

**EVALUATION OF ANTAGONISTIC POTENTIAL
OF PHYLLOPLANE *Bacillus subtilis* ISOLATES
AGAINST FOLIAR FUNGAL PATHOGENS**

THESIS

**Submitted to
Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola
in partial fulfilment of the requirements
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**MASTER OF SCIENCE
IN
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(PLANT PATHOLOGY)**

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DECLARATION OF STUDENT

I hereby declare that, the experimental work and its interpretation of the thesis entitled "**EVALUATION OF ANTAGONISTIC POTENTIAL OF PHYLLOPLANE *Bacillus subtilis* ISOLATES AGAINST FOLIAR FUNGAL PATHOGENS**" or part thereof has neither been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis / publication of any University or scientific organization. The source of materials used and all assistance received during the course of investigation have been duly acknowledged.

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CERTIFICATE

This is to certify that the thesis entitled “**EVALUATION OF ANTAGONISTIC POTENTIAL OF PHYLLOPLANE *Bacillus subtilis* ISOLATES AGAINST FOLIAR FUNGAL PATHOGENS**” submitted in partial fulfillment of the requirements for the degree of “**Master of Science in Agriculture (Plant Pathology)**” of Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola is a record of bonafide research work carried out by **MASRAM SAYALI DEVWRAT** under my guidance and supervision.

The subject of thesis has been approved by the Student’s Advisory Committee.

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D) List of Abbreviations

%	Per cent
/	per
@	At the rate
<	Lesser than
>	Greater than
μ	Micron
μm	Micrometre
°C	Degree Centigrade
Agril.	Agricultural
BOD	Biological oxygen demand
Bp	Base Pair
Bs	<i>Bacillus subtilis</i>
CAS	Career Advancement Scheme
Conc.	Concentration
DAI	Days after inoculation
DAS	Days after sowing
DNA	Deoxyribonucleic acid
Dr. PDKV, Akola	Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola
e.g.	For example
<i>et al.</i>	et alia (and others)
etc.	Etcetera
f. sp.	<i>formae specialis</i>
Fig.	Figure
Hr.	Hour (s)
<i>i.e.</i>	That is
Kg ha ⁻¹	Kilogram per hectre
Ltd.	Limited
Mbp	Million Base Pair
MgCl ₂	Magnesium chloride
ml	Millilitre (s)
Mm	Millimetre

mM	Mili Molar
NA	Nutrient agar
NaCl	Sodium Chloride
NaOCl	Sodium Hypochlorite
No.	Number
PBs	Phylloplane <i>Bacillus subtilis</i>
PDA	Potato dextrose agar
Pvt.	Private
Sig	Significant
Sr. No.	Serial number
<i>Taq</i>	<i>Thermus aquaticus</i>
U/ μ l	Unit per micro litre
UV	Ultra Violet
Viz.	Videlicet (namely)

(F) Thesis Abstract

- a) Title of the thesis : **EVALUATION OF ANTAGONISTIC POTENTIAL OF PHYLLOPLANE *Bacillus subtilis* ISOLATES AGAINST FOLIAR FUNGAL PATHOGENS**
- b) Full name of student : **Masram Sayali Devwrat**
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ABSTRACT

The *Bacillus* spp. are classified in the order Eubacteriales and family Bacillaceae. Almost all the species are gram positive and produces the endospores.

Bacillus subtilis are resistance only to Penicillin, amoxylin and ampicillin antibiotics. The antibiotics obtained from culture of certain *B. subtilis* strains are known to produce subtilin, bacillin, subtenolin, bacillomycin etc. Iturin antibiotics generated by numerous *B. subtilis* strains are compound that exhibit strong antifungal activity against most pathogenic fungi and several bacterial species.

Bacillus subtilis are exist as epiphytes or endophytes in environment and may provide plant with protection against pathogens attack by a blend of diverse mode of action.

The present study was undertaken to conduct collection of samples, isolation, identification, study of different character of *B. subtilis*, morphological variability, cultural variability, biochemical variability of selected isolates and *in vitro* evaluation against foliar fungal pathogens.

The isolates of the *Bacillus subtilis* from different six crops phylloplane such as cotton (PBs1), chilli (PBs2), sorghum(PBs3), pigeon pea (PBs4), soybean (PBs5), rice (PBs6). The “Evaluation of Antagonistic potential of phylloplane *Bacillus subtilis* isolates was carried out against foliar fungal pathogens.”

All the six isolates showed positive reaction to gram reaction, starch hydrolysis, H₂S and acid production, catalase test, gelatin liquifaction, starch hydrolysis production test except PBs3 showed negative reaction. All six isolates found negative for gas production, indole production, MR test and KOH test.

Isolates tested against various fungal pathogens by dual culture method. Minimum mycelial growth (16.83mm) with maximum mycelial growth inhibition (78.80%) of *Alternaria alternata*, *Curvularia lunata*, *Myrothecium roridum* and *Colletotrichum dematium* was recorded in *B. subtilis* isolate PBs1 followed by PBs6 (74.91%) and PBs2 isolates (73.28%). The other isolates of *B. subtilis* i.e. PBs3, PBs5 and PBs4 were found least effective against all the fungal pathogens tested.

CHAPTER I

INTRODUCTION

1.1 Background information

The *Bacillus spp.* are classified in the order eubacteriales and family Bacillaceae. Almost all the species are gram positive. Mostly gram positive *Bacillus* cells produce the spores may be cylindrical, ellipsoidal or spherical and located centrally, subterminally or terminally. Bacteria belonging to the genera *Bacillus* are considered to be safe microorganisms and hold the remarkable abilities of synthesizing a vast array of beneficial substances for agronomical and industrial purposes (Stein, 2005) and producing endospores, which warrant the prevalence of *Bacillus* under different environmental cues, its long-term storage and easy development of reliable formulations (Collins and Jacobsen, 2003).

Many of these *Bacilli* are soil-inhabiting bacteria and exist as epiphytes or endophytes (Sneath 1986; McSpadden Gardener, 2004) in environments as diverse as spermosphere (Walker et al., 1998), Brassica leaves (Leifert et al., 1992) or compost (Phae et al., 1990), and may provide plants with protection against pathogen attacks by a blend of diverse modes of action (Rampach and Kloepper ,1998; Shoda, 2000; Romero et al., 2004). These features have led to the increased devising and implementation of antimicrobial active biological products based on *Bacillus species* or their metabolites as alternative or supplementary methods to chemicals for plant disease control (Leifert et al., 1995; Raaijmakers et al., 2002; Schisler et al., 2004; Ongena et al., 2005).

Microorganisms from phylloplane have received considerable attention as potential protecting agent against foliar fungal pathogens. *B. subtilis* as bioagent have good prospectus in future for management of foliar fungal pathogens. The capacity and potential of the *Bacillus subtilis* to colonize the phylloplane can act as barrier for entrance of pathogen. Thus, objectives of present studies may be helpful for management of foliar disease through bacterial bioagent, *B. subtilis*.

Among bacterial biocontrol agents *Bacillus* genus encompasses a large genetic biodiversity. *Bacilli* are present in an extremely large palette of environments ranging from sea water to soil, and are even found in extreme environments like hot springs (Hoch et al., 1993). This bacterium could be one of the major sources of potential microbial biopesticides because it retains several valuable traits (Ongena & Jacques, 2008). *Bacilli*, such as *B. subtilis*, are a well-studied organism which facilitates their rational use. The US Food and Drug Administration (USFDA) has granted the "generally regarded as safe" (GRAS) status to *B. subtilis* which is thus recognized non-pathogenic (Harwood & Wipat, 1996). This of course essential regarding its application as a biopesticide. *Bacilli* have the capacity to produce spores (Piggot & Hilbert, 2004) which are extremely resistant dormant forms capable to withstand high temperatures, unfavourable pH, lack of nutrients or water, etc. They are produced by the bacteria when environmental conditions are unfavourable which probably helps these microorganisms to survive in the phylloplane.

1.2 Importance of study

Phylloplane isolates of *Bacillus subtilis* have been particularly useful as biocontrol agent increasing yields and stimulating plant growth and also responsible for increasing leaf area, chlorophyll concentration, NR activity (Nitrate Reductase) and total biomass production. It is most promising bio control agent against a range of plant pathogens under a variety of environmental conditions (Chen, et al.,1995). They are considered as biocontrol agent of foliar diseases (Mantecon, 2008).

Biological control is gaining momentum as an essential component. Amongst biological agents, at present *Bacillus subtilis* are receiving much attention. These bacteria have an ability to rapidly colonize the roots of host plants and compete with the soil borne pathogens for space and food. Keeping in view, present research work was undertaken to isolate and characterize the native strains of *B. subtilis* from phylloplane of crop plants to find out efficient strain of *B. subtilis* against foliar fungal pathogens.

1.3 Objectives of the study

Now days, regular use of biological systems in controlling plant disease has become more attractive due to added benefits of enhanced plant growth besides disease control. Such enhancement and disease control has been observed with *Bacillus subtilis*. Thus, in view of disease suppression ability of *Bacillus subtilis*, studies were carried out with the following objectives-

1. To study the antagonistic potential of phylloplane *Bacillus subtilis* isolates against foliar fungal pathogens.
2. To study the biochemical properties of phylloplane *Bacillus subtilis* isolates.

1.4 Hypothesis

Indiscriminate use of chemical pesticides for management of diseases has posed several serious problems such as pesticide residue, development of resistant strains, environmental pollution and adverse effect on beneficial microorganisms and created a greater concern over global food safety and security.

Biocontrol agents are proving an alternative to chemicals and exploration of new bioagents and their mass multiplication would be one of best option in managing the plant diseases. Such enhancement and disease control has been observed with *B. subtilis*. Due to strong antifungal activity, phylloplane *B. subtilis* used for management of foliar fungal pathogens of various crops.

1.5 Scope and limitation

The success of biocontrol strategies will depend to a large extent on the seeking and selection process of potential biological agents, which consider the pathogen to be the target and the cropping system.

These bacteria produce different extracellular enzymes like protease, amylase, lipase and cellulase. *Bacillus subtilis* beside its used as

industrially promising organism also used as biological control agent with special interest for basic and applied research. In agriculture it is found as a antifugal bioagent for beans, corn, wheat, and many other plants. Iturin antibiotics generated by numerous *B. subtilis* strains are compound that exhibit strong antifungal activity against most pathogenic fungi and several bacterial species (Maget-Dana and Peygpoux, 1994). Availability of effective strains of *B. subtilis* based on agroclimatic situation are some of the limitations in practical implementation of outcome of studies.

CHAPTER II

REVIEW OF LITERATURE

Studies on the “Evaluation of antagonistic potential of phylloplane *Bacillus subtilis* isolates against foliar fungal pathogens” were taken during 2018-2020. All the isolates of *B. subtilis* were characterized on the basis of morphological and biochemical analysis with their antagonistic potential against foliar fungal pathogens. The related information are reviewed in this chapter.

2.1 Isolation and morphological characteristics of *Bacillus subtilis*

Gurney and Quesnel (1980) observed morphology of *B. subtilis* var. nigar viz. circular, buffy white, malt textured colony with entire margin elevation.

Pretorius et al. (1986) recorded morphological characters of *Bacillus* sp. were chain, motility, round spores, ellipsoidal spores, cylindrical spores, central spores, subterminal, terminal, swollen spores vacuoles were used for numerical analysis.

Hiroshi and Mistsugu (1989) observed morphology of *B. subtilis* colonies were found to grow radially with almost regular branches with moist surface.

Wakita et al. (2001) observed that *B. subtilis* produce concentric ring-like colony.

Theoduloz et al. (2003) reported that *Bacillus subtilis* and *Bacillus licheniformis* strains, both are natural inhabitants of the tomato phylloplane.

Brian and McSpadden(2004) studied the population of culturable aerobic endospore forming bacteria present in the soybean, *Bacillus pumilus* and *Bacillus subtilis* reported to be most abundant bacteria cultured from the phylloplane.

Cazorla et al. (2007) reported that *B. subtilis* produced hydrolytic enzymes such as glucanases and antibiotics lipopeptides surfactins, fengycin or iturin.

Chen et al. (2008) reported three kinds of antibiotics isolated from *B. subtilis* and identified to the lipopeptide families, surfactin, iturin and fengycin. These compounds could function as biocontrol agents against a large spectrum of pathogens.

Islam et al. (2012) identified *B. subtilis* through its biochemical and physiological characteristics.

2.2 Isolation of foliar fungal pathogens for antagonistic studies

Obregon et al. (2013) studied the isolated *C. gloeosporioide* and grown *in vitro* on Potato dextrose agar (PDA) and pure culture was maintained in PDA slant. He also isolated *Ramularia*, *Cladosporium* and *Alternaria spp.* from safflower by using extraction of spore from lesion found on leaves.

Piyaboon et al. (2016) isolated *Myrothecium roridum* from leaf blight disease of water hyacinth by using tissue transplanting method.

Thilagam et al. (2017) reported five phytopathogenic fungi isolated and indentify from different plant parts. The fungal isolates were *Alternaria alternata* (Tomato early blight), *Fusarium oxysporum* (*Fusarium* wilt), *Fusarium solani* (Damping off and root rot) *Aspergillus flavus* (Ear rot) and *Colletotrichum spp.* (Anthracnose).

Chang et al. (2020) studied the isolated *Curvularia lunata* from Curvularia leaf spot disease on maize. The fungal isolates were subjected to morphological identification and 232 isolates were found 70% uncurved conidia and identified as *Curvularia lunata*.

2.3 Biochemical characteristics of *Bacillus subtilis* isolates

Willemse and Collinet (1980) used 24 biochemical test for a simple and rapid computer assisted technique for the identification of some selected *Bacillus sp.* The biochemical test shows VP positive test and 50

per cent gas production in glucose MR positive. Starch and gelatin hydrolysis negative.

Kathleen et al. (1983) reported that several organisms observed to be non cynogenic (*P. putida*, *Escherichia coli*, *Bacillus megaterium*, *B. subtilis*, and *Candida utilis*) gave negative results for cyanide production.

Gill et al. (1986) studied the morphological, physiological and biochemical characters namely growth at 7% NaCl, MR-VP test, Catalase test, Starch hydrolysis, Gelatin liquefaction, Acid production etc. They concluded that *B. subtilis* shows 100 per cent salt tolerance in broth and positive test for all above biochemical reaction.

Foldes et al. (2000) reported biochemical characters of *Bacillus* spp., isolates showed positive reaction to starch hydrolysis, gelatin liquefaction and negative reaction for indol production.

Zhang et al. (2008) reported that *Bacillus subtilis* isolates, showed positive reaction for starch hydrolysis, negative reaction to indole production.

Awais et al. (2010) studied morphological and biochemical tests of *Bacillus subtilis* isolates, showed positive reaction for gram staining, starch hydrolysis, catalase test, gelatin liquefaction, spore formation, oxidase test for acid production from glucose and negative reaction for gas production from glucose, indole production and MR test.

Khan et al. (2011) studied morphological and biochemical tests of *B. subtilis* and showed that *Bacillus subtilis* rod shaped, gram's positive and negative reaction to indol acetic acid production, positive to starch hydrolysis.

Karimi et al. (2012) carried out biochemical tests for *B. subtilis* and recorded positive reaction for starch hydrolysis.

Jadhav et al. (2014) recorded that the five isolates of *Bacillus subtilis* were rod shape and showed positive reaction for gram staining,

starch hydrolysis, gelatin liquefaction, H₂S production and phosphate solubilization.

XiaoHuaZhang et al. (2014) recorded that the *Bacillus subtilis* showed positive reaction to starch hydrolysis, gelatin liquefaction, nitrate reduction, catalase test, VP test, oxidase test and negative reaction to indole production.

Kapali et al. (2016) recorded *Bacillus subtilis* were found to be rod shaped spore forming motile bacteria. The isolates showed positive reaction to gram reaction, voges proskauer, citrate utilization, nitrate reduction, catalase test, oxidase test and negative reaction to IAA and methyl red test.

Tariq et al. (2016) carried out biochemical test of *Bacillus* spp., isolates showed positive reaction to starch hydrolysis, casein hydrolysis, gelatin liquefaction, voges proskauer, citrate utilization, nitrate reduction, catalase, oxidase test and negative reaction to indole test and methyl red test.

Rashmi et al(2017) reported biochemical character of bacillus spp., showed positive reaction to catalase test,MR test ,VP test and citrate test and some isolates showed negative reaction to indole test.

Mandla et al. (2017) reported *Bacillus subtilis* isolates, showed positive reaction for catalase test.

Singh et al. (2017) reported *Bacillus subtilis* isolates, showed positive reaction for gram reaction, VP test, nitrate reduction and negative reaction to MR test.

Zhenxiang et al. (2018) reported that the bacteria were gram positive spore chain *Bacillus*. The bacteria could only ferment glucose for acid production, but could not utilize lactose and maltose. The VP test for this bacteria was positive, while indole and methyl red tests were negative.

2.3 In vitro efficacy of *Bacillus subtilis* against foliar fungal pathogens

Backer et al. (1985) reported that the cultures of *Bacillus subtilis* and cell free filtrates thereof, provided better control of bean rust under greenhouse and coldframe condition. These bacterial cultures and filtrates prevented germination of the urediniospores and effective against all major races of bean rust on all bean cultivar tested.

Rytter et al. (1989) reported twelve strains of *Bacillus* isolated from the leaves of geranium cultivars and tested for their effect on spore germination of *Puccinia pelargonii-zonalis*, the causal agent of geranium rust. Of these, strain 3 of *B. subtilis*, isolated from a rust-infected geranium leaf, inhibited spore germination and reduced the incidence of rust pustules on inoculated leaves in the greenhouse.

Turner and Backman (1991) evaluated that treatment of peanut seeds with *B. subtilis* was associated with improved germination and emergence, increased nodulation by *Rhizobium spp.* AG4, and increased root growth.

Ferreira et al. (1991) studied that isolate of *Bacillus subtilis* from grapevine wood inhibited *in vitro* growth of *Eutypa lata*, the bacterium caused 91.4% inhibition of mycelial growth of *E. lata* and 100% inhibition of ascospore germination Douville and Boland (1992) studied the influence and mechanism of action of *Bacillus subtilis* on *Colletotrichum trifolli* causal agent of antracnose of Lucerne (*Medicago sativa*) *in vivo* and *in vitro*. *B. subtilis* reduced disease incidence and severity on lucern seedling from 16-50% and from 1.2-2 % respectively.

Dass and Teyegaga (1996) studied the antagonistic activity of *B. subtilis* against five wood decay fungi and five other fungi (*Candida sp.*, *Fusarium sp.*, *T. viride*, *P. digitatum*, *Curvularia geniculata*) on potato dextrose agar. All fungal species were inhibited by water soluble factors produced by the bacterium.

Selvarajan and Jayarajan (1996) screened eight fungi and three bacteria *in vitro* to select a suitable antagonist against chickpea root rot caused by *F. solani* and *Macrophomina phaseolina*. *T. viride*, *T. hamatum*, *Laetisaria*, *B. subtilis* and *P. fluorescens* formed inhibition zones

on PDA against both of the chickpea root rot pathogens. They reduced the sporulation of *F. solani* and sclerotial size, germination and germ tube number of *M. phaseolina*.

Jager and Korsten (1998) reported the most inhibitory bacteria belonged to the genus *Bacillus* and observed to be antagonistic *in vitro* against a range of plant pathogens viz. *Colletotrichum gloeosporioids*, *Fusarium oxysporum* etc.

Havega et al. (1999) reported *Bacillus subtilis*, effective in controlling avocado pre- and post-harvest anthracnose and the same was formulated for commercial use in packing houses. The formulated product resulted into significant control of anthracnose.

Perello et al. (2001) reported that the strong inhibiting effect of *B. subtilis*, isolated from wheat phylloplane *in vitro* against *Phoma* spp.

Kannan and Revathy (2002) reported the efficacy of powdered formulations of *Bacillus subtilis*, mixed with farmyard manure (FYM) in controlling *Phytophthora capsici* causing foot rot and wilt in pepper [*Piper nigrum*]. Bordeaux mixture, chlorothalonil, fosetyl-AL, metalaxyl, chlorothalonil and carbofuran were also applied alone or in combination with the formulations. All the treatments resulted in lower disease severity compared to the control. The highest control of foot rot and wilt was recorded with *T. viride* mixed with FYM + Bordeaux mixture and *T. viride* mixed with FYM treatment.

Ahmed et al. (2003) reported bacteria (*Bacillus subtilis* HS93 and *B. licheniformis* LS674) were isolated from repeatedly washed roots of pepper plants. In *in vitro* assays, HS93, LS674 and *T. harzianum* were antagonistic against *P. capsici* and *R. solani* and produced high levels of chitinase. Seed treatment and root drenching with bacterial suspensions of HS93 with 0.5% chitin was more effective against *Phytophthora* and *Rhizoctonia* root rot than addition of the organisms without chitin.

Bharathi et al. (2004) reported efficacy of 13 plant growth promoting antagonistic rhizobacterial strains evaluated against chilli fruit rot and dieback incited by *Colletotrichum capsici*. *Bacillus subtilis* was found to be effective in increasing the seed germination and seedling vigour.

Swain and Ray (2009) studied that the *B. subtilis* strain from cowdung inhibited the *in vitro* growth of fungi, *Fusarium oxysporum* (25-34%) and *Botryodiplodia theobromae* (100%) isolated from the postharvest rots of yam (*Dioscorea rotundata*) tuber.

Netu et al. (2008) evaluated that the *Bacillus subtilis* BN1 exhibited strong antagonistic activity against *Macrophomina phaseolina*, and other phytopathogens including *Fusarium oxysporum* and *Rhizoctonia solani*.

Archana et al. (2009) Studied that the 38% isolates showed competitive activity against *Fusarium oxysporum* and exhibit more than 50% mycelial inhibition in dual culture bioassay.

Salaheddin et al. (2010) evaluated that the *B. subtilis* management of bacterial blight of cotton under greenhouse and field condition.

Prapagdee et al. (2012) evaluate the antifungal efficacy of crude extracts of antifungal compounds produced from *Bacillus subtilis* SSE4 against fungal pathogen *Colletotrichum gloeosporioides*. Antifungal compounds in culture filtrate were extracted by ethyl acetate, hexane or dichloromethane and assessed for their efficacy to inhibit the growth of *C. gloeosporioides* on agar plates and for prevention of anthracnose disease in *Dendrobium*. The results showed that crude extracts of antifungal compounds extracted by all solvents were able to inhibit the growth of *C. gloeosporioides*.

Ashwini and Srividya (2013) observed that the treatment of the seed with co- inoculation of the pathogen with *Bacillus subtilis* culture

showed 65% reduction in disease incidence by the treatment as compared to the seed treated with pathogen alone (75%).

Yueju et al. (2014) observed that the *B subtilis* SG6 exhibited a high antifungal effect on the mycelial growth, sporulation of *F. graminearum* with the inhibition rate of 87.9% and 95.6%.

Salem et al. (2015) studied antagonism of *B. subtilis* strain towards fungal species the reduction percent of mycelial growth ranged from nil to as much as 51.4%.

Mangalanayaki and Durga (2016) revealed *Bacillus* spp. suppressed the mycelial growth of *Fusarium solani* causes *Fusarium* wilt of tomato in the range 55.7% by *Bacillus cereus* to 95.2% by *Bacillus subtilis*. *B. subtilis* strains inhibit the growth of a variety of fungal pathogens because of their ability to produce antibiotics such as zwittermicin, bacillomycin, fengycin, bacilysin and difficidin.

Neetu Kumari et al. (2017) recorded *Bacillus* strain isolated from the soil and its antagonistic activity was observed against several fungal pathogens. Isolated *Bacillus* strain inhibited the growth of *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Alternaria* sp., *Pencillium* sp., *Curvularia* sp. And *Fusarium* sp. *Bacillus* strain showed the maximum inhibition (72%) against *Pencillium* sp.

Sowanpreecha et al.(2018) recorded *Bacillus subtilis* N3 was able to inhibit the growth of *Curvularia lunata*, the causal agent of flower rusty spot disease in orchids.

Hazarika et al. (2019) recorded seven endophytic *B. subtilis* strain were isolated from sugarcane leaves and screened for its antifungal activity against 10 fungal isolates belonging to the genera *Alternaria*, *Curvularia*, *Fusarium*, *Cochliobolus*, *Neodeightonia*, *Phomopsis* and *Saccharicola* isolated from diseased leaves of sugarcane.

Kendre et al. (2020) tested phylloplane *Bacillus subtilis* isolates exhibited antagonistic potential in the range of 76.13- 85.59% against foliar pathogenic fungi.

CHAPTER III

MATERIAL AND METHODS

The present investigation entitled as “Evaluation of antagonistic potential of phylloplane *Bacillus subtilis* isolates against foliar fungal pathogens” was carried out during 2018-2020 at Department of Plant Pathology, Post Graduate Institute, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola.

The details of the materials used and methods or techniques followed during the course of present investigations are explained below.

3.1 Material used

3.1.1 Glassware

The glassware used were Petri plates, reagent bottles, micropipettes, conical flasks of different capacity, test tubes, pipette, beakers, slides.

3.1.2 Equipments

The laboratory equipments viz., Autoclave, pH meter, Hot Air Oven, Laminar Air Flow, Electronic balance, BOD incubator, Refrigerator, Colony counter and Microwave oven were used.

3.1.3 Miscellaneous material

The inoculation needle, double distilled water, spirit lamp, spirit, scarpel, forcep, polyethylene bags, glass slide, cover slip, dropper, handgloves, blotter paper, micropipette, Non absorbant cotton, cork borer, aluminium foil, permanent marker, tray, cello tapes, test tube stand, durham tube etc. were used during the studies.

3.2 Methods

3.2.1 Sterilization of glass wares, media, filter paper, water and other material

Petriplates, test tubes and conical flask of different capacities were used. The glasswares were sterilized in hot air oven at 180°C for one hour.

The media and distilled water were sterilized in autoclave at 15 psi for 15 minutes.

3.2.2 Preparation of culture media

3. 2.2.1 Nutrient Agar medium

Peptone	-	5.0 g
Sodium chloride	-	5.0 g
Yeast extract	-	2.0 g
Beef extract	-	3.0 g
Agar agar	-	20 g
Distilled water	-	1000 ml

3. 2.2.2 Potato Dextrose Agar

Peeled potato	:	200 g
Dextrose	:	20 g
Agar	:	20 g
Distilled water	:	1000 ml

3.2.3 Collection of phylloplane plant samples for isolation of *Bacillus subtilis*

Collected healthy crop plant samples from different field crops during kharif season from different location are given in Table 1.

Table 1. Details Of phylloplane samples collected from different locations

Sr. No.	Crops	Location	District	Phylloplane Samples Designated
1.	Cotton	Cotton Research Unit, Dr. PDKV, Akola	Akola	PBs1
2.	Chilli	Chilli Research Unit, Dr. PDKV, Akola	Akola	PBs2
3.	Sorghum	Sorghum Research Unit, Dr. PDKV, Akola	Akola	PBs3
4.	Pigeon pea	Pulses Research Unit, Dr. PDKV, Akola	Akola	PBs4
5.	Soybean	Pulses Research Unit, Dr. PDKV, Akola	Akola	PBs5
6.	Rice	Sakoli	Bhandara	PBs6

3.2.3 Collection of diseased samples for isolation for foliar fungal pathogens.

Collection of diseased crop plant samples from various field crops.

Table 2. Collection of diseased samples

Sr.No.	Crops	Diseased plant part used for isolation
1.	Chilli	Chilli seed
2.	Sorghum	Sorghum seed
3.	Chilli	Chilli fruit coat
4.	Cotton	Cotton leaves

3.3 Precaution to eliminate contamination

All isolation and inoculation work of microbial cultures was carried out aseptically under laminar air flow. The laminar air flow was sterilized by glowing ultraviolet lamp for ½ hr prior to commencement of the work.

3.4 Maintenance of bacterial culture

The respective bacterial cultures were maintained on Nutrient Agar medium at room temperature by adopting subsequent sub culturing at periodical, regular intervals. Seven day old cultures were used for further studies.

3.5 Isolation and maintenance of fungal culture

Fungal pathogens were isolated by tissues isolation method and cultures were maintained on PDA at room temperature by adopting subsequent sub culturing at periodical, regular intervals. Seven days old cultures was used for further studies.

3.6 Methodology for isolation of phylloplane *Bacillus subtilis*

For isolation of *Bacillus subtilis* Nutrient Agar medium was used.

Procedure –

1. The phylloplane *B. subtilis* were isolated from surface of healthy leaves collected from different field crops.
2. The collected leaves were gently rinsed in the sterilized distilled water for few seconds to remove dust particles and loosely adhering unassociated microbes.
3. The leaves were placed in 100 ml sterilized distilled water and vigourously shaken for 30 min.
4. Prepared serial dilutions from 10^{-1} to 10^{-10} .
5. Transferred 1 ml water from 10^{-7} dilution on Nutrient Agar media.
6. Incubated the plates for 48 hrs at room temperature.
7. Transferred representative of each bacterial colony to a fresh Nutrient Agar media plate to establish pure culture.

3.7 Morphological studies

The confirmation of the *Bacillus subtilis* isolates were performed with the following studies. Pure culture of 6 selected isolates were streaked on Nutrient Agar medium petriplate separately for colony

development. The individual colonies were examined for colony colour and shape.

Shape and colour : The shape and colour was observed on Nutrient Agar media.

Shape and colonies : Circular.

Elevation : Flat, Raised, Concave.

Colour : Yellow, Dull white.

3.8 Biochemical studies

Biochemical tests viz., starch hydrolysis, H₂S and IAA Production, KOH test, Gram's reaction, Acid and Gas production from Carbohydrates, Catalase test, Gelatin liquefaction etc. were carried out for biochemical confirmation of *Bacillus subtilis*.

All the isolates of *Bacillus subtilis* were also evaluated for plant growth promoting properties viz., IAA production etc.

3.8.1 Catalase test

During aerobic respiration in the presence of oxygen, microorganisms produce hydrogen peroxide (H₂O₂) which is lethal to the cell. The enzyme catalase present in some microorganisms breaks down hydrogen peroxide to water and oxygen and help them in their survival.

Medium :

Trypticase soy agar medium

Catalase test is performed by adding H₂O₂ to trypticase soy agar slant culture. Releases of free oxygen gas (O₂) bubbles is a positive catalase test.

3.8.2 Potassium hydroxide solubility test (KOH)

Two drops of potassium hydroxide were placed on a glass slide. A colony of culture was picked up from the medium with the help of inoculating needle and mixed with KOH drops for 10 seconds and raised the needle for 0.5 to 2 cm from thread which was treated as positive test.

3.8.3 Gram's reaction

Identification was made by Gram Staining and by studying the morphological characters of the isolates of *B. subtilis*.

Procedure :

- i) First a smear was prepared of bacterial cells by holding a clean slide by grasping at edges.
- ii) A loopful of bacterial suspension was transferred in the center of slide, with the help of wire loop.
- iii) The drop was smeared over slide and air dried.
- iv) Then dried smear was fixed by passing the slide 3-4 times rapidly over the flame.
- v) The smear was flooded with crystal violet for 30 seconds, washed in the tap water.
- vi) Then the smear was immersed in potassium iodide/ Lugol's iodine solution for 30 seconds washed in tap water then decolorized with 95% alcohol and rinsed with water.
- vii) Counterstained with saffranin for 10 second, again washed with tap water and air dried.

Drop of cedar wood oil was placed on the slide and examined the smear under oil immersion lense.

3.8.4 Starch hydrolysis

Starch is a complex carbohydrate (polysaccharide) composed of two constituents – amylose, a straight chain polymer of 200-300 glucose units, and amylopectin, a larger branched polymer with phosphate groups. The positive test indicates by the presences of amalyse enzymes, an exoenzymes that hydrolyses (cleaves) starch, into maltose (disaccharides) and some monosaccharides such as glucose.

Starch Agar : nutrient agar + 0.2% soluble starch

Test reagent :

Lugol's iodine

Bacterial culture was inoculated on starch agar plates and incubated for 7 days. After incubation, the plates were flooded with Lugol's iodine solution. Presence of starch hydrolysis indicated by the appearance of clear zone. Reddish zone indicated that the starch was partially hydrolysed to dextrin.

3.8.5 H₂S production

The activity of bacterium on sulphur containing amino acids frequently results in production and liberation of H₂S gas.

Medium :

Peptone water = peptone 1%, cystine 0.01%, NaCl 0.5%.

Lead acetate paper was prepared by moistening the filter paper in saturated solution of lead acetate.

Lead acetate paper was inserted in the tubes containing peptone water inoculated with bacterium and held by the plugs above the culture, without touching the medium. The tubes were incubated for 3 days. If filter paper strips turned black, it indicated the positive test for H₂S production.

3.8.6 Gelatine liquefaction

Gelatin is a protein produced by hydrolysis of collagen, a major component of connective tissue and tendons in humans and other animals. Hydrolysis (liquefaction) of gelatin is brought about by bacterium about by bacterium capable of producing a proteolytic exoenzyme known as gelatinase, which acts to hydrolyze this protein to amino acids.

Medium :

Nutrient gelatin = Nutrient broth + 1.5% gelatin

Bacterial cultures were inoculated through stab of nutrient gelatin tube and incubated for 7 days, uninoculated tubes serve as control and observed for liquefaction. Deep gelatin inoculated tubes that remain

liquefied produce gelatinase and shows positive test for gelatin hydrolysis and those tubes that remain solid demonstrate negative reaction for gelatin hydrolysis.

3.8.7 Indole production

Tryptophan, an essential amino acid, is oxidized by some bacteria by the enzyme tryptophanase resulting in formation indole test is performed by inoculating a bacterium into tryptone broth, the indole produced during the reaction is detected by adding Kovac's reagent which produces a cherry-red reagent layer.

Medium :

1% tryptone broth : 10 gm of peptone in one litre of distilled water.

Test reagent :

Kovac's reagent (P-di-mthylamino benzaldehyde 50 g amyl alcohol 750 ml, HCL 250ml).

Procedure :

The medium was distributed in test tubes and autoclaved. The bacterium was inoculated and incubated for 48 hrs. After 48 hrs of incubation, Kovac's reagent (1ml) was added in incubated test tube. The tubes were allowed to stand to permit the reagent to come to top.

Results :

Development of a cherry (deep) red colour on the top layer of the tube is a positive test for indole production. Absences of red colouration is indole negative.

3.8.8 Acid and Gas production from carbohydrates

Dextrose broth (Nutrient broth +0.5% dextrose) was prepared with test reagent (20 ml Bromo-Cresol purple). 10 ml dextrose broth distributed in each test tube and placed one Durham tube in inverted position in each test tube. After sterilization of the test tube it was inoculated with *B. subtilis* isolates and incubated for 3 days at room

temperature. If the colour of indication changes blue to yellow it indicated formation of acid and gas formation by its accumulation in Durham tube.

3.8.9 Methyl red (MR) Test

The test was performed to see the cleavage of glucose commonly used in the differentiation of organisms.

Medium = Glucose phosphate broth-Glucose 0.5%, K₂HPO₄ 0.5%, Peptone 0.5%, distilled water 1lit.

Test reagent :-

1) MR Test : 0.1g Methyl red dissolved in 300 ml of 95% ethanol and made up to 500 ml with distilled water.

Medium was distributed in the test tubes and incubated for 7 days at 27± 2°C temperature after inoculation. The test were performed in separate tubes.

Five drops of the indicator methyl red were added to 5 ml of culture. A red color described as a positive test for methyl red (MR). A yellow colouration was recorded as negative test.

3.9 In vitro efficacy of *Bacillus subtilis* against foliar fungal pathogens

The *Bacillus subtilis* isolates were tested for the antagonistic ability against foliar fungal pathogens *i.e.* *Alternaria alternata*, *Curvularia lunata*, *Myrothecium roridum* and *Colletotrichum dematum*.

3.9.1 In vitro efficacy of *Bacillus subtilis* against fungal pathogens by dual culture method

Six *Bacillus subtilis* isolates were tested for their efficacy against four foliar fungal pathogens. In sterilized petriplates autoclaved melted Potato dextrose agar was poured and allowed to solidify for obtaining leveled surface. These plates were inoculated with the culture of test fungi. In petri plates containing 20 ml NA medium (without antibiotics) and loopful of fresh bacterial culture was streaked three cm away from fungal mycelial disc at sides towards the edge of petriplates and then 5

mm disc of the fungal cultures were placed at the centre. The control plates were kept by placing culture disc grown under the same condition on plain PDA without streaking of bacterial isolates. Petri plates were incubated at $27\pm 2^{\circ}\text{C}$ seven days. The percent inhibition of test fungus with each bacterial isolates was calculated. Three replicated plates were maintained for each isolate. The percent growth inhibition was calculated using following formula.

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

Where,

I = Per cent inhibition

C = Growth of fungus in control (mm)

T = Growth of fungus in treatment (mm)

3.6 Statistical analysis

Statistical analysis was done by using CRD (Completely randomized design) method of analysis of variance Means were tested for significance and critical difference was used for comparing the differences between the treatments (Panse and Sukhatme, 1967).

CHAPTER IV

RESULTS AND DISCUSSION

The investigation on “Evaluation of antagonistic potential of phylloplane *Bacillus subtilis* isolates against foliar fungal pathogens” was carried out during 2018-2020. The results and its interpretation of the present studies are given in this chapter.

4.1. Collection of phylloplane samples and isolation of *Bacillus subtilis*

Plant samples were collected from phylloplane of kharif crops from different location of Dr. Panjabrao Deshmukh Krishi Vidyapeeth Akola. These samples were processed in the laboratory for isolation of *Bacillus subtilis* on Nutrient Agar medium by serial dilution method.

Table 3: Isolates of *Bacillus subtilis* obtained from different kharif crops

Sr. No.	Crops	Location	District	Phylloplane Samples Designated
1.	Cotton	Cotton Research Unit, Dr. PDKV, Akola	Akola	PBs1
2.	Chilli	Chilli Research Unit , Dr. PDKV, Akola	Akola	PBs2
3.	Sorghum	Sorghum Research Unit, Dr. PDKV, Akola	Akola	PBs3
4.	Pigeon pea	Pulses Research Unit, Dr. PDKV, Akola	Akola	PBs4
5.	Soybean	Pulses Research Unit, Dr. PDKV, Akola	Akola	PBs5
6.	Rice	Sakoli	Bhandara	PBs6

After three days of incubation, milky white colonies were observed on Nutrient Agar medium which were later picked and streaked on fresh Nutrient Agar medium for pure culture and used for further study.

Six isolates of *B. subtilis* were obtained of different field crops and were designated as PBs1, PBs2, PBs3, PBs4, PBs5 and PBs6 (Table 4).

Brian and McSpadden (2004) isolated and reported that *Bacillus subtilis* most abundant bacteria cultured from the phylloplane of soybean. Mohammadipour et al. (2009) studied the characterization of surfactin producing 290 phylloplane isolates of *B. subtilis* collected from different ecological zones of Iran. Similarly, Theodulos et al. (2003) reported that *Bacillus subtilis* as a natural inhabitants of the tomato phylloplane. These reviewed statement confirmed the existence of *B. subtilis* in the phylloplane of different crops.

4.2 Isolation of foliar fungal pathogens

Isolation of different foliar fungal pathogens from diseased phylloplane samples on PDA was done by tissue isolation method.

Table 4: Details of diseased sample used for Isolation of foliar fungal pathogens

Sr. No.	Crops	Diseased plant part used for isolation	Fungal pathogens Isolated
1.	Chilli	Chilli seed	<i>Colletotrichum dematium</i>
2.	Sorghum	Sorghum seed	<i>Curvularia lunata</i>
3.	Chilli	Chilli fruit coat	<i>Alternaria alternata</i>
4.	Cotton	Cotton leaves	<i>Myrothecium roridum</i>

4.3. Morphological characteristics of phylloplane *Bacillus subtilis* isolates

4.3.1 Shape

All the six isolates of phylloplane *Bacillus subtilis* were rod shaped when observed under microscope.

4.3.2 Colony colour

The isolates of phylloplane *Bacillus subtilis* that were grown on nutrient agar medium showed typical well separated white colonies with the colour variation from cream white to dirty white.

Morphological characterization was based on classical macroscopic techniques of colour, form, shape and elevation of pure colonies. Most colonies were able to grow within 2-3 days of incubation at $25 \pm 2^{\circ}\text{C}$.

Data presented in Table 5 and Plate 1 revealed that, all *Bacillus subtilis* isolates from phylloplane were gram positive and rod shape. Similar result have been earlier reported by Perez et al. (2000) and Toppo and Naik (2015). All the six isolates of *Bacillus subtilis* were found gram positive and rod shaped. These morphological characters confirm that the bacteria was *Bacillus subtilis*.

Table 5 Morphological characteristics of phylloplane *Bacillus subtilis* isolates

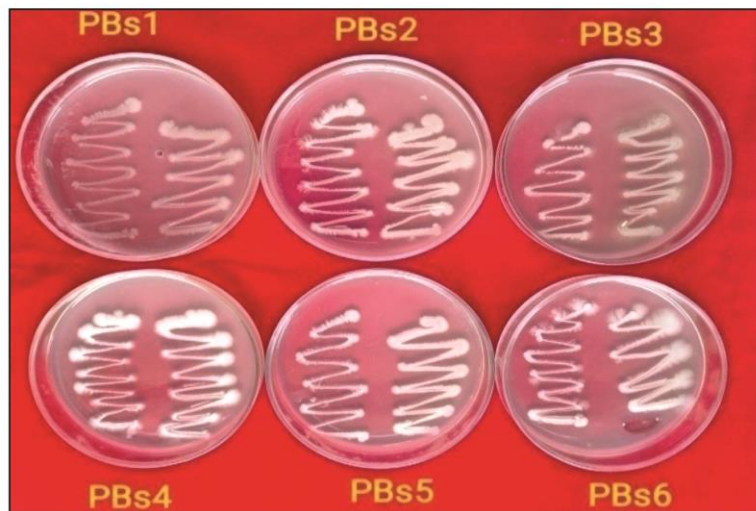
Isolates	Cell shape	Colony shape	Colony colour
PBs1	Rod	Irregular, dry, smooth, flat and lobate margin	Slightly off dirty white
PBs2	Rod	Circular, dry, smooth, flat and irregular with lobate margin	Dirty white
PBs3	Rod	Circular, wet smooth, concave	Dirty white
PBs4	Rod	Circular, wet smooth, concave	Slightly white
PBs5	Rod	Spreading, wet, smooth, flat and irregular with lobate margin	Off white
PBs6	Rod	Regular, wet, smooth, entire margin	Dull white



Colonies of *Bacillus subtilis* on NA medium

Pure culture of *Bacillus subtilis*

Plate 1. Morphological character of *Bacillus subtilis* isolates.



Phylloplane isolates of *Bacillus subtilis*

Plate 2. Isolation of *Bacillus subtilis* on nutrient agar medium.

4.4 Biochemical characteristics of *Bacillus subtilis* isolates

All six isolates of *B. subtilis* were subjected to the biochemical tests for their identification, some of the tests were performed for comparison of the characteristics among isolates. Six isolates tested with respect of Gram reaction, KOH, Acid and Gas production, Catalase, Starch hydrolysis, IAA and H₂S production were studied.

4.4.1. Catalase test

Catalytic activities of all the six isolates were found positive, when cultures were produced bubbles of oxygen within one minute after added H₂O₂ (Plate 3).

4.4.2. Potassium hydroxide solubility test (KOH Test)

All six isolates showed negative reaction to KOH test. All the six isolates had unable to form mucoid thread after added KOH (Plate 3).

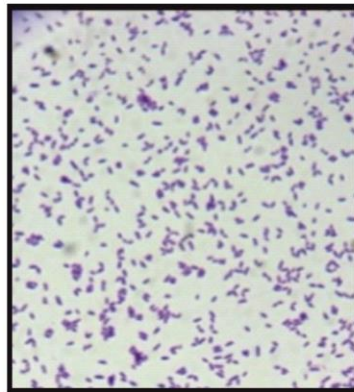
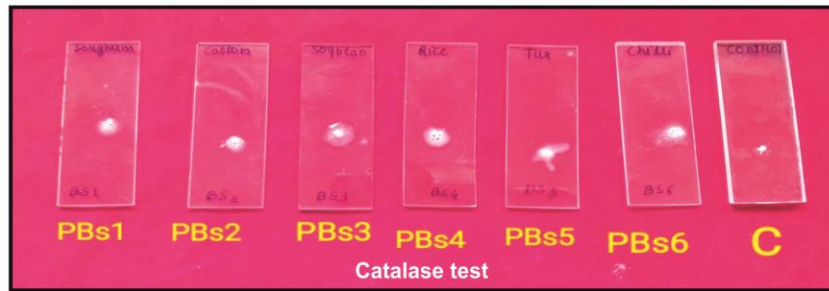
4.4.3. Gram reaction

Among all six isolates tested, all isolates were showed gram positive reaction.

Table 6. Biochemical properties of selected phylloplane *Bacillus subtilis* isolates

Sr. No	Character/ Properties	Reaction/ Isolates					
		PBs ₁	PBs ₂	PBs ₃	PBs ₄	PBs ₅	PBs ₆
1	Catalase test	+	+	+	+	+	+
2	KOH test	-	-	-	-	-	-
3	Gram test	+	+	+	+	+	+
4	Starch hydrolysis	+	+	-	+	+	+
5	H ₂ S Production	+	+	+	+	+	+
6	Gelatin liquefaction	+	+	+	+	+	+
7	IAA Production	-	-	-	-	-	-
8	Acid and Gas production	+	+	+	+	+	+
	Acid	+	+	+	+	+	+
	Gas	-	-	-	-	-	-
9	MR test	-	-	-	-	-	-

PBs- Phylloplane *Bacillus subtilis*, + positive, - negative reaction and R- Rod shape



Gram reaction

Plate 3. Biochemical test of *Bacillus subtilis* isolates

4.4.4. Starch hydrolysis

Among six isolates of phylloplane tested showed positive reaction to starch hydrolysis except from phylloplane isolate PBs3 forming a clear hallow around the colony. However other two isolates did not hydrolysed the starch (Plate 4).

4.4.5 H₂S gas production

All six isolates were tested for H₂S gas production, all the isolate found to produce H₂S gas (Plate 4).

4.4.6 Gelatin liquefaction

All six isolates were tested for gelatin liquefaction, all of these shows positive reaction (Plate 5).

4.4.7. IAA Production

The data from Table 6, revealed that all the isolates showed negative reaction to IAA production, development of cherry red colour after addition of Kovac's reagent into culture supernant confirmed IAA production, but all six isolates does not develop cherry red colour.

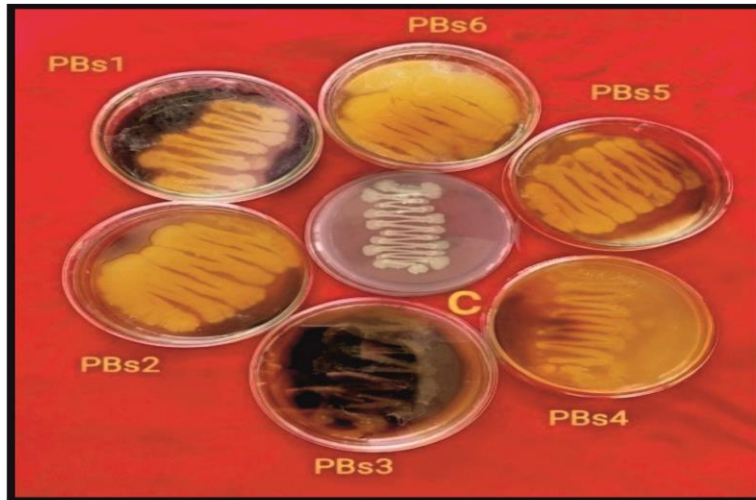
4.4.8 Acid and Gas production from carbohydrates

All six isolates were tested for acid and gas production, all the isolates found positive to produce acid but shows negative results for gas production (Plate 6).

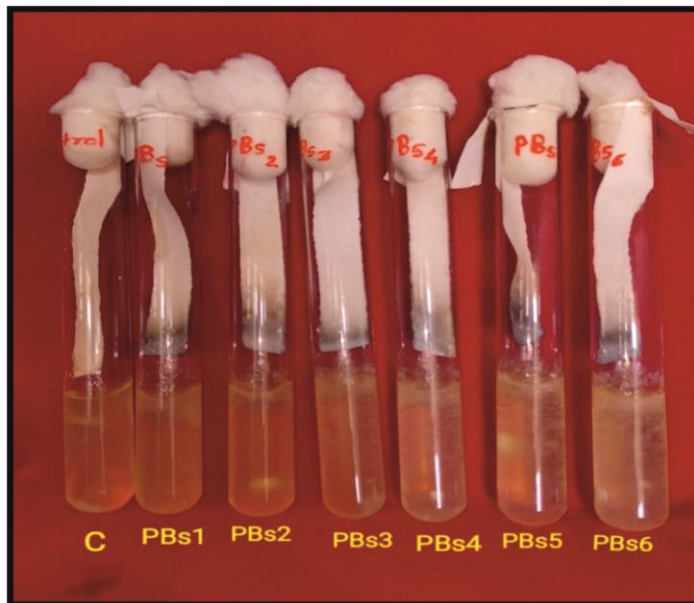
4.4.9. Methyl red test

All six isolates of phylloplane were tested for Methyl red test. All isolates showed negative reaction to Methyl red test.

Data presented in the Table 6 and Plate 3, 4, 5 and 6 indicated that all the isolates of phylloplane *Bacillus subtilis* showed positive reaction in respect of Gram reaction, starch hydrolysis, gelatin liquefaction, H₂S production, catalase test and acid production . Negative reaction recorded regarding with phylloplane *Bacillus subtilis* for KOH, MR test, indole production and Gas production.



Starch hydrolysis

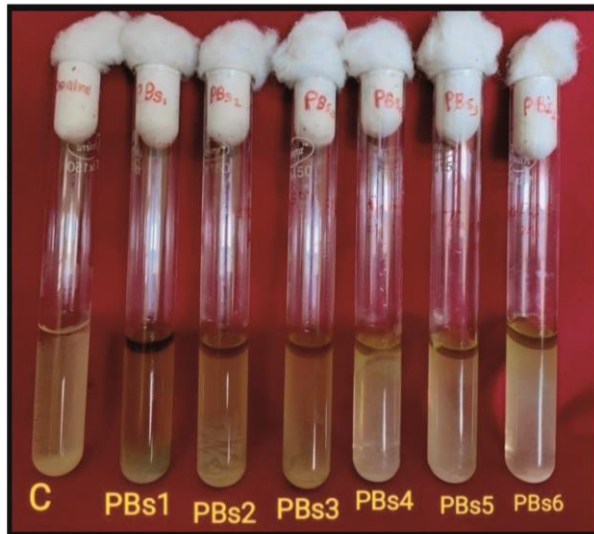


H₂S production

Plate 4. Biochemical test of *Bacillus subtilis* isolates

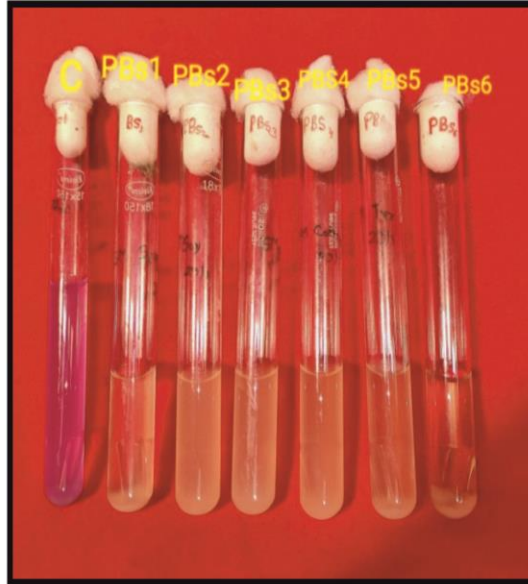


Gelatin liquefaction



IAA production

Plate 5. Biochemical test of *Bacillus subtilis* isolates



Acid and Gas production



Methyl red test

Plate 6. Biochemical test of *Bacillus subtilis* isolates

Positive reaction with *B. subtilis* for starch hydrolysis, gelatin liquefaction, H₂S production, casein hydrolysis, catalase test and acid production reported by Abbo et al. (2014), Karimi et al. (2012), Jadhav et al. (2014), Jha et al. (2016), Tariq et al. (2016). Avsar et al. (2017), Mandla et al. (2017) and Zhenxiang et al. (2018). Negative reaction regarding KOH test, gas production, starch hydrolysis and indol production reported by Abbo et al.(2014).

Willemse and Collinet (1980) and Khan et al. (2011) also observed negative reaction of *B. subtilis* to these tests. The positive and negative result for biochemical tests of different *B. sibtillis* isolates reported by various authors confirmed the morphological and biochemical identification of the isolates under study. Abbo et al. (2014) and Jha et al. (2016) recorded positive reaction with *Bacillus subtilis* for catalase, H₂S production and acid production, starch hydrolysis, phosphate solubilization and gelatin liquefaction. However, they observed negative reaction for KOH test, gas and indole production and starch hydrolysis.

4.5 Efficacy of *Bacillus subtilis* isolates against foliar fungal pathogens by dual culture method

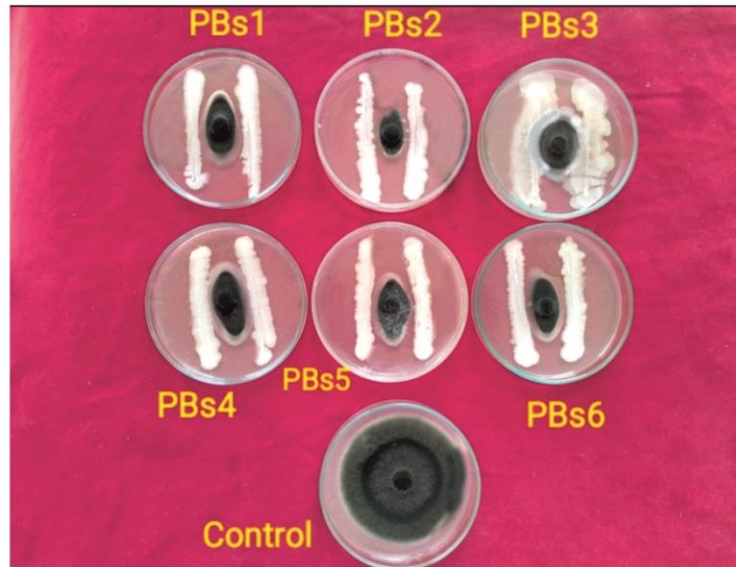
Data presented in the Table. 7 and 8 graphically represented in fig 1 and 2, Plate 7 and 8, indicated that all the isolates control the growth of *Alternaria alternata*, *Curvularia lunata*, *Myrothecium roridum* and *Colletotrichum dematium*. Per cent growth inhibition ranges between 70.39 – 78.80% in PBs isolates.

However, minimum mycelial growth inhibition (16.83 mm) with maximum mycelial growth inhibition (78.80%) of *Alternaria alternata*, *Curvularia lunata*, *Myrothecium roridum* and *Colletotrichum dematium* was recorded in phylloplane *B. subtilis* isolate PBs1 followed by PBs6 (19.91mm, 74.05%), PBs2 (21.24mm, 73.28%) and PBs3 (22.91mm, 71.13%) respectively.

The other isolates of *B. subtilis* i.e.PBs5 and PBs4 were found least effective against all the fungal pathogens.

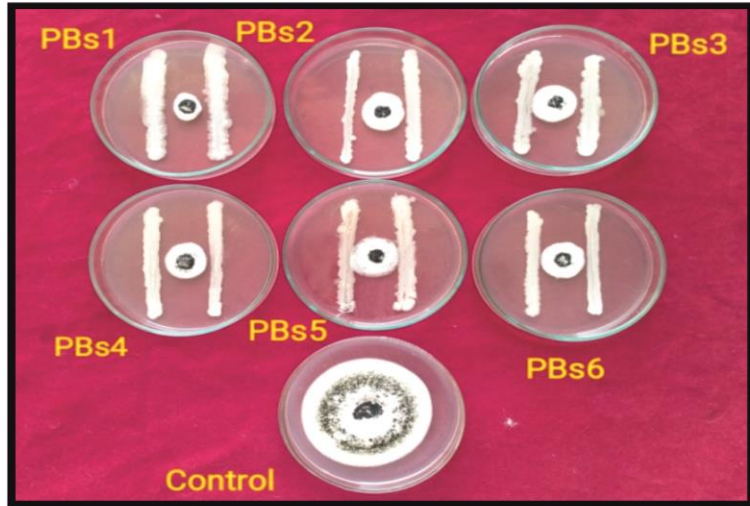


Alternaria alternata

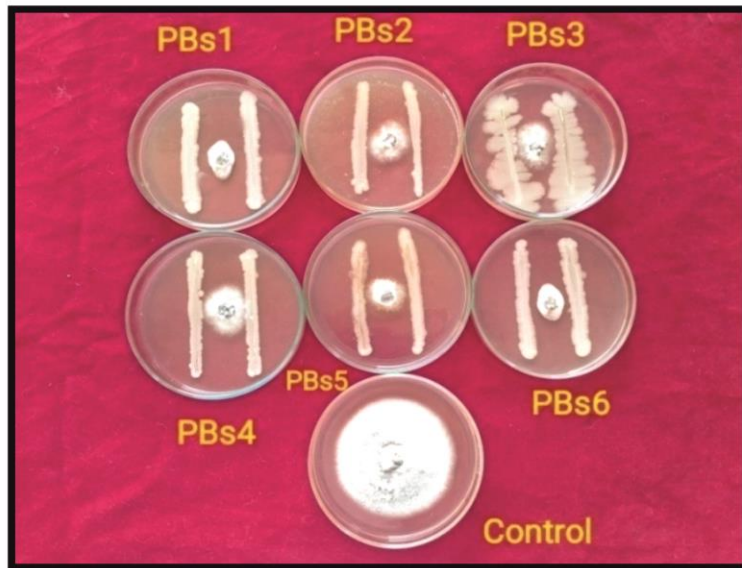


Curvularia lunata

Plate 7. Efficacy of polyloplane *Bacillus subtilis* isolates against foliar fungal pathogens



Myrothecium roridum



Colletotrichum dematium

Plate 8. Efficacy of polyphloplane *Bacillus subtilis* isolates against foliar fungal pathogens

Table 7. Efficacy of phylloplane *Bacillus subtilis* isolates against foliar fungal pathogens

HOST	Isolates	<i>Alternaria alternata</i>		<i>Curvularia lunata</i>		<i>Myrothecium rorodium</i>		<i>Colletotrichum dematium</i>	
		Av.radial mycelial growth (mm)	Per cent growth inhibition	Av.radial mycelial growth (mm)	Per cent growth inhibition	Av.radial mycelial growth (mm)	Per cent growth inhibition	Av.radial mycelial growth (mm)	Per cent growth inhibition
Cotton	PBs 1	18.00	76.00	17.00	78.75	16.33	79.06	16.00	81.39
Chilli	PBs 2	20.33	72.89	21.66	72.92	22.33	71.37	20.66	75.97
Sorghum	PBs 3	24.33	67.56	22.66	71.67	23.00	70.51	21.66	74.81
Pigeon pea	PBs 4	22.00	70.66	23.66	70.42	24.33	68.80	24.33	71.70
Soybean	PBs 5	21.66	71.12	22.33	72.08	23.66	69.66	26.00	69.76
Rice	PBs 6	20.00	73.33	18.00	73.96	22.00	71.79	19.66	77.13
Control	-	70	00.00	80.00	00.00	78.00	00.00	86.00	00.00
	F Test	Sig.	-	Sig.	-	Sig.	-	Sig.	-
	SE(m)±	1.11	-	1.72	-	2.04	-	1.45	-
	CD(P=0.01)	3.39	-	5.26	-	6.26	-	4.45	-

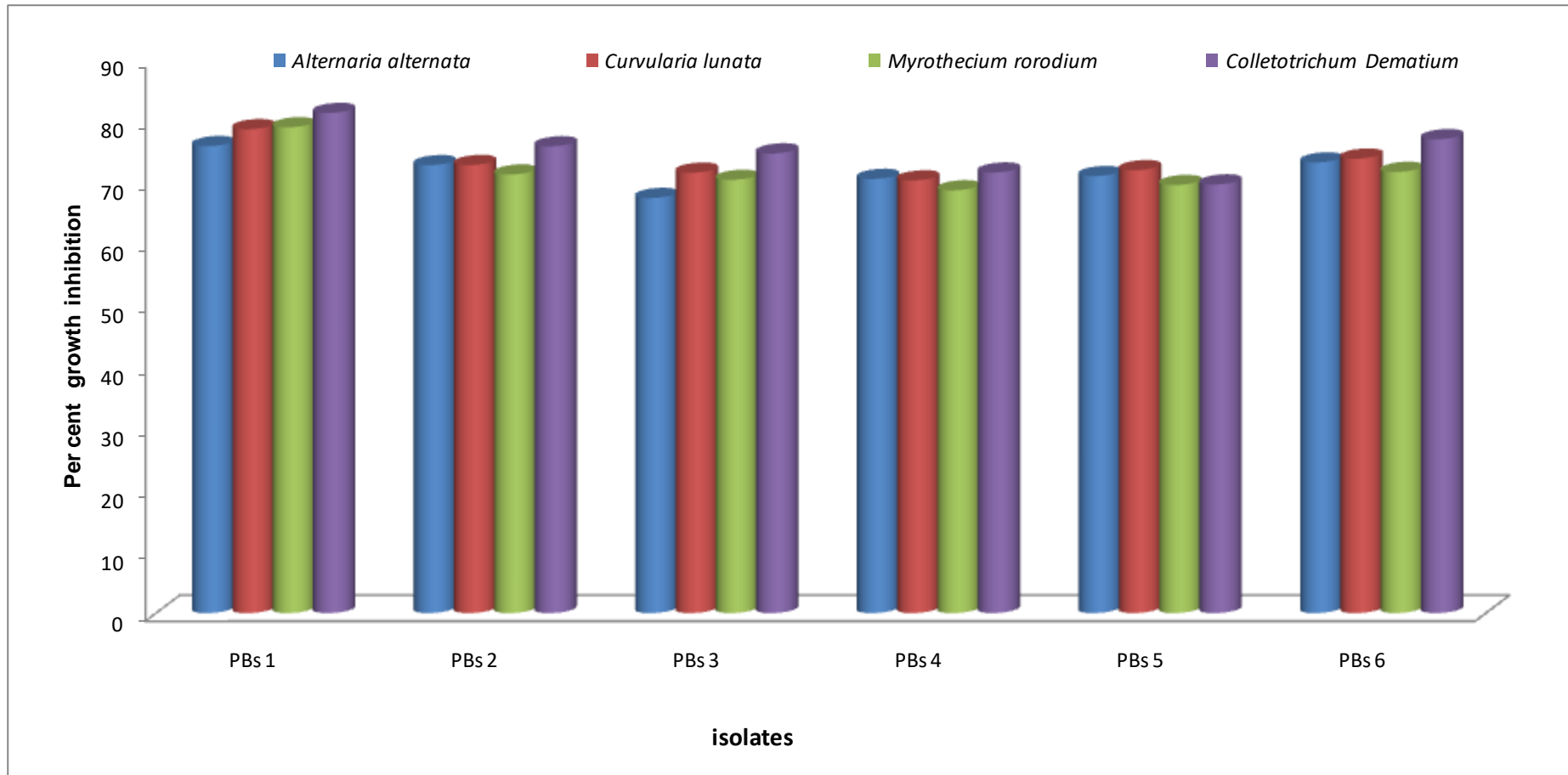


Fig. 1 Efficacy of polyloplane *Bacillus subtilis* isolates against foliar fungal pathogens

Table 8. Efficacy of phylloplane *Bacillus subtilis* isolates against foliar fungal pathogens.

Host	Isolates	Mean of mycelia growth of fungi tested (mm)	Mean of mycelia growth of inhibition of fungi tested (%)
Cotton	PBs1	16.83	78.80
Chilli	PBs2	21.24	73.28
Sorghum	PBs3	22.91	71.13
Pigeon pea	PBs4	23.58	70.39
Soybeam	PBs5	23.41	70.65
Rice	PBs6	19.91	74.05
Control		79.75	

The result of present investigations are in agreement with the finding of Ghosh et al. (2014) who reported *B. subtilis* screened against *Fusarium moniliforme* var. *subglutinans* for their antagonistic potentiality by dual culture plate method. *B. subtilis* strongly inhibited the growth of mycelium and spore germination of the pathogen. The non-volatile metabolites of *B. subtilis* exhibited 72.00% of inhibition of radial growth. Amaresan et al. (2012) who reported *B. subtilis* isolates were effective against *Colletotrichum capsici* in chilli. However, the suppression of mycelial growth of *Colletotrichum gloeosporioides* causing anthracnose in *Dendrobium* with crude extract of antifungal compound produced by *B. subtilis* was also reported by Prapagdee et al. (2012).

The inhibition of *Colletotrichum lindemuthianum* causing anthracnose of cowpea with different strains of *B. subtilis* (Bs-21, Bs-22 and Bs-23) was recorded by Adebajo and Bankole (2004). Laha and Venkantaraman (2001), Muralidharan et al. (2004) and Rajbir and Sinha(2004) reported inhibition of *Curvularia lunata* causing black kernel in rice with *B. subtilis* (97.77%). Mycelial growth inhibitions of *Curvularia geniculata* with antifungal compound produced by *B. subtilis* was also reported by Dass and Teyegaga (1996). These results are in accordance with present finding in respect of mycelial growth inhibition of various foliar fungi tested.

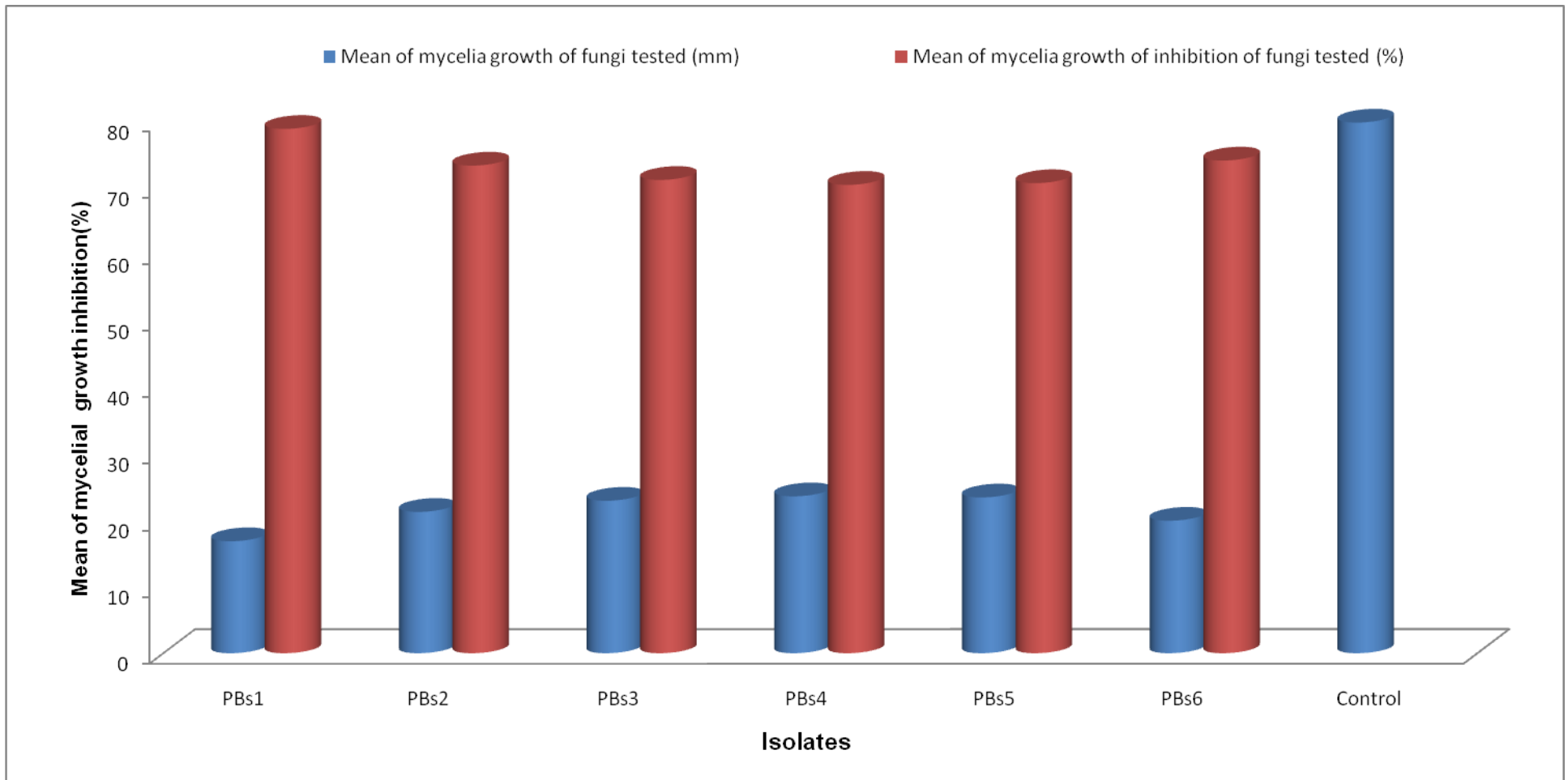


Fig. 2. Efficacy of phylloplane *Bacillus subtilis* isolates against foliar fungal pathogens (mean of mycelial growth inhibition)

CHAPTER V

SUMMARY AND CONCLUSIONS

The *Bacillus* spp. are classified in the order Eubacteriales and family Bacillaceae. Almost all the species are gram positive and produces the endospores may be cylindrical, ellipsoidal or spherical and located centrally, subterminally or terminally.

Bacillus subtilis showed resistance only to Penicillin, amoxylin and ampicillin antibiotics. The antibiotics obtained from culture of certain *B. subtilis* strains are known to produce subtilin, bacillin, subtenolin, bacillomycin etc. Iturin antibiotics generated by numerous *B. subtilis* strains are compound that exhibit strong antifungal activity against most pathogenic fungi and several bacterial species.

The present investigations entitled “Evaluation of antagonistic potential of phylloplane *Bacillus subtilis* isolates against foliar fungal pathogens” were undertaken to assess the *Bacillus* isolates on the basis of morphological and biochemical character. It was found that distinct variability existed among the *Bacillus* isolates recovered from the phylloplane of different kharif crops.

All total six isolates of *B. subtilis* were selected on the basis of their morphological and biochemical character. These isolates were designated as PBs1, PBs2, PBs3, PBs4, PBs5 and PBs6. All six isolates were Gram positive and rod shaped. The colonies were dull white or light yellow on Nutrient Agar medium. All the isolates observed positive reaction to Catalase test, Gelatin liquefaction, H₂S production, Starch hydrolysis production test except PBs3 showed negative reaction and positive to Acid reaction. All six isolates found negative for gas production test, methyl red test, indole production and KOH test.

Efficacy of phylloplane *B. subtilis* tested by dual culture method against *Alternaria alternata*, *Curvularia lunata*, *Myrothecium roridum* and *Colletotrichum dematium*. All six isolates of *B. subtilis* was found effective against foliar fungal pathogens. However, minimum mycelial

growth inhibition (16.83 mm) with maximum mycelial growth inhibition (78.80%) of *Alternaria alternata*, *Curvularia lunata*, *Myrothecium roridum* and *Colletotrichum dematium*. was recorded in phylloplane *B. subtilis* isolate PBs1 followed by PBs6 (19.91mm, 74.05%), PBs2 (21.24mm, 73.28%), PBs3 (22.91mm, 71.13%) PBs5 (23.41mm, 70.65%) and PBs4 (23.58mm, 70.39%) respectively. The other isolates of *B. subtilis* i.e., PBs5 and PBs4 were found least effective against all the fungal pathogen tested.

Conclusions

1. The phylloplane isolates of *Bacillus subtilis* obtained from various crop plants were rod shaped and gram positive reaction.
2. All isolates of phylloplane *Bacillus subtilis* showed positive reaction towards Catalase test, gelatin liquefaction, H₂S production, Indole production, Acid production and starch hydrolysis production test except isolate PBs3 showed negative reaction.
3. All six isolates shown negative reaction to KOH test, Methyl Red test and Gas production,.
4. Out of six isolates, the maximum mycelial growth inhibition of fungal pathogens was observed in isolate PBs1 (78.80%) followed by PBs6 (74.05%) and PBs2 (73.28%).
5. Overall, it is concluded that phylloplane *Bacillus subtilis* isolates PBs1 was found most effective against *Alternaria alternata*, *Curvularia lunata*, *Myrothecium roridum* and *Colletotrichum dematium*.

CHAPTER VI

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APPENDIX-I

1) Media

i) Nutrient Agar

Beef extract	-	3 g
Yeast extract	-	2 g
Peptone	-	5 g
Sodium chloride	-	5 g
Agar agar	-	20 g
Distilled water	-	1000 ml

ii) Potato Dextros Agar

Peeled potato	-	200 g
Dextrose	-	20 g
Agar agar	-	20 g
Distilled water	-	1000 ml