. One loop of 48 hrs old culture of bacterial isolates were streaked one cm from the outer side of 9 cm PDA plates and a mycelial disc (8 mm diameter) of five day old culture of *Fusarium verticillioides* was placed at the centre of plates, 2.5 cm apart from the bacteria. The petridishes inoculated with pathogen alone were kept as control. The plates were incubated at room temperature (28 ± 2ºC) for 10 days. After 10 days of incubation, the pathogen growth was measured in all the petri dishes separately and calculated as per the formula given below [22].

$$I = \frac{C - T}{C} \times 100$$

Where,

I = Per cent inhibition over control
C = Radial growth of pathogen in control (mm)
T = Radial growth of pathogen in treatment (mm)

3. RESULTS AND DISCUSSION

3.1 Isolation of Antagonists from the Rhizosphere Soil

Ten isolates of *Bacillus subtilis* (Plate 1) were isolated separately from the rhizosp here region of maize plants collected from different parts of Telangana (Table 1).

3.2 Gram's Reaction

The Gram reaction was studied for the isolated bacteria. All 10 isolates of *Bacillus subtilis* were found to show positive result with purple colour. Endospore staining confirmed *Bacillus subtilis* by showing rod shaped green colour spore forming cells under microscopic observation (Plate 2). Based on the microscopic and cultural characteristics, Preeti and Rawat [23] also identified four isolates as *Pseudomonas* spp. and others as *Bacillus subtilis*.

Table 1. *Bacillus* spp. isolated from the rhizosphere soils of different crops collected from different parts of Telangana

Isolate	Place of collection	District
B-ISO-1	Allipuram	Khammam
B-ISO-2	Kodumuru	Khammam
B-ISO-3	Raghavapuram	Khammam
B-ISO-4	Arepally	Warangal
B-ISO-5	Oglapur	Warangal
B-ISO-6	Balanaiktanda	Warangal
B-ISO-7	Gundlapalli	Karimnagar
B-ISO-8	Timmapur	Karimnagr
B-ISO-9	Wyra	Khammam
B-ISO-10	Rajendranagar	Rangareddy

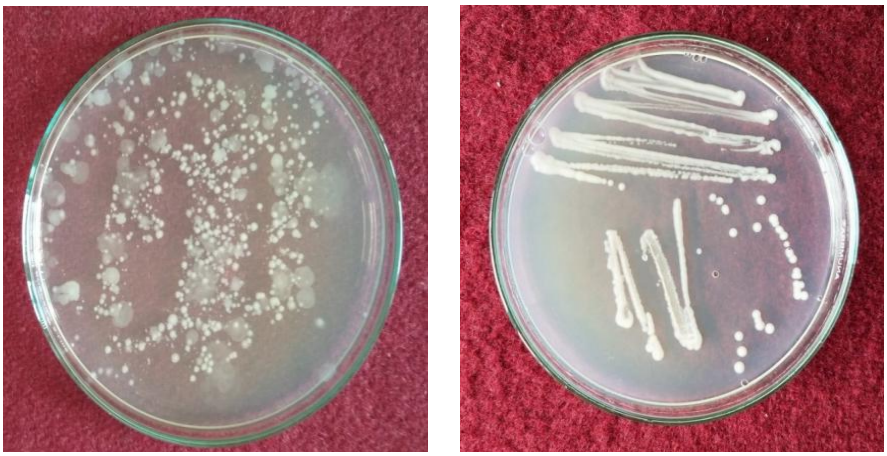
Table 2. Efficacy of isolates of *Bacillus subtilis* against mycelial growth of *Fusarium verticillioides* (F-ISO-7) *In vitro*

S. No	Isolate	Mycelial growth (cm)* at 10 DAI	Growth reduction over control (%)
1	B-ISO-1	4.32	49.3
2	B-ISO-2	3.17	62.8
3	B-ISO-3	3.13	63.3
4	B-ISO-4	5.22	38.8
5	B-ISO-5	4.80	43.7
6	B-ISO-6	3.30	61.3
7	B-ISO-7	5.41	36.5
8	B-ISO-8	5.61	34.2
9	B-ISO-9	3.40	60.1
10	B-ISO-10	5.33	37.5
11	Control	8.53	-
CD (P=0.05)		0.034	-
SE(m) ±		0.012	-
C. V.		0.56	-

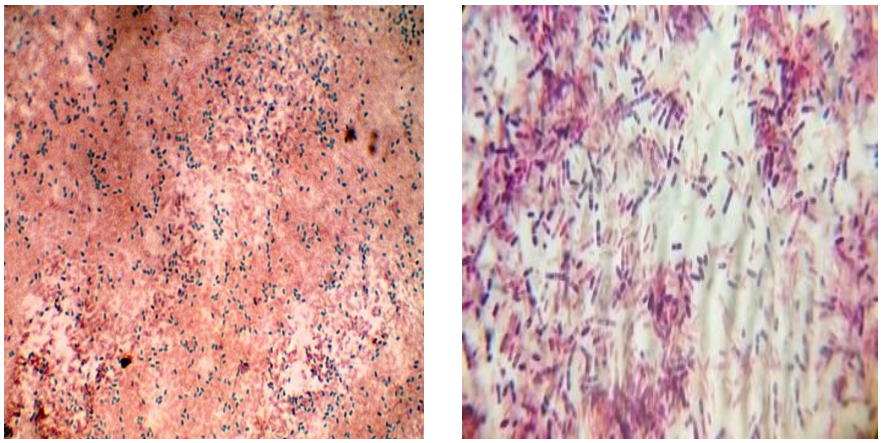
**Mean of five replications; DAI – Days after incubation*

1. B-ISO-1 2. B-ISO-2 3. B-ISO-3 4.B-ISO-4 5. B-ISO-5
6. B-ISO-6 7. B-ISO-7 8. B-ISO-8 9. B-ISO-9 10. B-ISO-10

Plate 1. *Bacillus* spp. isolated from the different rhizosp here soils



(a) Isolation of *Bacillus* spp. from soil by serial dilution method



(b) Gram staining **(c) Endospore staining**

Plate 2.

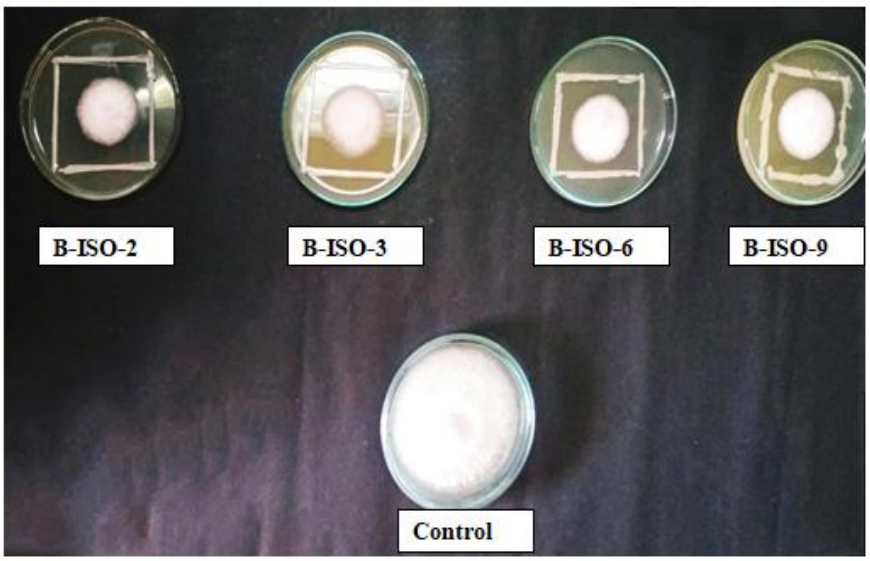


Plate 3. Efficacy of isolates of *Bacillus subtilis* against *Fusarim verticillioides* (F-ISO-7) *in vitro*

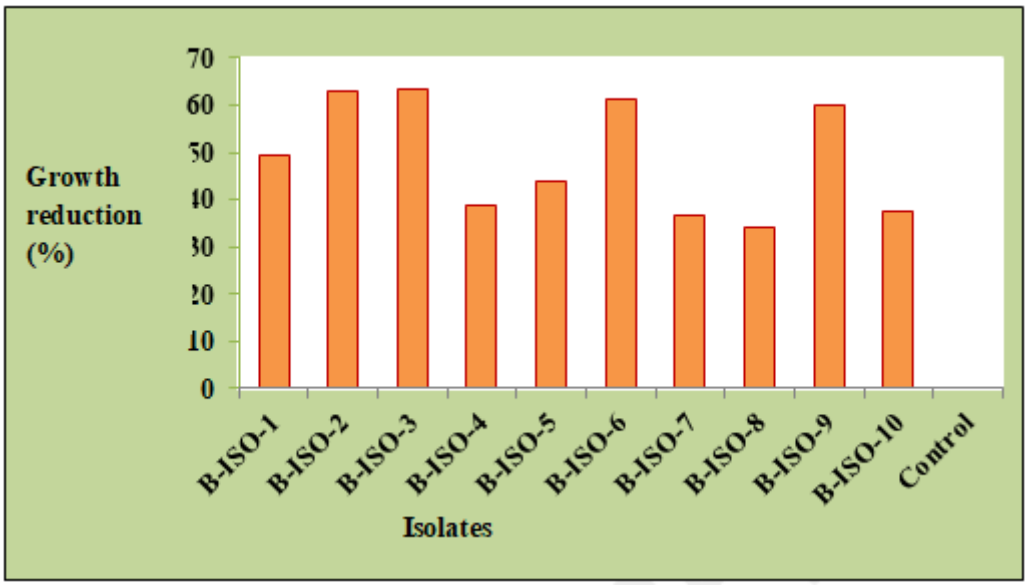


Fig. 1. Efficacy of isolates of *B. subtilis* against mycelial growth of *F. verticillioides*(F-IS0-7) *in vitro*

3.3 Efficacy of Isolates of *Bacillus subtilis* against *Fusarim verticillioides*

Out of ten isolates of *Bacillus subtilis* tested for their antagonistic activity against *Fusarim verticillioides* by dual culture technique (Plate 3), B-ISO-3 and B-ISO-2 were found to record significantly highest per cent reduction of mycelial growth 63.3 and 62.8 % respectively, followed by B-ISO-9 which recorded 61.3%t reduction of mycelial growth over control. The lowest per cent reduction of mycelial growth was recorded with the isolate B-IS0-8 (34.2 %) over control (Table 2) (Fig. 1).

Cavaglieri et al. [24] reported antibiosis produced by 10 *Bacillus* strains on *Fusarimverticillioides* M7075 ranged between 28-78%, *Bacillus* sp.3and *Bacillus* sp. CE 1 produced the greatest antfungal activity. Franscisco et al. [25] reported that *B. pumilus* and *B. liquefaciens* also recorded significantly higher inhibitory effects and strong growth inhibition on *F. verticilliodes*. Zaim et al. [26] recorded the antifungal activity of five isolates of *Bacillus* spp. *viz*., Rb29, Rb6, Rb12, Rb4, and Rb15 on two isolates of *F. oxysporum*f.sp. *ciceris.* The inhibitory effect against FOC1 ranged from 25.63 to 71.11% and on FOC2, from 28.43 to 60.65% *in vitro.* He also suggested that local isolates of *Bacillus* spp. have a prospective use as biological control agents to protect chickpea plants against chickpea wilt caused by *F. oxysporumf.* sp. *ciceris.* Sukanya et al. [27] reported *Bacillus subtilis* isolate BAS114 with highest inhibitory activity against *Fusariumoxysporum* in dual culture.

4. CONCLUSION

All the *Bacillus* strains used *in vitro* experiments inhibited the *Fusarium vertcillioides* mycelia growth. However, the degree of antagonism of the strains for a *F. verticilliodes* pathogen was varied and the mycelia growth inhibition degree depended on the *Bacillus* spp encourages us for more specific selection and field use. Conducted research justify the use of the *Bacillus subtilis* strainsin biological control of diseases caused by phytopathogenic microorganisms.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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