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Antagonistic effect of the newly isolated PGPR *Bacillus* spp. on *Fusarium oxysporum*

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Abstract: Soil borne plant pathogens cause annual economic losses in most of the crops. In nature microbial interactions involve competition, hyper parasitism or antibiosis and these phenomena play an important role in striking ecological balance and keeping several plant pathogens in check. It is observed that plant growth promoting rhizobacteria (PGPR) influence the growth and yield of many plants. In this study, isolates of *Bacillus*, one of the important PGPR, were isolated from different rhizosphere soils. Seven isolates of *B. megaterium* JUMB1, JUMB2, JUMB3, JUMB4, JUMB5, JUMB6 and JUMB7 were screened *in vitro* for their plant growth promoting traits like production of indole acetic acid (IAA), ammonia, HCN, phosphate, siderophore and evaluated for the ability to suppress fusarial growth. All the isolates were able to produce IAA, ammonia, HCN and siderophore but none of the isolates solubilized phosphorous. Production of IAA and siderophore was highest in the isolate JUMB3 (127 µg/ml and 124% respectively) and lowest in JUMB7 (35µg/ml and 44% respectively). *In-vitro* screening for antagonism against *F. oxysporum* revealed significant inhibitory effects on mycelial radial growth by all the seven isolates. Among seven isolates JUBM5 showed highest inhibition of 3.25, 0.22 and 0.21 cm in well diffusion, streak and point inoculation method respectively.

Key words: PGPR*, Bacillus,* antagonistic effect*, Fusarium oxysporum.*

1. Introduction

Fungal plant pathogens are among the most important factors that cause serious losses to agricultural products annually (Ekundayo et al. 2011). Management of fungal diseases using antagonistic microorganisms, known as biological control, has been the focus of intense research worldwide (Killani et al. 2011). Biological control of plant pathogens is considered as a viable alternative method to chemical control. Nonpathogenic soil bacteria, the plant growth promoting rhizobacteria (PGPR) living in association with roots of higher plants enhance the adaptive potential of the hosts and increase their growth through a number of mechanisms (Gholami et al. 2009; Zongzheng et al. 2009; Heydari and Pessarakli 2010; Killani et al. 2011; Farhana et al. 2011; Saharan and Nehra 2011). Gram-positive bacteria offer a biological solution through formulations as they form heat- and desiccation-resistant spores (Emmert and Handelsman 1999). Among them, *Bacillus* spp. is recognized as a powerful tool. Species of *Bacillus* are able to synthesize more than 60 different types of antibiotics which also act as plant growth promoters (Zongzheng et al. 2009; Girish et al. 2010; Nihorimbere et al. 2010). *Bacillus* spp. have showed significant inhibitory activity against many plant pathogens including *Ceratocystis ulmi* (Gregory et al. 1986), *Puccinia pelargonii-zonalis* (Rytter et al. 1989), *Euthypa lata* (Ferreira et al. 1991), *Fusarium moniliforme* (Agarry et al. 2005), *Phytophthora capsici* (Jo 2005), *P. cinnamomi* (Aryanta and Guest 2006), *Colletotrichum gloeosporoides*, *Botrytis cinerea, Monilinia laxa*, *Sclerotium rolfsii* (Prapagdee et al. 2008), *Colletotrichum musae* (Alvindia and Natsuaki 2009) and *F. oxysporum* (Nikam et al. 2011). Species of

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Bacillus have also been known to produce compounds which promote plant growth directly or indirectly *viz*., hydrogen cyanide (HCN), siderophores, indole acetic acid (IAA), solubilize phosphorous and antifungal activity (Godinho et al. 2010; Saharan and Nehra 2011; Sharafzadeh 2012; Wahyudi et al. 2011; Ibiene et al. 2012).

Fusarium is a well distributed large genus of filamentous saprophytic fungus affecting plant, animal and human health as they enter the food chain (Agrios 1988; Smith et al. 1988). They produce toxins, fumonisins and trichothecenes. *F. oxysporum* has a variety of hosts that include sugarcane, garden beans, cowpeas, potatoes, banana, water melon, prickly pear, tomato, cucumber, pepper, muskmelon, tobacco, cucurbits, sweet potatoes, asparagus, vanilla, strawberry and cotton (Naik et al. 2010; Nikam et al. 2011).

Evidence shows the intervention of rhizosphere microorganisms as protective agents against soil-borne plant pathogens (Lowe et al., 2012). In many instances a correlation has been established between antagonist *in vitro* and protection in the field (Pal and Gardner 2006). The present study has been taken up with the objective to isolate and characterize the isolated *Bacillus* spp., for their plant growth promoting by various mechanism and their antagonistic effect on *F. oxysporum.*

2 Materials and Methods

2.1 Isolation

Soil samples from the rhizospheres of rice, chilly, ragi and beans from regions around Bangalore were collected. *Bacillus* species were isolated using dilution method (Alexander 1965) and stored on nutrient agar (NA) at 4^{o}C. Isolates were identified as belonging to the genus *Bacillus* biochemically as per Holt et al. (1994).

2.2 Plant growth promoting characteristics of *Bacillus* isolates

The isolated *Bacillus* colonies were analyzed for their plant growth promoting characteristics *viz.,* production of IAA, ammonia, siderophores, HCN and their ability to solublize phosphates.

2.2.1 IAA Analysis

a) Qualitative Analysis

Isolates were cultured on NA medium amended with L-Trp and overlaid with cellulose membrane and incubated for 48h (Brick et al. 1991). Salkowski's reagent was added on the cellulose membrane after 48h of incubation. Pink colouration indicated production of IAA. The results were also analyzed visually on a three point scale (+ - low; ++ - medium and +++ - high).

b) Quantitative Analysis

Production of IAA was analyzed as per Patten and Glick (2002). Spectroscopic analysis was done at 520 nm and quantified using a tryptophan standard curve.

2.2.2 Phosphate Solubilization

The ability of the isolates to solubilize tri-calcium phosphate was observed as per (Wahyudi et al. 2011). Pikovskaya's Agar was inoculated with the isolates and incubated at 36±2°C for five days. Formation of halo indicated phosphate solubilization.

2.2.3 Production of Ammonia

Bacterial isolates were tested for ammonia production in peptone water using Nessler's reagent (Joseph et al. 2007). Development of brown yellow color was a positive test for ammonia production.

2.2.4 Production of hydrogen cyanide

All the *Bacillus* isolates were screened for HCN production using nutrient broth amended with glycine (Lorck 2004).

2.2.5 Siderophore detection in liquid medium

From overnight cultures, a sample of 10 µL of cell suspension was inoculated onto Luria Bertani (LB) broth (pH 7.2) supplemented with iron (10µmol/L $FeCl_3$) and tryptophan (10µmol/L) without any exogenous amino acids. After 48 h of incubation, the bacterial cells were pelleted by centrifugation at 4,000 rpm for 15 min at 4°C. Absorbance spectra were determined from 200 to 800 nm using a UV-Visible spectrophotometer (Hu and Xu 2011). The siderophore production by the *Bacillus* isolates was determined using King's B broth as per Van Peer et al. (1990). A reference was prepared using, uninoculated King's B broth medium. Both the test and reference were read at 380 nm and percent siderophore units in the culture filtrate were calculated.

$$\text{Percent Siderophore Units} = \frac{A_r - A_s}{A_r} \times 100$$

Where, A_r = Absorbance of reference at 380 nm and A_s =Absorbance of test sample at 380 nm.

2.2.6 Hydrogen Sulphide Production

SIM agar medium tubes were stab inoculated by *Bacillus* isolates and incubated for 24-48h at 37±2°C (Clarke, 1953). Tubes were observed for presence or absence of black coloration along the line of stab inoculation indicating hydrogen sulphide production.

2.3 Production of hydrolytic enzymes

The isolates of *Bacillus* spp. possessing the growth promoting characteristics were further characterized biochemically through tests for the production of oxidase, amylase, catalase, protease, gelatinase, urease, lipase, cellulase. The isolates were also tested for their ability to carry out nitrate reduction and carbohydrate fermentation. The biochemical characterization has been carried out as per Cappucino and Sherman (1992).

2.4 Isolation of *Fusarium oxysporum*

Fungi were isolated from garden soil by serial dilution method (Alexander 1965). *Fusarium* species were isolated based on its colony and morphological characteristics (Singh et al. 1991). Isolated colonies were sub cultured onto potato dextrose agar (PDA) slants and stored at 4 ^{o}C. Six day old cultures were used throughout the study.

2.5 Mycelial growth inhibition

Identified bacterial isolates were tested for mycelial growth of *F. oxysporum* inhibition by well diffusion, streak method and point inoculation methods.

2.5.1 Well diffusion method

Fungal spore suspensions were prepared in 0.85% sterile saline. Ten-mL melted solid water-agar medium was allowed to solidify in sterile petriplates. In 10-mL of sterile melted SDA cooled to 30-40 ^{o}C, one millimeter of fungal spore suspension was added mixed well and poured onto solidified water-agar medium plates. A well was punched on the solidified agar and 50 µL *Bacillus* suspensions was put into the well except for the controls that had 50 µL sterilized saline instead. The plates were incubated at 28±2^{o}C for seven days. After seven days fungal inhibition ring were detected and recorded as per Zongzheng et al. (2009).

2.5.2 Streak method

Bacillus isolates were streaked on nutrient agar medium and incubated at 35±2^{o}C for 24h, after which plates were streaked with six day old culture of *F. oxysporum* opposite to bacteria by point inoculation. The control plate was point inoculated with *F. oxysporum*. The plates were sealed with parafilm and kept at 28±2°C for five days (Nelson 2004). Antagonistic activity was investigated for four to seven days after incubation at room temperature (28±2°C).

2.5.3 Point Inoculation method

Each of the bacterial isolates was point inoculated at four sides, 3cm from the center of the plate and incubated at 35$\pm$2^{o} C for 24 h. After 24h, six day old culture of *F. oxysporum* was point inoculated in the center of the plate. Control plate was only point inoculated with *F. oxysporum*. The plates were sealed with parafilm and incubated at 28±2°C for 4-5 days (Kumar et al. 2002). Antagonistic activity was investigated for four to seven days after incubation at room temperature (28±2°C).

2.6 Statistical Analysis

All the experiments were carried out in triplicates. Means of all the parameters were calculated along with standard error (SE) and standard deviation (SD). Results were expressed as Mean$\pm$SD.

3 Results and Discussions

3.1 Isolation

Nineteen rhizosphere samples from various plant *viz*., ragi, brinjal, paddy, tomato, beans, mango, chilly, lady's finger and marigold were collected from different regions in and around Bangalore (Karnataka) and Krishnagiri (Tamil nadu). Results of serial dilution showed various colonies of different nature. Colonies with gram positive bacilli, showed endospore formation produced catalase, fermented mannitol and negative for VP test were considered for further study. Seven isolates were selected for the further studies and named as JUBM1; JUBM2; JUBM3; JUBM4; JUBM5; JUBM6 and JUBM7. Isolates of *Bacillus* spp. from soil were identified as *B. megaterium* based on the morphological and biochemical tests as per Bergey's manual of Determinative Bacteriology (Holt et al. 1994)**.**

3.2 Plant Growth Promoting Characteristics of *Bacillus* Isolates

3.2.1 Production of IAA

a) Qualitative Analysis of IAA

After 48h of incubation, the cellulose membrane was removed and treated with Salkowski's

reagent. Observations showed that the membrane turned pink indicating the production of IAA. Cellulose membrane placed on cultures of all the isolated species of *Bacillus* showed pink coloration but with varying intensity. Visual observation showed that JUBM 5 showed maximum colorations whereas JUBM 7 culture showed the least (Table 1).

Table 1: Shows the qualitative analysis of indole acetic acid based on visual observation

Bacillus isolates	JUBM1	JUBM2	JUBM3	JUBM4	JUBM5	JUBM6	JUBM7
Intensity	++	++	++	++	+++	+++	+

Note: **+** - low intensity, **++** - medium intensity, **+++** - high intensity

b) Quantitative Analysis of Indole Acetic Acid (IAA)

When the culture extracts of all the *Bacillus* isolates were mixed with 2 ml of Salkowski's reagent and incubated at room temperature 28±2°C for 30 min it was observed that all the isolated *Bacillus* spp. produced IAA but in varying quantities. It was observed that JUBM 1, 2, 3, 4, 5, 6 and 7 synthesized 65, 127,132, 65, 217,150 and 35µg/ml respectively as shown in the **Fig 1**.

Diverse bacterial species possess the ability to produce IAA (Ashrafuzzaman et al. 2009; Saharan and Nehra 2011). It functions as an important signal molecule in the regulation of plant development and indirectly by influencing bacterial amino cyclopropane-1-carboxylate (ACC) deaminase activity (Ryu and patten 2008; Wahyudi et al. 2011). Production of IAA has been shown in species of *Bacillus, Pseudomonas, Azotobacter, Azospirillium, Phosphobacteria, Glucanoacetobacter, Aspergillus niger* and *Penicillium*. It has been observed that the role of bacterial IAA in different plant-microbe interactions highlights the fact that bacteria use this phytohormone to interact with plants as part of their colonization strategy, including phytostimulation and circumvention of basal plant defense mechanisms (Ahmad et al. 2008; Samuel and Muthukkaruppan 2011; Patel et al. 2012).

It has been reported that *B. megaterium* from tea rhizosphere produces IAA and thus helps in plant growth promotion (Chakraborty et al. 2006). Tryptophan increases production of IAA in *B. amyloliquefaciens* FZB42 (Idris et al. 2007). IAA production, even in the culture without tryptophan supplementation has also been reported (Wahyudi et al. 2011). It has also been reported by Patten and Glick (2002) that the enzyme indolepyruvic decarboxylase (IPDC) is the principal enzyme which determines IAA biosynthesis and stimulates the development of the root system of the host plant (Erturk et al. 2010; Patel et al. 2012). It has been reported that IAA production by PGPR can vary among different species and strains and is also influenced by culture condition, growth stage and substrate availability (Ashrafuzzaman et al. 2009).

3.2.2 Phosphate solubilization

It was observed that Pikovaskaya's agar, when inoculated with the isolates of *Bacillus* did not show any clear zone of hydrolysis around and the bacterial growth, which indicated their inability to solubilize phosphates. Ashrafuzzaman et al. (2009) showed that phosphate were not able to solubilize by all the seven *Bacillus* isolates, isolated from rice rhizosphere and Wahyudi et al. (2011) showed that one out of twelve isolates of *Bacillus* from soybean rhizosphere were not able to solubilize the phosphorus.

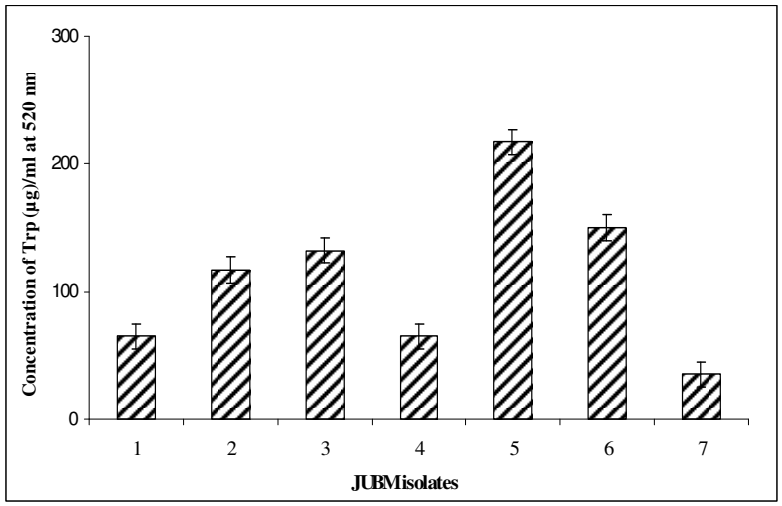


Figure 1: Shows the quantification (µg/ml) of indole acetic acid in the various isolates of *Bacillus* species

3.2.3 Production of Ammonia

Development of yellow-brown color was observed after addition of Nessler's reagent indicating a positive test for ammonia production. It has been reported that ammonia production indirectly influences the plant growth. *B. subtilis* strain MA-2 and *Pseudomonas fluorescens* strain MA-4 was efficient in ammonia production and significantly increased biomass of medicinal and aromatic plant such as *Geranium* (Mishra et al. 2010). However, ammonia production was observed less frequently in *Azotobacter* isolates. Ammonia production was detected in 95% of the isolates from the rhizosphere of rice, mangrove and effluent contaminated soil influencing plant growth promotion (Joseph et al. 2007; Samuel and Muthukkaruppan 2011).

3.2.4 Production of Hydrogen Cyanide (HCN)

Production of HCN was indicated by change in color of the filter paper to red. It is reported that HCN indirectly influences plant growth promotion. Isolate from rhizosphere of rice, mangrove, chicken pea and effluent contaminated soil showed HCN production (Joseph et al. 2007; Samuel and Muthukkaruppan 2011).

3.2.5 Siderophore Production

Siderophore production of a particular organism has been studied with minimal medium supplemented with amino acids, which have suggested that siderophore production is substrate dependent. From our results, growth reaction was significantly affected by the pH of the medium. When iron concentrations are in micro-molar concentration, siderophore-dependent, high affinity Fe-uptake systems are sufficient for obtaining iron from the environment. A strong absorption peak was observed between 350-400nm wavelengths.

Spectrophotometric estimation of siderophore production at 380 nm was carried out for seven days at 24h interval. In the course of siderophore production, maximum siderophore secretions by all *Bacillus* isolates was recorded on the 2nd and 3rd day, thereafter, a decline in percent siderophores production was observed. Of all the isolates JUMB3 showed highest siderophore production of 124% followed by JUMB1, JUMB5, JUMB2, JUMB4, JUMB6 and JUMB7 which showed 119, 119, 104, 87, 76 and 44 respectively (Figure 2).

The siderophores are produced by various bacteria and fungi. Siderophores are usually classified by the ligands used to chelate the ferric iron. The major groups of siderophores include the catecholates (phenolates), hydroxamates and carboxylates (e.g. derivatives of citric acid). Soil bacterial isolates including *Azotobacter vinelandii* MAC 259, *Pseudomonas* and *Bacillus cereus* UW 85 produce siderophores which can be used as efficient rhizobacteria to increase the crop yield (Husen 2003). The bacterial growth as well as siderophore production is stimulated by ammonium sulphate and amino acids. However, the optimum siderophore yield is obtained using urea (Sayyed et al. 2005).

Siderophores directly stimulate the biosynthesis of other antimicrobial compounds by increasing the availability of these minerals to the bacteria, would suppress the growth of pathogenic organisms *viz., F. oxysporum* and *R. solani*, function as stress factors in inducing host resistance (Haas and Defago 2005; Joseph et al. 2007; Wahyudi et al. 2011). *B. megaterium* from tea rhizosphere produces siderophores which helps in plant growth promotion and disease reduction (Chakraborty et al. 2006).

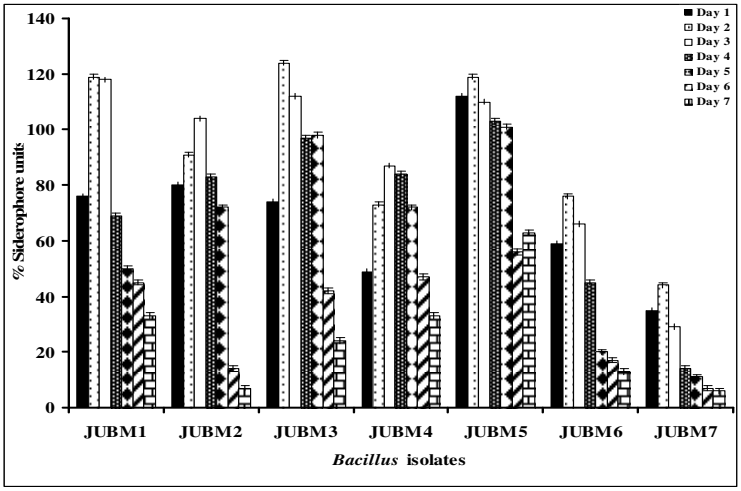


Figure 2: Shows the production of siderophores by the isolated species of *Bacillus* isolates from day1-7 after inoculation. Bars indicate standard error

3.3 Characterization of *Bacillus*

The bacterial isolates were gram-positive rods. Cultures on NA showed irregular, entire cream colored colonies. The isolates were positive for citrate, gelatin, starch, casein hydrolysis and production of catalase, organic acids and oxidase. They also utilize carbon sources viz., mannitol, glucose and lactose. They were negative for voges-proskauer test, production of indole, hydrogen sulphide and utilization of lipids and cellulose.

3.4 Isolation of *Fusarium oxysporum*

Fungal identification was done based on colony (macroscopic) morphology and microscopic characteristics as per Singh et al. (1991). On PDA, fungal colony appeared as cream colored cottony growth and was pink on the reverse side. Microscopic observation showed *F. oxysporum* septate, dark hyphae with spherical, slightly curved, unicellular and hyaline microconidia macroconidia were falcate and three- four septate.

3.5 Antagonistic assay

A comparative study between three different types of assay was carried out to evaluate the antagonistic

effects of *Bacillus* using the well diffusion, streak and point methods (Figure 3).

3.5.1 Well diffusion method

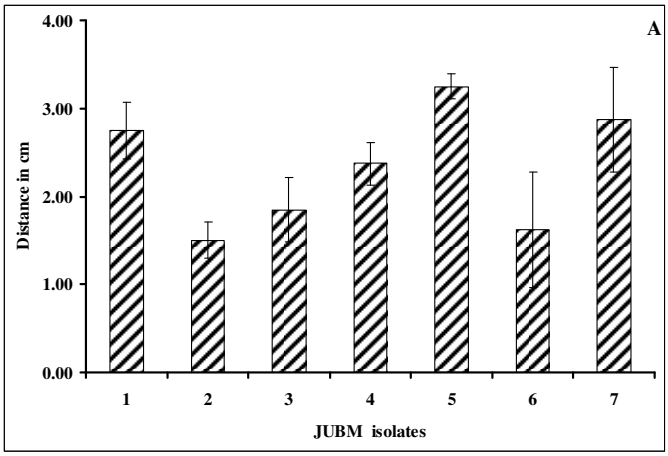
Bacillus isolates showed the certain degree of antagonism to *F. oxysporum.* The antifungal activity of *Bacillus* isolates are shown in Figure 3A. When *Bacillus* isolates were inoculated simultaneously with the fungal pathogen, they established mycelia contact after fifth day but this was later stopped on day seven with the average growth diameter measuring 2.75, 1.50, 1.85, 2.38, 3.25, 1.63 and 2.88cm in JUBM 1, 2, 3, 4, 5, 6 and 7 respectively. Statistical analysis showed that JUBM5 had highest antagonism whereas JUBM7 showed the least.

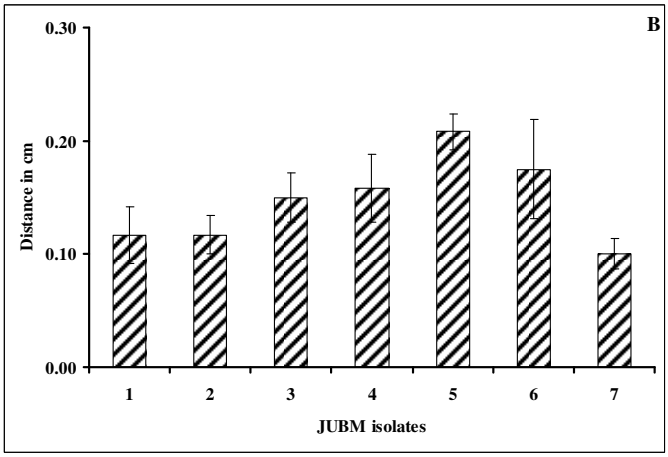
3.5.2 Streak method

The average diameter size indicates the antagonistic activity of *Bacillus* isolates against *F. oxysporum by* streak method on SDA medium *in-vitro*. The antifungal activity of *Bacillus* isolates were shown in Figure 3B. Statistical analysis revealed that *Bacillus* isolates inhibited growth to an average zone of inhibition of 0.12, 0.14, 0.15, 0.14, 0.22, 0.14 and 0.13cm in diameter, respectively after fifth day of inoculation. Among seven isolates JUBM 5 showed maximum inhibition as per statistical analysis.

3.5.3 Point Inoculation method

The *Bacillu*s isolates were evaluated for their antagonistic effect against *F. oxysporum* in-*vitro.* A graph was plotted to obtain significant results. JUBM5 showed maximum inhibition and JUBM7 showed least inhibitory effect as per statistical analysis. The antifungal activity of *Bacillus* isolates were shown in Figure 3C.





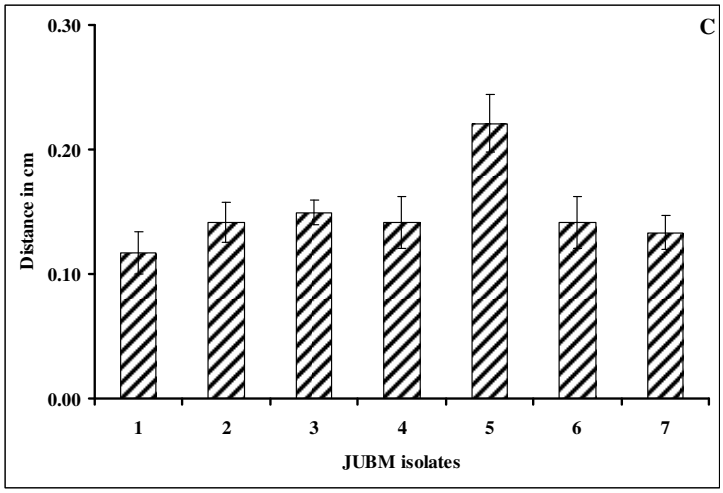


Figure 3: Shows the antagonism of the isolated *Bacillus* species against *Fusarium oxysporum* by well diffusion assay (A); streak method (B) and point inoculation assay (C)

4. Conclusion

To conclude, *Bacillus* isolates controlled *F. oxysporum* growth irrespective of the antagonistic method used. The clear zone of inhibition produced in the *in-vitro* experiment is an indicative of antibiosis by the biocontrol agent against the fungal pathogens. *In vitro* evaluation of the antagonist's showed strong antagonistic activity in well diffusion assay compare to streak assay and point inoculation. Present investigation studies on the usage of biocontrol agents for the management of *F. oxysporum* revealed that, all the *Bacillus* isolates reduced the growth of *F. oxysporum*. Among them, JUBM5 effectively reduced growth of *F. oxysporum* showed highest inhibition of 3.25, 0.22 and 0.21 cm respectively.

This result was contrary to that of Killani et al. (2011), who reported that *B. subtilis* successfully inhibited the growth of all the root-/soil-borne fungal pathogens isolated from cowpea *in-vitro*. *B. subtilis* significantly reduced the growth of *F. oxysporum* f. sp. *vanillae in vitro* under green house and field conditions as reported by Naik et al. (2010). *B. subtilis* SY1 had a certain degree of antagonism to all fungal pathogens and host plants improved after inoculation. In the seedling test, sprout tendency, accumulative germination percentage, sprout index and vigour index of seeds increased 24, 24, 35 and 64%, respectively (Zongzheng et al. 2009).

The biological control of plant diseases is one of the viable alternatives to chemical control in sustainable agriculture (Killani et al. 2011). The various reasons for the inhibitory activity of *Bacillus* may have been the active enzymes, toxic metabolites from *Bacillus* spp. that can damage the fungal cellular walls. Bacterial exo-chitinases and glucanases may have an important antagonist role against fungi (Machado et al. 2010).

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