

**ANTAGONISTIC ACTIVITY OF
Bacillus subtilis AGAINST *Fusarium udum***

THESIS

**Submitted to
Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola
in partial fulfilment of the requirements
for the Degree of**

**MASTER OF SCIENCE
IN
AGRICULTURE
(PLANT PATHOLOGY)**

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Enrolment Number – KK/186

2021

DECLARATION OF STUDENT

I hereby declare that the experimental work and its interpretation of the thesis entitled “**Antagonistic activity of *Bacillus subtilis* against *Fusarium udum***” 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 sources of material 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 “**Antagonistic activity of *Bacillus subtilis* against *Fusarium udum***” submitted in partial fulfillment of the requirement 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 **Avhale Ganesh Sheshrao** under my guidance and supervision.

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

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ACKNOWLEDGEMENT

Words may be more powerful than swords, but they are generates of great movement and revolts and revolution. Still words many times are not adequate enough to express the sentiments which bloom in the secret chamber of heart and minute modes of mind.

Acknowledgement is written at last, placed at first and read the least, but still it is the only opportunity to thank one and all who are responsible in completion of the task in time during the course of study and research work.

Accomplishment of this thesis is the result of benevolence of almighty, blessings of my parents, motivation of my teachers and help of my friends.

At the very outset, whole heartedly I wish to express my fidelity and deep sense of gratitude to the chairman of my advisory committee Dr. V. R. Gupta, Associate Professor (CAS), Department of Plant Pathology, College of Horticulture, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola for his inspiring guidance, timely advice, constant supervision, constructive criticism, vivid encouragement & affectionate dealing through the period of investigation & during preparation of the manuscript. I feel elated to express my heartfelt thanks for his immense interest, transcendent suggestions, affectionate encouragement and adroitness in handling the research work in all its stages.

I own inadequate words to express my deep sense of gratitude & profound indebtedness to members of my advisory committee Dr. M. V. Totawar Associate Professor, Department of Plant Pathology, Dr. S. T. Ingle, Associate Professor (CAS) of Plant Pathology, Dr. PDKV, Akola and Dr. S. S. Hadole, Associate Professor (CAS), Department of Soil Science and Agriculture Chemistry, Post Graduate Institute, Dr. PDKV, Akola for their valuable advice, thought provoking and inspiring guidance, kind help and co-operation during the course of study and research work.

I cordially offer my profound gratitude to Dr. S. S. Mane, Professor and Head, Department of Plant Pathology for his constant

encouragement, valuable comments, timely help and co-operation during the course of my study.

My inexplicable gratitude goes to Dr. M. B. Nagdeve, Dean (Agri.) and Dr. D. B. Undirwade, Associate Dean, Post Graduate Institute, Dr. PDKV, Akola for the kind help of the administration during my degree programme. I equally owe my sense of gratitude to Dr. S. T. Ingle, Associate Professor (CAS), Shri. P. R. Padghan, Assistant Professor and Shri. Ratan Tajane, Attendent, Department of Plant Pathology, Post Graduate Institute, Dr. PDKV, Akola for their kind co- operation, valuable guidance, vibrating enthusiasm, diligence and sterling character value a lot of me.

From my mere existence on earth to present situation, my every step of life is moulded mud by my Father Shri. Sheshrao Ramrao Avhale and Mother Sau. Indu Sheshrao Avhale I will forever remain indebted to them. Their parenting brings every time the best in every effort of my venture. My way in this world paves through the path carved out by my parents whom, I wish to idolize throughout my life. I also acknowledge each and every chunk of help, guidance and suggestions, from my Sister Sharda I wish to express my special thanks to my Wife Ashwini for her unconditional love and motivational support for the success of my work.

I wish to express my heartiest thanks to my friends Yogesh, Mangesh, Akash, Akshay, Pankaj, Sunny, Shubham, Akshaykumar, Ghanshyam, Vishal for their help and suggestions during the course of study and research work.

I was thankful to PGI, Dr. PDKV, Akola for giving me the opportunity to pursue my post graduation successfully, for providing the facilities to conduct my Research work and for giving financial support in the form of stipend during the course of study.

Finally a word of appreciation goes to Shri digitals for scrupulous printing of this thesis in time. Any omission in this brief acknowledgement does not mean lack of gratitude.

Place: Akola
Date: / /2021

(AVHALE GANESH SHESHRAO)
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(D) List of Abbreviations

@	-	at the rate of
%	-	per cent
/	-	Per
Av	-	Average
CD	-	Critical difference
cm	-	Centimetre
°C	-	Degree Celsius
et al.	-	et alia (And others)
etc	-	Etcetera
viz.	-	Videlicet (Namely)
Fig.	-	Figures
Sig.	-	Significant
i.e.	-	id est (that is)
m	-	Metre
µm	-	Micrometre
ml	-	Milli litre
pw	-	Powder
liq	-	Liquid
pc	-	Pure culture
No.	-	Number
g	-	Gram
ha	-	Hectare
<i>In vivo</i>	-	In field
<i>In vitro</i>	-	In laboratory
SE(m)±	-	Standard error of mean
TGMR	-	Threshed grain mold rating
SBM	-	Standard Blotter paper method
CCA	-	Colloidal chitin agar medium
dSm	-	deciSiemens per meter
EC	-	Electrical conductivity
pH	-	Potential of Hydrogen
cfu	-	colony forming unit
BS	-	<i>Bacillus subtilis</i>

Fourty soil samples were collected i.e. 9 from Akola, 9 from Buldhana, 9 from Amravati, 9 from Washim, 2 from Nagpur, 1 from Chandrapur, 1 from Gadchiroli from different places of fields during June to September 2019. Ten soil samples contained *Bacillus* isolates by exhibiting zone of solubilization on CCA.

Hydrogen ion concentration and electrical conductivity of soil samples containing *B. subtilis* having chitin degrading ability on CCA was ranged from 8.1 to 8.6 and 0.21 to 0.52 dSm⁻¹ respectively having isolate with maximum solubilizing zone. Soil samples showed least pH and EC i.e. 8.1 and 0.21 dSm⁻¹ respectively

Isolate BS3 show maximum population (54.33X10⁷ cfu/g) followed by BS1 (50.66X10⁷ cfu/g). The least population of 34.10x10⁷ cfu/g of soil was recorded in BS9 followed by BS8 38.10x10⁷ cfu/g.

Biochemical tests were carried out to identified the *Bacillus subtilis* and results revealed that the strains collected from different regions were *B. subtilis* strains. Obtained from various test that all the isolates tested positive for Gram reaction, negative for KOH, positive for catalase test, gelatin liquification, growth in 5% NaCl, Arginine dehydrolase, H₂S production and negative for methyl test.

Among ten isolates of *Bacillus* BS3 (Medshi) allowing only 6.92 mm mycelial growth of *Fusarium udum* with 92.31% proved best over all other isolates however was at par with BS1 i.e. Patur isolate.

Maximum effect was noted due to BS3 with 9.57 mm growth of *R. bataticola* and 89.37% inhibition. Minimum ability was noted due to BS9 with 27.59% inhibition of test pathogen.

Highest efficiency with 88.97% inhibition of *S. rolfsii* was also noted due to BS3. where the pathogen could grew only upto 9.92 mm as against 90 mm without antagonist followed by BS1 with 88.49% growth inhibition of pathogen.

Imidacloprid exhibited more than 50% inhibition in almost all isolates of *B. subtilis* as compared to control indicating detrimental against

Bacillus. Isolate BS3 recorded highest CFU i.e. 60.37×10^7 which was being lowest i.e. 48.26×10^7 in BS9.

Thiomethoxom was also found non tolerable to isolates as less than 50% viable colonies in this chemical were observed. Isolate BS3 was least sensitive to Thiomethoxom with 54.76% inhibition.

Combined product of Carboxin + Thiram and Thiram alone was completely toxic to all isolates of *Bacillus* while Carbendazim exhibited maximum inhibition of 70.14% in BS9.

Among all the isolates, BS3 showed least sensitivity to the weedicides whereas BS9 exhibited highest sensitivity.

Root length, shoot length and seedling vigour index was also highest due to isolate BS3 i.e. 8.44 cm, 19.07 cm and 2082 respectively. Compared to 6.0 cm, 11.44 cm and 1046 root length, shoot length and seedling vigour index in control.

CHAPTER I

INTRODUCTION

1.1 Background information

Pigeon pea (*Cajanus cajan* (L.) Millsp.) is an important pulse crop grown in Kharif season. Arhar crop in Maharashtra during 2018-2019 was about 9.71 lakh tonne of total production. It is one of the extensively used pulses in India as an important source of protein in human diet. Generally, it is grown all over the country, but it is cultivated extensively in Bihar, Uttar Pradesh, Maharashtra, Tamilnadu, Andhra Pradesh, Karnataka, West Bengal and Gujarat. It suffers from various diseases viz. wilt (*Fusarium udum*) root rot (*Macrophomina phaseolina*), Stem canker (*Phoma cajani* and *Collectotrichum capsici*), bacterial blight (*Xanthomonas axonopodis* pv. *cajani*) and the sterility mosaic virus. The wilt caused by *Fusarium udum* Butler is one of most serious and oldest known disease (Butler, 1906) and it is known to cause heavy losses every year in India. The incidence of disease has been reported from 30 to 60 percent at flowering and at crop maturity stages (Kannaiyan et al. 1981).

Wilt caused by *Fusarium udum* is the economically devastating fungal disease of Redgram and is common throughout India. It is prevalent in Andhra Pradesh, Maharashtra, Madhya Pradesh, Uttar Pradesh and Bihar. Redgram is susceptible to wilt pathogen throughout its development stages. However, the symptoms are more pronounced and the damage is greater at flowering and pod formation stage. Wilting of seedlings and grown up plants as if they have suffered from water shortage although there is plenty of moisture in the field, is the main symptom. Wilting is characterized by gradual, sometimes sudden yellowing, withering and drying of leaves followed by drying of the entire plant or some of its branches. A voluminous work has been done on *F. udum* Butler in India and abroad. The pathogen is a soil and seed borne. The genus *Fusarium* has wide host range and survives for long time in field in the absence of host plant. Therefore, chemical control is not satisfactory, adequate and non-economical as a long-term solution. Considering, the crop health and

economic losses, the alternative to this is to explore the possibility of improving genetical disease resistance and integration of chemical and biological control, which can be successfully adopted in modern agriculture. some time up to 100% in susceptible cultivars (Kumar and Upadhya,2013).

The disease is caused by soil inhabiting *Fusarium udum* and hence not easy to manage once the pathogen penetrates the host systemically. The application of chemical fungicides through soil drenching is not easy practically and hence no body is using this practice of management of wilt. Host plant resistance and use of biocontrol agents then remains only solution for this devastating disease. Various biocontrol agents are known to affect the growth of *Fusarium udum*, one among them is *Bacillus subtilis* which has antagonistic activity against *Fusarium udum*, however the efficacy of this bioagents differ from location to location. With a view to find out most effective *Bacillus subtilis* prevalent in the soil of Vidarbha, the present study is proposed to undertake.

1.2 Importance of Study

Increasing sensibility and pressure of public and environmental agencies against the application of chemical based pesticides and their long lasting adverse effects on ecosystems and human health, has motivated the search for non hazardous alternatives. The most trustworthy substitute of chemical pesticides is considered as biocontrol agents. These agents could be formulations of bacteria, fungi, viruses, plant extracts or antibiotics.

Chitinases belongs to the class of hydrolytic enzymes with a potential to inhibit or degrade the chitin containing pathogens like fungi, insects and their larva's. The use of chitinases as a bio control agent is one of the attractive and environmentally safe strategies. Chitinases are needed by fungi to disrupt their existing cell walls during cell division and also played an important role in some plants being essential for the inhibition of fungal diseases. Microbial production of chitinase has captured worldwide

attention of both industrial and scientific environments, because of its wide spectrum of application.

1.3 Scope and Limitations

Biocontrol has a vast potential for the management of soil borne pathogens. Owing to the mechanism of disease control i.e. antibiosis, competition and hyperparasitism, there is very less risk of developing resistance in the pathogen against *Bacillus subtilis*. Moreover there are numerous native rhizospheric organisms which can be exploited as a new potential biocontrol against soil borne pathogens.

As *Bacillus subtilis* is living organism and they are exposed in environment the survival, efficiency and their required number in rhizosphere is affected by number of factors which governs the performance of these organisms. However, the efficacy of repeatedly used bioagent may lost because of continuous multiplication in lab conditions. Now a day's use of new chemicals for crop management is also governing the growth & performance of bioagents. Having a tolerant strain with all desirable character, its multiplication & application is a limiting factor for use of biocontrol agents. We can expect to see new biological control products becoming available to farmers as a biofungicides. The success of the product will depend on our ability to manage the rhizosphere to enhance survival and competitiveness of these beneficial microorganisms.

1.4 Objectives of study

1. To know the status of *Bacillus subtilis* in the soils of different pigeonpea growing districts of Vidarbha.
2. To evaluate the comparative antagonistic activity of *Bacillus subtilis* present in soils of Vidarbha against *Fusarium udum*.

1.5 Hypothesis

Bacillus subtilis from rhizosphere have received considerable attention as potential protecting agents against soil borne pathogens. *Bacillus subtilis* as bioagent have good prospectsus in future for management of wilt of pigeaonpea. The use of this rhizobacteria (*Bacillus*

subtilis) against *Fusarium udum* causing wilt of pigeonpea helps in reduction of *Fusarium udum* in pigeonpea rhizosphere by either acting as barrier for entrance of pathogen or suppressing the growth of pathogen by various means. Thus, objectives of present studies may be helpful for management of wilt disease through more superior and aggressive native isolate of *Bacillus subtilis*.

CHAPTER II

REVIEW OF LITERATURE

An attempt has been made to present a review of related past work, on pigeon pea wilt disease caused by *Fusarium udum* and the *Bacillus subtilis* for its management at different places by some research workers.

2.1 Background information

Butler (1906) was the first to describe the causal agent of wilt disease of pigeonpea in India, which resembles with the character of the genus *Fusarium*.

Butler (1910) identified and named the causal agent of wilt of pigeonpea for the first time in India as *Fusarium udum*. The wilt symptoms were observed after a month of inoculation under pot condition.

Patil and Gokhale (1965) noticed that the continuous cultivation of pigeonpea for 2-3 years in same field leads to more than 50 percent losses of plants due to wilt disease. They recorded that the wilt disease is more conspicuous in adult stage compared to seedling stage.

Kannaiyan and Nene (1981) obtained 100, 67.1 and 29.6 percent grain yield reduction in pigeonpea when wilt occurred at prepod stage, pod maturity and at pre-harvest stage respectively.

Okiror and Kimani (1997) reported the consistent differences in virulence and aggressiveness of *F. udum* among several isolates tested under glasshouse and laboratory condition.

Madhukeshwara and Seshadri (2001) tested the virulence behavior of six isolates of *Fusarium udum* by test tube and modified root injury method on a set of host differential of pigeonpea. They observed that all the isolates differed in their virulence behavior showing their distinct nature in pathogenicity on a set of host differentials.

Jalander and Gachande (2015) observed the growth and pigmentation of *Fusarium oxysporum f. sp. udum* of seven isolates isolated

from seven different varieties of pigeonpea using Czapek's dox agar(CDA), potato dextrose agar (PDA) and glucose nitrate agar(GNA) and found variability in pigmentation, more on CDA and growth rate of the pathogen isolates per day were high in case of GNA and colonies grown on CDA and GNA showed maximum fluffy growth and on PDA it was appressed.

2.2 Rhizospheric microorganisms

Agnihotru (1953) studied rhizosphere microflora of some important crop plant like pigeonpea, cluster bean, cotton sorghum, sesamum, french bean and sunhemp & found more microbial population in the rhizosphere than in the distant soil. Bacterial population was much greater followed by fungi and actinomycetes.

Harman et al. (1980) isolated *Trichoderma hamatum* and *Trichoderma spp.* from *Rhizoctonia* suppressive soil.

Bruce and Highley (1991) isolated fifteen *Trichoderma*, two *Penicillium* and one *Aspergillus* isolates and tested against *Trametes versicolor* and *Neolentinus lepideus* (brown and white rot basidiomycetes i.e. wood decay fungi) by agar interaction tests and by measuring the toxicity of culture filtrates. Three fungi produced the greatest inhibitory effect (2 *Trichoderma* and 1 *Aspergillus* isolate) against both target fungi but *Aspergillus* filtrate produced greater inhibition

Gaur and Sharma (1991) obtained 34 fungi, 6 bacteria and 4 actinomycetes from rhizosphere soil of pigeonpea and tested them against *Fusarium udum* on Czapek's sucrose nitrate agar medium. They observed that *Trichoderma viride* was the most effective followed by *Aspergillus niger*, *Streptomyces sp.*, *Penicillium sp.* and *Bacillus sp.* and found that presence of these bioagent plays role in disease resistance and susceptibility of a variety and decrease wilt incidence &

Foldes et al. (2000) used *Bacillus* strains isolated from the rhizosphere of cereals as natural biocontrol agents.

Pandey and Upadhyay (2000) found that rhizosphere of healthy pigeonpea (cv. Bihar) plants were heavily colonized by *A. niger* and

Penicillium sp. while *F. udum* dominated the rhizosphere of diseased plants. There was mixed population of *Aspergillus* spp., *Penicillium* spp., and *F. udum* in non rhizosphere soil of pigeonpea. Isolate of *Trichoderma harzianum*, *Gliocladium virens* and *T. viride* exhibited strong antagonism by inhibiting hyphal growth of *F. udum*.

Siddiqui and Shakeel (2009) studied the biocontrol of wilt disease complex of pigeon pea using 21 isolates of fluorescent *Pseudomonas* isolated from pathogen suppressive soils and found that isolates Pf 718, Pf 719, Pf 736 and Pa737 of *P. aeruginosa* caused 79, 84, 87 and 93% reductions in *Meloidogyne incognita* respectively and showed inhibition in the growth of *F. udum*. Among the Isolates isolate Pf736 caused 309, 9 and 78% increase in seedling growth, phosphate solubilization and IAA production, moderate HCN production also caused greater increase in plant growth and higher reduction in nematode multiplication and wilting index along with Rhizobium.

Nehra et al. (2012) tested 310 pigeonpea (*Cajanus cajan* (L.) Millsp.) rhizobacteria isolated from cultivated soils of north-east India (Alwar district, Rajasthan). Among the total isolates, 25 isolates were confirmed as *Pseudomonas fluorescense* based on the biochemical tests, such as arginine hydrolysis, catalase activity, production of fluorescing compounds, gelatin liquefaction and growth at 4°C and 42°C. Twelve isolates of *P. fluorescense* exhibited strong antifungal activity against *F. udum*.

Rana et al. (2014) isolated thirty *Pseudomonas* spp. and twenty *Bacillus* spp. from soil of pigeon pea field. Among them, five isolates of *Pseudomonas* spp. (Pf05, Pf14, Pf19, Pf23, Pf25) and four isolates of *Bacillus* spp. (Bc01, Bc09, Bc14, Bc20) were found as potential biocontrol agents against the *Fusarium* wilt, Under pot condition, Pf14 and Bc20 isolates treated pigeon pea seeds showed 59% and 50% increase in seedling growth and under field condition, Pf14 treated seeds resulted in higher grain yield.

Avinash et al. (2019) conducted an experiment was conducted to isolate and check the pathogenicity of *Fusarium* strains

isolated from the wilted Redgram plant. The results revealed that the isolate RGF1 has shown significant amount of disease incident in pre emergent and post emergence of plant. Further studies were carried out to control the wilt disease incident through the help of biocontrol agents isolated from rhizosphere soil of Redgram. The results shows that the treatment which includes consortium of microorganisms had controlled significant amount of disease incident.

Dukare et al. (2020) A total of 53 CDB was isolated from the pigeon pea rhizosphere. Among them, a total 26 bacterial isolates was obtained using serial dilutions method and remaining 27 were from the enrichment culture technique. Isolates differing in colony morphology and characteristic were picked, purified, and used in further stages of the experiment.

Mugiastuti et al. (2020) characterized four endophytic bacterial isolates and found that they have the potential to be developed as biopesticides to control maize disease, especially *R. solani* and *Pantoea sp.* *Bacillus sp.*, endophytic from the root (BK.A1; BK.A3; PP.A5) and *Bacillus sp.* endophytic from the stem (PPD.B2) can suppress the growth of *R.solani* by more than 50%, have a strong antagonistic index against *Pantoea sp.* (> 4), and can produce protease and lipase enzyme, and phosphate solubilization.

2.3 Chitinase activity of *Bacillus subtilis*

Nandakumar et al. (2007) evaluated the chitinase producing ability of *Pseudomonas fluorescens* strains viz., PF1, PB2 and FP7 in a culture medium with and without a chitin source and observed that addition of 1% (v/v) chitin in culture medium increased the bacterial population and chitinase activity. Isolate FP7 responded well to the addition of chitin by producing 31.2% increased chitinase in culture as

Singh et al. (2013) evaluated biocontrol potential of chitinase from different sources i.e. bacteria, fungi, plant, insect, etc and observed that inhibition or killing of harmful pests by biocontrol agents is environmentally safe and without creating any soil pollution

Surywanshi et al. (2013) treated *Trichoderma viride* with different conc. of ethyl methane sulfonate (EMS) and obtained mutants TVME1a, TVME1b, TVME1c, TVME2a, TVME2b, TVME2c, TVME3a, TVME3b, TVME3c, TVME4a, TVME4b and TVME4c which were further tested for chitinase productivity and found that mutants TVME4c, TVME4b and TVME3c were highest producer of chitinase enzyme.

Farag et al. (2014) isolated five fungi from different soil sample from Al-Jouf city, Saudi Arabia. Among them *Aspergillus terreus* was found to be a good chitinase producer when 2% of shrimp-shell powder was used as a sole carbon source in the fermentation medium. They studied properties of the crude chitinase and investigated the antifungal activity of crude *A. terreus* chitinase against *Aspergillus niger*, *Aspergillus oryzae*, *Penicillium oxysporium*, *Rhizoctonia solani*, *Fusarium oxysporium*, *Rhizopus* sp. and *Mucor* sp. and found that chitinase found to inhibit the growth of tested phytopathogenic fungi.

Lestari et al. (2017) Extracted and characterized extracellular chitinase from *Bacillus subtilis* B 298 According to production curve of the enzyme, it was known that incubation time which gave the highest chitinase activity of 15 hours with activity of 6.937 U/mL respectively. Effect of various temperatures on chitinase activity showed that optimum activity was achieved at 40°C with an activity of 5.764 U/mL respectively. Meanwhile, the optimum pH for chitinase activity was achieved at pH of 5.0 with an activity of 6.813 U/mL respectively.

Aggarwal et al. (2017) obtained a clear chitin hydrolysis halo (mm) formed around the bacterial colonies of actively growing 24-h bacterial culture in Luria Bertani (LB) broth was spot inoculated in the center of 1% CCA plate and incubated at 28 °C for 48–72 h. Promising CDB were chosen further based on the size of chitin-degrading zones in media.

Berini et al. (2018) opinioned that due to their mycolytic activities, chitinase producing bacteria prevents the proliferation of phytopathogens and holds great potential in regard to their development as

biofungicides. Biocontrol ability of such bacteria has been reported against many crop pathogens.

2.4 Biochemical studies activity of *B. subtilis*

Mary Balle Allen (1953) studied the effect of laboratory culture on certain biochemical characteristics of thermophilic aerobic spore forming bacteria, *subtilis* shows hydrolysis of starch. hydrolysis of gelatine and recorded observation on morphological characteristics of *B subtilis* as they are rod. 3-6 0.7-1.0 u spore are characteristically oval to cylindrical terminal and do not swell. The sporangium growth may be single or in pair or in short chain, colony types were highly variable one a finely fimbriate edge. smooth glossy non spreading colonies were common.

Gurney and Quesnel (1980) recorded observation on morphology of *B.subtilis* var niger Circular colony, buff-white. malt textured colony with irrigated margin elevation were noted.

Wilemse (1980) used 24 biochemical test for a and rapid computer assisted technique for the identification of sane selected *Bacillus* sp. The biochemical test shows VP postve test and 50 per cent gas production in glucose MR positive-Starch and gelabn hydrolysis negative.

Gill et al. (1986) studied about the morphological physiological and biochemical characters namely growth at 7 per cent NaCl, MR-VP test . catalase test , starch hydrolysis . gelatine liquification , acid production etc. They concluded that *B. subtilus* shows 100 per cent salt tolerance in growth and positive test for all above biochemical reaction.

Pretorius et al. (1986) recorded observation on morphological characters of *Bacillus* sp. The characters are chain. motility, round spores. ellipsoidal spores. cylindrical spores, central spores, sub-terminal, terminal spores.

Foldes et al. (2000) observed biochemical characters of *Bacillus* sp. isolates as positive reaction to starch hydrolysis. gelatin liquefaction and negative reaction for indole production.

Brain (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 of soybean.

Annamalai et al. (2009) studied morphological, and biochemical tests of *Bacillus subtilis* isolates, showed positive reaction for gram staining, starch hydrolysis, gelatin liquefaction and negative reaction for indole production.

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

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

Mohammadipour et al. (2009) study the characterization of chitin producing 290 phylloplane isolates of *Bacillus subtilis* collected from different ecological zones of Iran. Characterization was performed using blood agar, PCR. drop-collapse. and reverse-phase high-performance liquid chromatography (HPLC) analyses.

Awais et al. (2010) studied morphological and biochemical tests of *Bacillus subtilis* isolates and obtained positive reaction for gram staining, starch hydrolysis, catalase test, gelatin liquefaction and negative reaction for gas production from glucose, indole-production NR test.

Barman et al. (2011) obtained positive reaction for gram staining, starch hydrolysis, catalase test, gelatin liquefaction and negative reaction for Gas production from glucose, indole—production and MR test of B.S. isolates

Khan et al. (2011) studied morphological and biochemical tests of *B. subtilis* and showed that *Bacillus subtilis* is rod shaped. gram's

+ve and —ve reaction to indole acetic acid production, +ve to starch hydrolysis.

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Gaur et al.(2012) studied thermostable lipolytic enzyme of *B. subtilis* isolates estimated by qulative and quantitative experiments. The isolates were identified as *Bacillus* sp. by morphological,biochemical and molecular characterization.

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

Khusro et al. (2014) recorded *Bacillus subtilis* strain KPA as positive to catalase test and negative reaction t indole production and MR test.

Jha et al. (2016) found *Bacillus subtilis* to be gram-positive rod-shaped spore forming motile bacteria.

Avsar et al. (2017) reported positive reaction to gelatin liquefaction and catalase test of *Bacillus* sp.

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

Singh et al. (2017) found that *Bacillus subtilis* isolates showed positive reaction for gram staining and negative reaction to MR test.

Zhenxiang et al. (2018) reported that the isolates od *Bacillus subtilis* showed negative reaction towards indole and methyl red test.

2.5 Antagonistic activity of *Bacillus subtilis*

Goudar and Kulkarni (2000) studied the antagonistic nature of *Trichoderma viride*, *T. harzianum*, *Aspergillus niger*, *A. flavus*, *Bacillus subtilis*, *Pseudomonas fluorescense*, *Penicillium* spp. and *Streptomyces* spp. against *F. udum* and found that percent inhibition was more in *T. viride*

(87.03%) followed by *T. harzianum* (85.40%), *P. fluorescence* (81.87%) and *Bacillus subtilis* (72.23%).

Kaswate et al. (2003) revealed that *Trichoderma viride* was most effective in inhibiting various isolates of *Rhizoctonia bataticola* (100%) followed by *B. subtilis* (87.41%) and *P. fluorescens* (73.98%) against *Rhizoctonia bataticola*.

Siddiqui and Shakeel (2007) isolated twenty unidentified *Bacillus* species from pathogen-suppressive soils of pigeonpea field for control of wilt disease complex caused by *Heterodera cajani*, *Meloidogyne incognita* and *F. udum*. Among them five isolates (B602, B603, B605, B615 and B618) were considered to have potential antifungal activity & inhibitory effect.

Umashankar and Kumar (2013) screened five isolated *Bacillus* (BCOAH2, BCOAH3, BCOAH5, BCOAH8 and BCOAH7) and four isolated *Pseudomonas* (BCOAH9, BCOAH4, BCOAH6 and BCOAH1) against *Fusarium* and found that the bacterial isolates were highly antagonistic and inhibited the growth of *Fusarium*. Further they found that isolate BCOAH6 and BCOAH5 showed 56.30 and 52.90% inhibition of *Fusarium*.

Jadhav et al. (2014) recorded five isolates of *Bacillus subtilis* from rhizospheric soil of wilt infected pigeonpea plants, viz. BSI, BS2, BS3, BS4 and BS5. These isolates were evaluated for their antagonistic ability to reduce incidence of Pigeonpea wilt. Isolates Bs5 was found most effective against *Fusarium udum* which recorded 48.12% myceli growth inhibition in dual culture technique.

Rajasekhar et al. (2016) evaluated most promising antagonists *Trichoderma harzianum* (TH), *Pseudomonas flouescens* (PF), *Rhizobium* (Rh) and *Bacillus subtilis* (BS) in their study for plant health management of pigeonpea in different combinations to make consortia. Results in this study indicated that the consortia having, treatments T7 (TH+PF+BS+Rh) (86%), T2 (TH+BS) (82%) and T5 (PF+Rh) (77%) have shown remarkable wilt disease reduction. These results showed role of

biological consortia to play in integrated disease management and plant health management of pigeonpea.

Banakar et al. (2017) evaluated five bioagents *T. viride*, *T. harzianum*, *T. virens*, *P. fluorescens* and *B. subtilis* against foot rot of tomato caused by *Sclerotium rolfsii* and maximum mycelial inhibition was observed in *Trichoderma virens* (67%), followed by *T. viride* (61%) and *T. harzianum* (44%).

Huang et al. (2017) stated that *Bacillus subtilis* SL-44 has a great potential as biocontrol agent against *Rhizoctonia solani* on pepper plants.

Sumi et al. (2018) isolated five native bioagents viz. *Trichoderma harzianum*, *T. viride*, *T. koningii*, *Bacillus subtilis* and *Pseudomonas fluorescens* from the rhizosphere of pigeon pea crop using serial dilution technique. The bioagents were screened in vitro using dual culture plate technique to find the potential antagonists against *Sclerotium rolfsii*. Out of the three *Trichoderma* species and two bacterial antagonists, *Trichoderma harzianum* recorded the highest inhibition of *Sclerotium rolfsii* (57.70%), followed by *T. viride* with 43.00%, *Bacillus subtilis* (39.20%) and *Pseudomonas fluorescens* (38%), while the lowest inhibition was recorded from *T. koningii* with 37.70%.

Arsia et al. (2018) tested four antagonists viz; *Trichoderma vridii* (TV-S1) *T. harginum* (TH-S2), *Bacillus subillis* (BST-S1) and *Pseudomonas fleuroscence* (PSF-S2) against *Fusarium udum*. Among the antagonists PSF-S2 was found most effective with highest mycelial inhibition (77.21%) of the test pathogen followed by BST-S1 with 73.48% and TH-S2 with 66.60% inhibition and the lowest inhibition was found with TV-S1 with mycelial inhibition of 65.95 per cent

Al-Fadhal et al. (2019) observed that the 2 bacteria (*Pseudomonas* spp. NS-1 and *Bacillus* spp. NS-22) reduced considerably the *Fusarium* propagules in crop rhizosphere and controlled wilt disease incidence. *Bacillus* spp. and *Pseudomonas* spp. are the most ubiquitous soil bacteria that serve as phytostimulator and bio-protector in crop

rhizosphere. Their antifungal metabolites are known to suppress many soilborne phytopathogens such as *Rhizctonia solani* and *F. solani*

Ghante et al. (2019) screened *Trichoderma viride*, *T. harzianum*, *T. hamatum*, *T. longibrachiatum*, *T. (Gliocladium) virens*, *T. koningii*, *Aspergillus Niger*, *Trichoderma lignorum*, *Pseudomonas fluorescens* and *Bacillus subtilis* *in vitro* for their antifungal activities against *F. udum* by using dual culture technique. *Trichoderma viride* was found most effective with least linear mycelial growth (10.50 mm). Among bacterial isolates *B. subtilis* was superior over *Pseudomonas fluorescens*.

Sammer (2019) opined that the biological strategy of using *B. subtilis* and *T. harzianum* in controlling *R. solani* and *F. graminearum* in wheat could be improved by using fungicides at low rates.

2.6 Compatibility of *Bacillus subtilis* with chemicals

Singh and Dube (2010) evaluated the compatibility of different fungicides (Metalaxyl, Ridomil MZ, Captan, Difolatan, Thirum and Bavistin) at 0.3 and 0.6% with *Pseudomonas fluorescens* and found that *P. fluorescens* was compatible

Ahiladevi and Prakasam (2013) reported that tolerance concentration for *B. subtilis* were maximum in Carbendazim (5000mg/l) followed by Captan (3200 mg/l) whereas, Mancozeb was most inhibitory and .

Madhuri et al (2013) studied the effect of herbicides on *S. rolfsii*, *R. solani* and *F.udum* and revealed that the herbicides have certain non- target effects on plant pathogens present in the soil may influence plant pathogens interactions through their effect on the plant, or on the surrounding soil organisms including symbiotic interactions.

Nusarat et al. (2014) isolated and characterized microorganism that were capable of degrading Imidacloprid between 48-72 hours after incubation in minimal media.

Shetti et al. (2014) studied the biodegradation ability of soil bacterium *Bacillus weihenstephanesis* to Imidacloprid. The result showed

that *Bacillus* was able to degrade 78% Imidacloprid within four weeks of incubation.

Basamma and Kulkarni (2017) assessed compatibility of bacterial biocontrol agent *Bacillus subtilis* with commonly used chemical fungicides viz., Carbendazim, Mancozeb, MetalaxylIMZ, Wettable sulphur, Hexaconazole, Difenconazole, Tebuconazole and Kresoxim methyl at different concentrations of 50, 100, 250, 500, 1000, 2000, 3000 and 5000 mg/lit for solid formulation fungicides and μ /lit for liquid formulation fungicides respectively. The compatibility tests revealed that among the solid formulation fungicides, the *B. subtilis* showed more tolerance to Carbendazim and among the liquid formulation fungicides Hexaconazole and Kresoxim methyl showed maximum compatibility upto 3000 μ / l concentration. The fungicides viz., Carbendazim, Difenconazole, Hexaconazole and Kresoxim methyl were compatible with *B. subtilis* at concentrations which were recommended for were plant disease management.

Raghavendra and Gundappagol (2017) showed that the herbicide treatments inhibited the development of microbial populations in the soil, and the degree of inhibition was closely related to the mode of application as pre emergence or post emergence and varied with the types of herbicide. Weed free check recorded the highest number of microorganisms at all the different growth stages and differed in herbicides treated plots. The study suggested that the herbicide application to soil of chickpea cultivation cause transient impacts on microbial population growth, when applied at recommended field application rate.

Harshita et al. (2019) recorded that two potential bacterial antagonists viz. *Bacillus subtilis* and *Pseudomonas fluorescens* were compatible with different concentrations (50 ppm, 100 ppm, 200 ppm) of Carbendazim and Topsin M in vitro.

2.7 Growth promoting ability of *B. subtilis*

Baki and Anderson (1973) recorded germination percentage of seeds and vigour index using the formulas. Vigour index=Percent Germination × seedling length (shoot length+ root length).

Podile and Dube (1988) observed enhancement in plant growth and yield of five crops through seed bacterization with an antibiotic producing strain of *Bacillus subtilis* AF1.

Jacob and Sivaprakasam (1993) tested the effectiveness of seed treatment with the fungal antagonists *T. harzianum* and *T. viride* as well as the bacterial antagonist *Bacillus subtilis* against root rot of black gram caused by *Macrophomina phaseolina*. The seedlings showed lower infection and longer shoots and no significant different in the length of the roots.

Sumitha and Gaikwad (1995) showed that antagonists *T. harzianum* and *Bacillus subtilis* produced no adverse effects on pigeonpea seed germination. Seed coated with antagonists germinated better than untreated seeds and produced longer

Junges et al. (2013) confirmed the the results in which bioprimering with *B. subtilis* in combination with polymer coating showed increase in germination by reduction of dead seeds, weak and abnormal seedlings in maize

Keote et al. (2014) used bioagents against chick pea wilt under In-vitro and green house (pot culture) conditions. Under Dual culture bioagents *Trichoderma viride*, *Bacillus subtilis* and *Pseudomonas fluorescens* showed significant results.

Mane and Yadav (2015) obtained 48.22% growth inhibition of *Fusarium oxysporum* F. sp. in vitro due to B.S. under biocontrol efficacy

Shifa et al. (2015) found that among the various biocontrol agents tested *Bacillus subtilis* strain G-1 was the most effective in inhibiting the mycelial growth of *S. rolfsii* and recorded an inhibition of 28% of

groundnut seeds when treated with *B. subtilis* G-1 showed significant increases in root length, shoot length and seedling vigour.

Sathya (2016) opined that the isolated strain of *Bacillus amyloliquifaciens* plays a unique role in plant growth promoting activities in jute and showed the best effect in seed germination, seedling growth, highest pathogen inhibition and reduction of stem rot disease severity in the green house test.

From the successive in vitro experiments and in vivo bioassay, 2 promising bacterial strains (NS-1 and NS-22) that exhibited strong chitinolytic activities and mycolytic action were biochemically characterized and identified as *Pseudomonas* spp. NS-1 (NCBI GenBank accession number- KT175909) and *Bacillus* spp. NS-22 (NCBI GenBank accession number-MH425454), using 16S rDNA sequencing. In planta, disease severities for *Pseudomonas* spp. NS-1- and *Bacillus* spp. NS-22-treated plants were only about 14.62 and 23.08%, respectively, as compared to pathogen-only control (67.34%) at the end of 4 weeks. Additionally, these bacteria improved plant germination and fresh and dry plant biomasses under pathogen-challenged conditions. Further, both the strains were able to cause a considerable amount of reduction in the fungal biomass, as revealed by bacteria-pathogen interaction studies in liquid culture. Scanning electron microscopy studies also revealed the strong antifungal activities of these 2 chitinolytic bacteria on the pathogen hyphae and mycelia. The curling, bulging, breakdown, and other dysfunctions in fungal hyphae were induced by the biocontrol bacteria. In nutshell, the present exclusive investigation demonstrated mycolytic potential and biocontrol prospects of the 2 promising bacteria (*Pseudomonas* spp. NS-1 and *Bacillus* spp. NS-22) for the sustainable *Fusarium wilt* management and as a phytostimulator in pigeon pea.

Dukare et al. (2020) identified and evaluated 53 native chitinolytic rhizobacteria as biocontrol potential against pigeon pea wilt disease caused by *Fusarium udum*.

CHAPTER III

MATERIAL AND METHODS

The present investigation was carried out during 2018-19 at Department of Plant Pathology, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola. The details of the material used and methods followed are described in this chapter.

3.1 Material

Following material were used for experimentation.

3.1.1 Glass wares and Plastic wares

During the course of investigation following glass wares and plastic wares were used Glass Petri plate's (Borosil and Schott Duran, Germany), test tubes (Borosil, Germany), conical flasks of 250ml, 500ml and 1000ml (Schott Duran, Germany), funnel (Schott Duran, Germany), beaker (Schott Duran, Germany), glass pipette (Schott Duran, Germany), measuring cylinder (Schott Duran, Germany), glass cover slip, glass rod, polypropylene Petri plates and eppendorf tubes etc.

3.1.2 Equipments

Standard laboratory equipments used for different experiments were Autoclave (Equitron, India), BOD incubator (Sanco, India), laminar airflow (Klenzaid, India), student microscope (Olympus, India), refrigerator (LG, India), hot air oven (Bio-techniques, India), digital weighing balance (Anamed, India), Bunsen burner, digital camera (Kodak, India), double distillation unit (JSGW, India), soil sterilization tank (locally made), etc.

3.1.3 Chemicals

Crab shell chitin for preparation of media, Streptomycin sulphate, Mercuric chloride, Spirit etc. were obtained from Department of Plant Pathology, Post Graduate Institute, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola.

3.1.4 Seeds

Seeds of PKV-Tara was obtained from Pulses Research Unit Dr. PDKV, Akola for experimentation.

3.1.5 Other materials

Blotter paper, Paper towel, non-absorbent cotton, muslin cloth, polyethylene bags, cork borer (5 mm), inoculation needle, micropipette, dissection needle, forceps, paper bags, butter paper bags, pencil, permanent marker, cello tapes, Whatman filter paper (4 mm), tags, polyethylene sheets, test tube stand, tray, hand sprayer, wash bottle, thread, wooden sticks, potato, rubber band, scissors, etc were used during study.

3.2 Methods

3.2.1 Collection of healthy and wilt infected soil sample

Healthy and wilt infected soil sample were collected from different locations of Vidarbha region from root zones of healthy and infected plants from 5 different locations of a field i.e. 4 from corner side and one from centre at the depth of 15-20 cm. Composite samples were prepared by mixing soil samples collected from respective field and were stored in polythene bags or plastic containers which were properly tied and tagged with the sample label for further study.

3.2.2 Sterilization of glassware and media

During entire course of investigation glasswares viz., petriplates, pipettes, flasks, etc were sterilized in hot air oven at 180°C for 1 hr. before its use, whereas distilled water and media were sterilized in autoclave at 15 lbs for 15 minutes.

3.2.3 Precautions to eliminate contamination

All isolation work and inoculation of microbial culture was carried out in laminar air flow. The laminar flow was sterilized by glowing ultra violet light for ½ hr prior to commencement of work. The working surface of laminar flow and side glasses were surface sterilized with

denatured spirit. Moreover, other such necessary care was taken to maintain and carryout work under aseptic condition.

3.2.4 Preparation of colloidal chitin agar medium (CCA)

The colloidal chitin agar medium was prepared by using colloidal chitin which was obtained as per the method given by Roberts and Selintrenikoff (1988).

- 1) Five g of chitin powder (HiMedia Laboratories Pvt. Ltd., Mumbai) was added slowly into 60 ml of concentrated HCl (Sd. Fine Chemicals Ltd., Mumbai) and left for vigorous shaking for overnight at 40⁰C.
- 2) The mixture was added to two liters of ice-cold 95 per cent ethanol with rapid stirring and kept overnight at room temperature of 25⁰C.
- 3) The precipitate was collected by centrifugation at 5,000 rpm for 20 minutes at 4⁰C and then washed with sterile distilled water until the pH of the colloidal chitin turned neutral (pH 7.0).
- 4) Five percent colloidal chitin solution was prepared and stored at 4⁰C for further use.
- 5) The colloidal chitin thus obtained was used for preparation of media.

Following materials were used for preparing media.

Colloidal chitin	-	5g
Na ₂ HPO ₄	-	2g
KH ₂ PO ₄	-	1g
NaCl	-	0.5g
NH ₄ Cl	-	1g
MgSO ₄ 7H ₂ O	-	0.5g
CaCl ₂ 2H ₂ O	-	0.5g
Yeast extract	-	0.5g
Agar Agar	-	20g
Distilled water	-	1000 ml

- All materials were mixed and dissolved by indirect heating (in boiling water) and volume was made up to one litre. The medium was distributed and poured in conical flask then plugged with non-absorbent cotton and autoclaved at 15 lbs. pressure for 15 minutes.

3.2.5 Screening of micro-organisms on CCA media by serial dilution method

Soil samples collected from different places were used for isolation of fungi and bacteria by serial dilution method. Tubes containing 9 ml distilled water were sterilized in autoclave at 1.04 kg/ cm² for 15 minutes. One gram composite soil sample of respective location was added to 9 ml water in tube under aseptic conditions, mixed thoroughly to make 1:10 dilution. From this tube 1 ml suspension was taken with sterilized pipette and added to 9ml water in second tube, again one ml suspension from 2nd tube was added to 3rd tube. From third to fourth and likewise five dilutions were prepared and 1ml suspension per plate was added in 3 sterilized glass petriplates. Approximately 20 ml sterilized warm CCA (at approx. 45°C) was poured in to each plate and the plates were gently rotated. After solidification of medium, plates were incubated at room temperature for 7 days. Bacterial isolates exhibiting a clear zone (degradation of chitin) around the colony were picked and the fungal purified by frequent subculturing on NA media adopted by Velusamy and Das (2014).

3.3 Preparation of nutrient agar medium (NA)

The medium was prepared by following ingredients

Beef extract - 3g
Peptone - 5g
Agar - 20g
Distilled water - 1000ml

Beef extract and peptone were mixed in 500ml water and agar was dissolved in another 500 ml water by heating Both the parts were mixed and its volume was made to 1 litre. The medium was distributed in

flasks. The slants were used for maintenance of culture and the medium in flasks for purification of bacteria.

3.3.1 Identification of *Bacillus subtilis*

Organisms were identified after mounting on slides under microscope. Gram staining and morphology were taken into consideration for preliminary identification of *Bacillus subtilis*.

3.3.1.1 Gram reaction

Initial identification was made by gram staining and by studying the morphological characters of the isolates of *Bacillus subtilis*. First a smear was prepared of bacterial cells by holding a clean slide by grasping at edges. A loopful of bacterial suspension was transferred in the center of slide, with the help of wire loop. The drop was smeared over slide and air dried. Then dried smear was fixed by passing the slide 3-4 times rapidly over the flame. The smear was flooded with Crystal violet for 30 seconds, washed in the tap water. The smear was immersed in Lugol's iodine solution for 30 seconds. washed in tap water, then decolorized with 95% alcohol and rinsed with water 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 lens.

3.4 Biochemical tests of *Bacillus subtilis* isolates

3.4.1 Gelatin liquefaction

The test indicated utilization of protein and production of proteolytic enzymes by bacterium. Nutrient gelatin medium used i.e Nutrient broth + 1.5% gelatin. Bacterial culture were inoculated through stab of a nutrient gelatin tube incubated for 3 days. Uninoculated tubes served as control and observed for liquefaction.

3.4.2 Catalase test

A loop full of 24 hours old culture of the test bacterium was placed on the clean glass slide and to this a drop of 3% hydrogen peroxide (H_2O_2) was mixed and allowed to react for few minutes and observed for the production of gas bubbles.

3.4.3 Potassium hydroxide (KOH) solubility test

Two drop of 3% potassium hydroxide (KOH) was placed on a glass slide. A loop full of culture was picked up the medium with help of inoculating needle and mixed with KOH drop for 10 seconds and raised the needle at 0.5 to 2 cm above formation of thread was treated as positive test.

3.4.4 Arginine Dihydrolise

An inoculum from a pure culture was transferred aseptically to a sterile tube of arginine dihydrolase broth The inoculated tube was incubated at 35-37 °C for 24 hours and the preliminary results were determined. The microbe first used the glucose present to cause the pH to drop. This was indicated by a change from purple to yellow. Once the medium has been acidified, the enzyme arginine dihydrolase is activated. The culture was incubated for an additional 24 hours at 35-37 °C to allow the microbe to use the arginine. The final results are then obtained by observing the tube at 48 hours. Change back to purple from yellow indicated positive test for arginine dihydrolase. Failure to turn yellow at 24 hours or to revert back to purple at 48 hours indicated negative result

3.4.5 Sodium Chloride (NaCl)

Medium was prepared by mixing Heart digest (10 g), enzymatic digest of animal tissue (10 g), NaCl (65 g), dextrose (1 g) and bromocresol purple (0.016 g), in 1000 ml. water. One or two colonies from an 18- to 24-hour culture were incubated into 6.5% NaCl broth. The tubes were incubated at 35-37 °C in ambient air for 48 hours. Examined the tubes for turbidity after 24 hours again at 48 and 72 hours for negative. Visible turbidity in the broth, with or without a color change from purple to yellow indicated positive results. Turbidity alone indicated positive test while No turbidity and no color change after 72 hours of incubation indicated negative test.

3.4.6 Methyl Red

MR-VP broth was used for both MR Test and VP test. Only the addition of reagent differed and both tests were carried out consecutively. Inoculated two tubes containing MR-VP Broth with a pure culture of the microorganisms under investigation. Incubate at 35 °C up to 4 days. Add about 5 drops of the methyl red indicator solution to the first tube (for Voges-Proskauer test, Barrit's reagent was added to another tube). A positive reaction was indicated by the change in color of the medium to red within a few minutes.

3.4.7 H₂S production

Ten ml of SIM agar media (beef extract-3 g, peptone-30 g, ferrous ammonium sulphate-0.2 g, sodium thiosulphate 0.025 g and distilled water) was poured in each test tubes and autoclave at 15 psi for 15 min. A loop full culture of *Bacillus subtilis* was inoculate by means of stab inoculation in each test tube, then lead acetate paper was inserted in these tubes and hold by the plugs above the culture without touching the medium. The tubes were inoculated at 30⁰C for three days. If filter paper strips turns black, it indicated the positive test for H₂S production.

3.5 Isolation of test organisms

3.5.1 Preparation of PDA

The medium was prepared by following ingredients

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

- Peeled potatoes were sliced into pieces and boiled in 600 ml water till properly cooked.
- The starch extract was strained through muslin cloth and measured. In remaining water, dextrose and agar were mixed.
- Potato starch extract was added to it and volume was made to 1 litre.

- The medium was distributed in flasks and sterilized in autoclave.
- This media was used for purification of fungi from colloidal chitin agar media whereas slants were used for maintenance of culture

3.5.2 Isolation

Soil samples were collected from rhizosphere of different crops. Test tubes with 9 ml distilled water were sterilized in autoclave for preparation of water blank.

Procedure:-

- 1) Dilution blank was labeled 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} and 3 plates marked with marking pencil.
- 2) Prepared initial dilution by adding 1 g of air dry soil sample from composite sample into 9 ml distilled sterilized water and dilution blank labeled 10^{-1} . Thus diluting original sample 10 times (1:10).
- 3) The dilution was vigorously shaken to obtain uniform distribution and release of microorganism from adhering soil particles.
- 4) The soil particles were allowed to settle and from the first dilution transferred 1 ml of suspension to the dilution blank number of 10^{-2} by sterile pipette shaken well for minutes.
- 5) 1 ml suspension was transferred from 10^{-2} to 10^{-3} dilution blank with a fresh sterile pipette.
- 6) The procedure was repeated till the original sample had been diluted to 10^{-7} using every time a fresh sterile pipette.
- 7) 1 ml of suspension was transferred from the appropriate dilution (10^{-6} and 10^{-7}) with respective pipette to sterile Petriplates. Three Petriplates were used for each dilution.
- 8) Approximately 20 ml melted medium was added to each Petridish, containing the diluted sample. The content of each dish was rotated gently to distribute the cell through out the medium.
- 9) The plates were allowed to solidify and plates were incubated in an inverted position for 48hr at $28\pm 2^{\circ}\text{C}$. The 6th and 7th dilution were

plated in Petriplates containing selective medium for isolating *Bacillus subtilis*.

- 10) Well separated individual colonies with whitish colour and cream colour pigments were marked.
- 11) The individual colonies were picked up with sterilized loop and transferred on fresh NA medium plate to establish pure culture.

3.6 Study of antagonistic activity

Antagonistic activities of isolated *Bacillus subtilis* against pathogens causing root infection in pigeonpea (*F. udum*, *R. bataticola* and *S. rolfsii*) was studied in lab by dual culture technique

3.6.1 Dual culture Technique

Dual culture technique as described by Vincent (1927) was adopted for estimating the ability of *Bacillus subtilis* isolates as an antagonist against *Fusarium udum*, *Rhizoctonia bataticola* and *Sclerotium rolfsii*.

- The tests were carried out on PDA medium in triplicate. The mycelia disc (5mm) was taken from 7 days old culture of pathogen individually and transferred to centre of agar plate and bacterial isolate was streaked by inoculating needle to both side of pathogen at equal distance
- The plate were incubated at 28 ± 2 °C for 5 days and then inhibition of mycelial growth of pathogen was measured.
- Antagonistic effect was expressed as zone of inhibition by subtracting colony diameter of the fungus from the control. Control plates were run without antagonistic organism.

3.7 Preparation of carrier based inoculam of *B. subtilis*

The best performing isolates of *B. subtilis* was used for preparing carrier based inoculam.

The *B. subtilis* multiplied on Nutrient Broth. 5-7 days old culture from respective broth was used for preparation of carrier based

inoculam. The bacterial growth obtained after incubation were mixed with the talc powder in 1:2 proportion i.e., 100 ml broth culture of *B. subtilis* were mixed in 200g of sterilized talcum powder, the count were maintained as per BIS and dried in shade. After overnight drying the formulation having *B. subtilis* were packed in polythene bag.

The bags were sealed and kept for further use, seeds of pigeonpea variety PKV-Tara was treated with these formulations at recommended doses.

3.8 Effect of *Bacillus subtilis* isolates on seedling vigour index by paper towel method

3.8.1 Procedure for germination test

1. Paper towel was spread on flat surface and was moisten with water until it was thoroughly damp. Pigeonpea seeds of PKV-Tara was treated with isolates of *Bacillus subtilis* individually @10 g/kg of seed.
2. One hundred seeds from each treatment were placed on paper towel keeping 10 seeds in a row. Ten such rows were placed on paper, for each treatment 3 replication were made.
3. Second moistened towel paper was taken and was carefully placed over the first paper leaving the seeds sandwiched between two towels.
4. The paper were rolled up with the seeds in between and placed in sealed container that retained the moisture. The container were placed in an area of relatively stable temprature
5. After required germination period (10 days after placing) seeds on paper towel were removed from the container and unwrapped carefully so that the fragile shoots were not destroyed.
6. The seedling were observed for germination percent, root length, shoot length and seedling vigour index was calculated by following way.

$$\text{Germination percent} = \frac{\text{Number of seed germinated}}{\text{Number of seed sown}} \times 100$$

Vigour index = Percent Germination × seedling length (shoot length+ root length).

Given by Abdul Baki and Anderson (1973)

3.8.2 Preparation of carrier based cultures of isolates of *Bacillus subtilis*

3.9 Sensitivity of *Bacillus subtilis* isolates to Pesticides (Poison food technique)

Poison food technique was employed to test the sensitivity of isolates of *Bacillus subtilis* to commonly used fungicides, weedicides & insecticides

Following chemicals were used for poison food technique

Sr. No.	Name of Chemicals	Conc.
	Fungicides	
1.	Thiram	0.3%
2.	Carbendazim	0.1%
3.	Carboxin + Thiram (Combined product)	0.3%
	Insecticides	
4.	Imidacloprid	0.4%
5.	Thiomethoxom	0.4%
	Weedicides	
6.	Imazethapyr	0.3%
7.	Pendimethalin	0.7%

- The above fungicide, weedicide and insecticide with their respective concentrations were incorporated in Nutrient agar Medium before pouring in plates.

- Five plates of each fungicide, weedicide and insecticide were inoculated with 1ml bacterial suspension from 7th dilution of carrier based inoculants. The plates were incubated for 1-2 days. Control plates were run without chemicals. CFU were measured after 24-48hr of incubation and sensitivity was judged based on population in control.
- The inhibition of bioagent was calculated by following formula.

$$\% \text{ growth inhibition} = \frac{\text{No. of colonies in control} - \text{No. of colonies in treatment}}{\text{No. of colonies in control}} \times 100$$

3.10 Statistical analysis

Statistical analysis was done by using 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

RESULT AND DISCUSSION

The research work on “Antagonistic activity of *Bacillus subtilis* against *Fusarium udum*” was carried out during *Kharif* 2018-19 in Department of Plant Pathology, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola.

The observations recorded and the data generated through different experimentation is interpreted and presented in this chapter. Bioagents are the important component of integrated pest/disease management. Microbial pesticides are emerging as major driving force to the chemicals, as indiscriminate and excessive use of chemicals disturb the eco system. In this study native biocontrol agents were screened on the basis of their ability of chitin degradation and tested against soil borne pathogens of pigeon pea by dual culture method.

4.1 Isolation of *Bacillus subtilis* from healthy and wilt infected soil sample from different places of Vidarbha

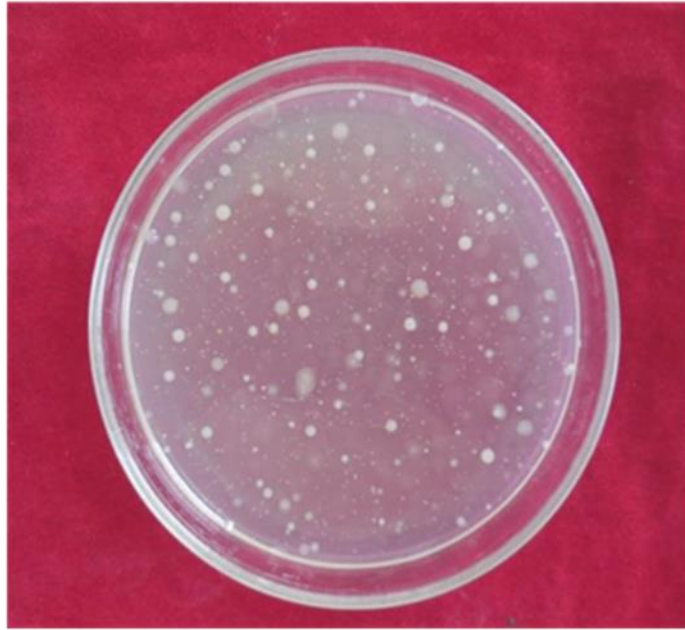
During the study soil samples from the rhizosphere of pigeon pea healthy and wilt infected plants were collected from different places of Vidarbha.

Forty soil samples were collected i.e. 9 from Akola, 9 from Buldhana, 9 from Amravati, 9 from Washim, 2 from Nagpur, 1 from Chandrapur, 1 from Gadchiroli from different places of fields during June to September 2018 (Table 1).

These collected soil samples were used to isolate *Bacillus subtilis* by serial dilution technique on selective media. The isolated *B. subtilis* were assessed for chitin degrading ability. For this Colloidal Chitin Agar media was used and the serial dilution method was employed. Data revealed that out of 40 soil samples, 10 samples contained *Bacillus subtilis* exhibiting chitin degradation ability on CCA media while the remaining 30 were empty. Colonies exhibiting clear zone on colloidal chitin agar media were picked up, purified & reconfirmed as *B. subtilis* based on their morphological and cultural characteristics on media.

Table 1. Soil samples exhibiting zone of solubilization on CCA media

Sr. No.	Dist.	Location	Zone of solubilization observed
1.	Akola	Akot	√
		Balapur	X
		Barshitakli	X
		Borgaon Manju	X
		Lakhanwada	X
		Murtijapur	√
		Patur	√
		Shirla	X
		Wadala	X
2.	Amravati	Advi	X
		Amravati	√
		Bhatkuli	X
		Daryapur	X
		Loni	X
		Ner	X
		Nimbhora	X
		Paratwada	X
		Pedhi	X
Uprai	X		
3.	Buldhana	Amdapur	X
		Chikhali	√
		Janephal	√
		Kolara	X
		Lakhanwada	X
		Lavala	X
		Mehkar	√
		Sakharkhelda	X
		Undri	X
4.	Chandrapur	Chandrapur	X
5.	Gadchiroli	Gadchiroli	X
6.	Washim	Kamargaon	X
		Karanj	X
		Malegaon	X
		Mangurpur	√
		Medshi	√
		Risod	√
		Tiwali	X
		Vyad	X
		Wakad	X
7.	Nagpur	Katol	X
		Nagpur	X



Colonies of *Bacillus subtilis*



Pure culture plate

Plate 1. Isolation of *Bacillus subtilis*

4.2 *Bacillus subtilis* identified based on morphological and cultural characteristics

From the collected soil samples isolates of ten *Bacillus subtilis* were obtained. Based on their morphological characters the isolates were identified

The *Bacillus subtilis* exhibited their ability of chitin degradation and solubilization zone ranged from 5 to 9 mm with the maximum by *Bacillus subtilis* from Patur and Medshi 9 mm and the least of 5 mm by BS8 & BS9 i.e. isolate from Mehkar & Janephal zone range. The selection of *Bacillus subtilis* through potential of chitinase from bacteria were also made by Shubakov and Kucheryavkh (2004), Nandkumar et al. (2007) and Jayalakshmi et al. (2009) and Singh et al. (2013) observed high inhibitory ability against pathogens. Narayanan et al. (2013), Suryawanshi et al. (2013) and Farag et al. (2014) made their studies on the basis of chitinase activity of *A. terreus*, *Trichoderma viride* and *A. flavus* respectively and the same were proved efficient antagonist against pathogens. The similar basis was adopted for selecting the bioagents under present studies.

Table 2. *Bacillus subtilis* isolated from soil samples

Sr. No.	Location of samples	Zone of solubilization (mm)	Labelled as
1	Patur	9	BS1
2	Murtijapur	7	BS2
3	Medshi	9	BS3
4	Risod	7	BS4
5	Mangrurpur	7	BS5
6	Akot	7	BS6
7	Amravati	6	BS7
8	Mehkar	5	BS8
9	Janephal	5	BS9
10	Chikhli	6	BS10

4.3 Hydrogen ion concentration and electrical conductivity

4.3.1 Hydrogen ion concentration and electrical conductivity of use soil samples containing chitin degradily *B. subtilis*

Data presented in Table 3 showed that the pH and EC of the soil samples which contained *B. subtilis* having chitin degrading ability on CCA was ranged from 8.1 to 8.6 and 0.21 to 0.52 dSm⁻¹ respectively having isolate with maximum solubilizing zone. Soil samples showed least pH and EC i.e. 8.1 and 0.21 dSm⁻¹ respectively whereas Janephal having isolate with minimum zone of solubilization sample exhibited more pH of 8.5 and less EC of 0.29 dSm⁻¹. Although the trend indicated maximum zone of zone solubilisation with least PH and EC, it could not be confirmed because of lack of literature on this aspect, however it can be considered PH and EC plays an important role in supporting the activity of bioagents.

Table 3. Hydrogen ion concentration, electrical conductivity and population of *B. subtilis*

Sr. No	Location of Samples	pH	EC (dSm ⁻¹)	Population of <i>B. subtilis</i> (cfu x 10 ⁷)
1.	BS1	8.2	0.40	50.66
2.	BS2	8.3	0.52	50.33
3.	BS3	8.1	0.21	54.33
4.	BS4	8.3	0.30	41.66
5.	BS5	8.4	0.41	44.00
6.	BS6	8.3	0.39	44.33
7.	BS7	8.5	0.34	39.00
8.	BS8	8.5	0.31	38.10
9.	BS9	8.6	0.29	34.10
10.	BS10	8.5	0.40	41.66

The population of the *Bacillus subtilis* on its respective media also varied and seen to be fluctuated according to the ability of chitin degradation. Isolate BS3 show maximum population (54.33×10^7 cfu/g) followed by BS1 (50.66×10^7 cfu/g) which were having maximum zone of solubilisation on CCA media i.e. 9mm. the least population of 34.10×10^7 cfu/g of soil was recorded in BS9 followed by BS8 38.10×10^7 cfu/g where the zone of solubilisation was also least i.e 5mm. this indicated that the population of the bioagent varied depending upon their aggressiveness or ability of having chitin degradation which on, although since logically true, needs more data for confirmation.

4.4 Biochemical Characterization of *Bacillus subtilis*

Biochemical tests were carried out to identified the *Bacillus subtilis* and results revealed that the strains collected from different regions were *B. subtilis* strains.

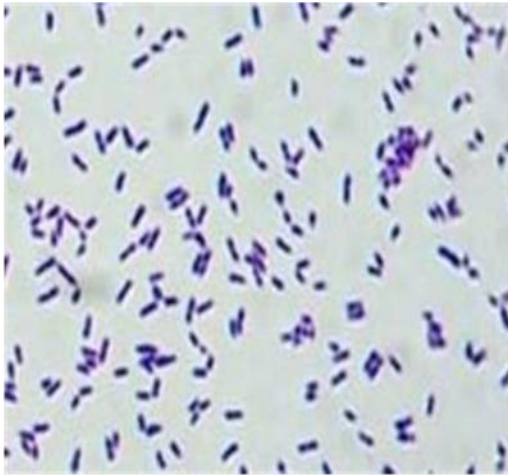
Obtained from various test are presented in table 4 and plate 2a and 2b revealed that all the isolates tested positive for Gram reaction, negative for KOH, positive for catalase test, gelatin liquification, growth in 5% NaCl, Arginine dehydrolase, H₂S production and negative for methyl test.

Gill et al. (1986), Foldes et al. (2000) Annamalai et al. (2009), Zhang et al. (2008) Awais et al. (2010), Barman et al. (2011), Khan et al.(2011), Karimi et al. (2012), Khusro et al. (2014), Jha et al. (2016), Ausar et al. (2017), Mandla et al. (2017), Singh et al. (2017) and Zhenxiang et al.(2018) are some of the workers who reported similar observation in one or other biochemical test for *Bacillus subtilis*. Their findings and the result of present study supported the confirmation of isolates as *Bacillus subtilis*.

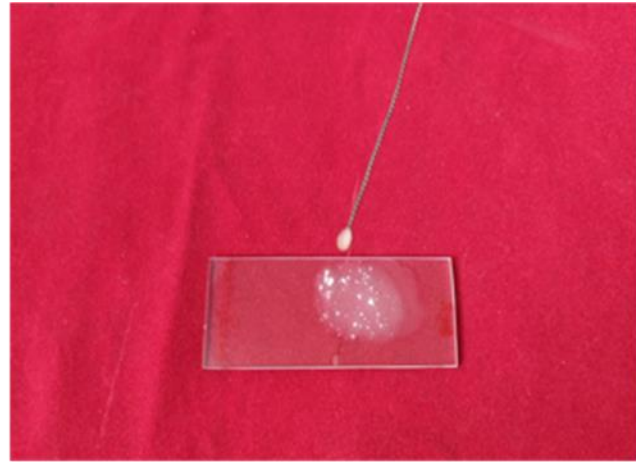
Table 4. Biochemical test of *Bacillus subtilis*

Sr. No.	Isolates	Control	BS1	BS2	BS3	BS4	BS5	BS6	BS7	BS8	BS9	BS10
1	Gram reaction	-	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
2	KOH	-	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
3	Catalase test	-	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
4	Gelatin liguifiction	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
5	Growth in 5%Nacl	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
6	Arginine Dihydrolase	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
7	Methyl Red test	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
8	H2s production	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve

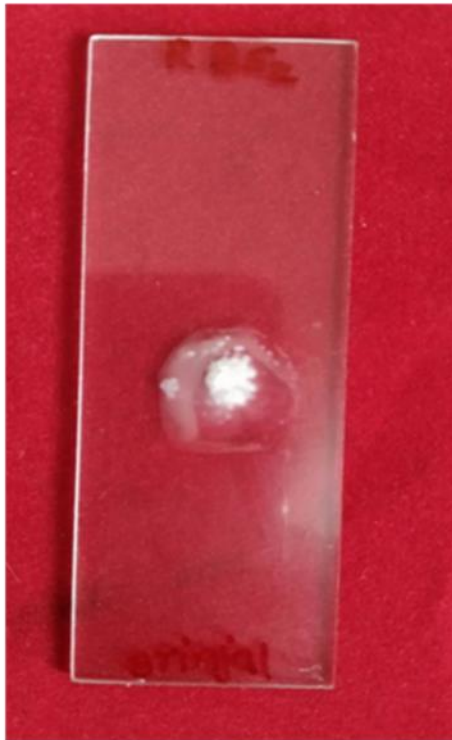
-ve =Negative; +ve = Positive



Gram reaction



KOH Test



Catalase Test



Gelatin liquification

Plat 2a. Biochemical Characterization of *Bacillus subtilis*



Growth in 5% NaCl



Arginine Dihydrolase



Methyl red test



H2S Production Test

Plate 2b. Biochemical Characterization of *Bacillus subtilis*

4.5 *In vitro* Efficacy of *Bacillus* isolates against *F. udum*, *R. bataticola* and *S. rolfsii*

Tested bacterial antagonists are able to minimize growth of fungus causing disease in pigeon pea. Among ten isolates of *Bacillus* BS3 (Medshi) allowing only 6.92 mm mycelial growth with 92.31% proved based over all other isolates however was at par with BS1 i.e. Patur isolates. Both the isolates exhibited maximum zone of solubilization on CCA media also. Least inhibition of *Fusarium udum* was observed in isolate BS7 80.73%. All other isolates also inhibited the growth of *F. udum* significantly over control.(Plate 3a)

Table 5. Efficacy of *Bacillus* isolates against *F. udum*, *R. bataticola* and *S. rolfsii*

Isolates	<i>F. udum</i>		<i>R. bataticola</i>		<i>S. rolfsii</i>	
	Colony diameter (mm)	Percent Growth Inhibition	Colony diameter (mm)	Percent Growth Inhibition	Colony diameter (mm)	Percent Growth Inhibition
BS1	7.22	91.98	10.14	88.73	10.36	88.48
BS2	8.03	91.08	10.36	88.49	11.59	87.13
BS3	6.92	92.31	9.57	89.37	9.92	88.97
BS4	8.51	90.14	14.36	84.04	14.48	83.91
BS5	9.51	89.84	18.33	79.63	18.66	79.26
BS6	8.85	90.16	16.36	81.82	16.66	81.49
BS7	10.14	80.73	22.59	74.90	22.11	75.44
BS8	10.85	87.94	26.25	70.83	19.92	74.90
BS9	11.36	87.37	27.59	69.35	27.89	69.01
BS10	9.05	89.39	19.59	78.24	19.66	78.15
Control	90.00	00	90.00	00	90.00	00
Ftest	Sig	-	Sig	-	Sig	-
SE(m)±	0.13	-	0.24	-	0.29	-
CD(P=0.01)	0.61	-	1.15	-	1.36	-

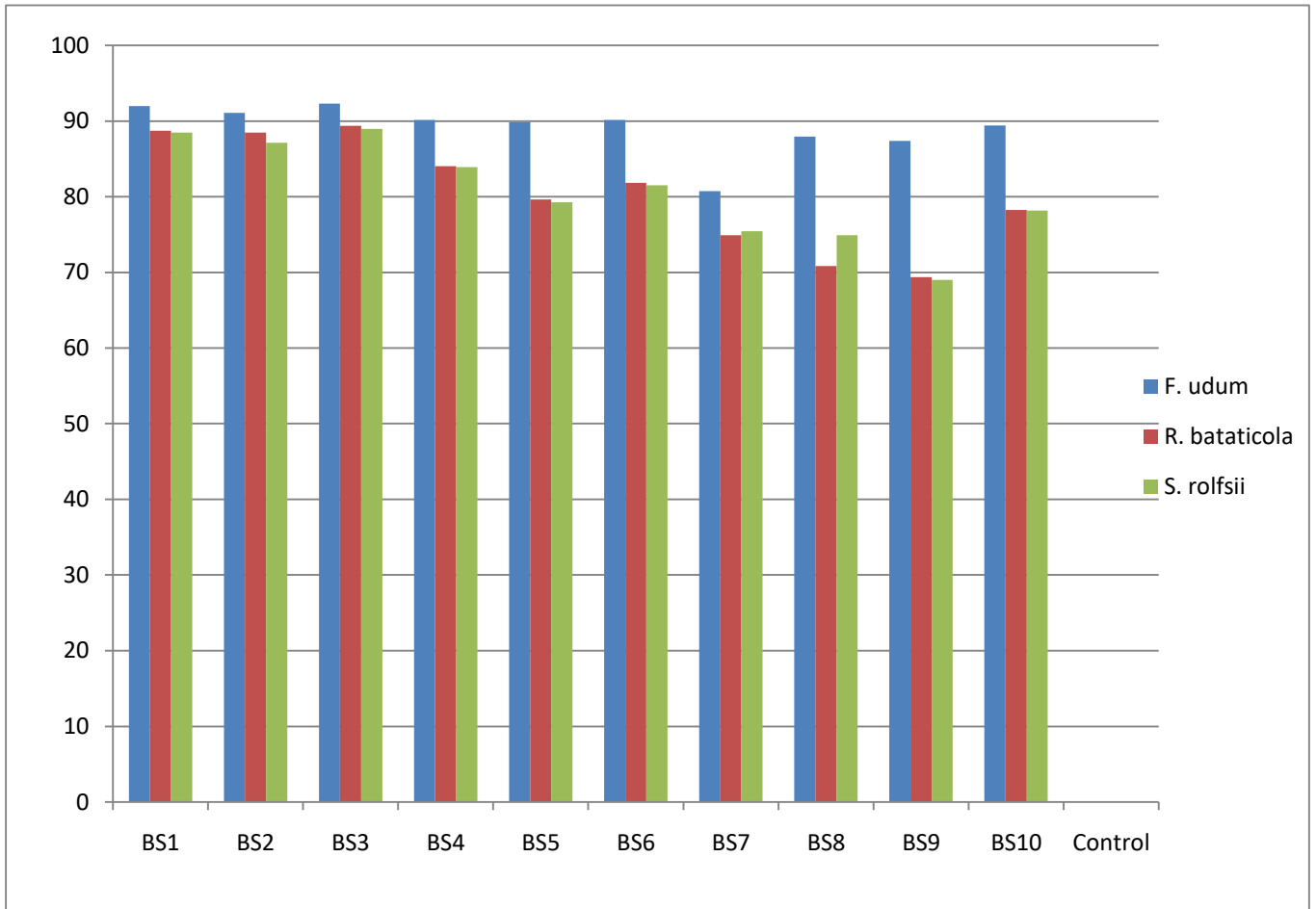


Fig. 1 Efficacy of *Bacillus* isolates against *F. udum*, *R. bataticola* and *S. rolfsii*



Control

Plate 3a. Efficacy of *Bacillus subtilis* against *F. udum*



Control

Plate 3b. Efficacy of *Bacillus subtilis* against *R. bataticola*



Control

Plat 3c. Efficacy of *Bacillus subtilis* against *S.rolsfii*

Similar trend of efficiency was achieved against *R. bataticola* due to *Bacillus* isolates. Maximum effect was noted due to BS3 with 9.57 mm growth of pathogen and 89.37% inhibition. Minimum ability was noted due to BS9 with 27.59% inhibition of test pathogen. Isolate BS3 and BS1 were at par with each other in inhibiting the growth of *R. bataticola*.(Plate 3b)

Highest efficiency with 88.97% inhibition of *S. rolfsii* was also noted due to BS3. Where the pathogen could grow only upto 9.92 mm as against 90 mm without antagonist followed by BS1 with 88.49% growth inhibition of pathogen.(Plate 3c)

All the tested isolates proved antagonistic inhibiting activity of the growth of *F. udum*, *R. bataticola* and *S. rolfsii in vitro*. The isolated having a more zone of solubilization on CCA media i.e. more chitinase activity perform better than isolates having comparatively less zone of solubilization on CCA media i.e. less chitinase activity.

The antagonistic activity *Bacillus subtilis* against *F. udum*, *R. bataticola* and *S. rolfsii* is very well known and recorded by various workers like Gonder and Kulkarni (2000), Kaswate et al. (2003), Siddiqui and Shakeel (2007), Jadhav et al. (2014), Arsia et al.(2018), Dukre et al. (2020) and Berini et al. (2018).The bioagents and their isolates vary from location to location and so as their efficacy Finding a suitable highly aggressive isolate of a potent biocontrol agent needs to be focused for a particular region/location which yielded a significant achievement in the form of isolate BS3 of *Bacillus* collected from Washim and Akola district. The results of the present studies can be taken as an rapid method screening the bioagents for their antagonistic activity based on zone of solubilization on CCA media as it was found directly correlated with the performance of the isolates in suppressing the growth of *F. udum*, *R. bataticola* and *S. rolfsii in vitro*.

4.6 Sensitivity of *Bacillus subtilis* to commonly used pesticides

4.6.1 *In vitro* effect of pesticides on growth of *Bacillus* isolates

Now days most of the seeds obtained from seed companies are treated with various seed dressing chemicals i.e. fungicides insecticides etc. with this view selected antagonists were tried against Pesticides at their recommended doses and their effect was assayed by Poison food technique.

Bacillus generally use as seed treater for reducing some soil/seed borne pathogens, colony forming units affected due to pesticides are noted .

Imidacloprid exhibited more than 50% inhibition almost all isolates of *B. subtilis* as compared to control indicating detrimental against *Bacillus*. Isolate BS3 recorded highest CFU i.e. 60.37×10^7 which was being lowest i.e. 48.26×10^7 in BS9. Thiomethoxom was also found non tolerable to isolates as less than 50% viable colonies in this chemical were observed. Isolate BS3 was least sensitive to Thiomethoxom with 54.76% inhibition. Isolate BS3 was found significantly superior over all others isolates in sustaining the effect of both the insecticides.(Plate 4)

Combined product of Carboxin + Thiram and Thiram alone was completely toxic to all isolates of *Bacillus* while Carbendazim exhibited maximum inhibition of 70.14% in BS9. The least sensitive isolate to this fungicide was also exhibited by BS3 with 42.97% inhibition. Only which was significantly or superior over all other isolates.(Plate 4)

Weedicides were found to be comparatively less hazardous to *Bacillus* isolates than insecticides and fungicides.

Pendimethalin in most cases supported more than 50% growth of *Bacillus* isolates. The maximum inhibition in this weedicide was 66.43%, while in imazethypr it was 65.69% in BS9 isolates. Among all the isolates, BS3 showed least sensitivity to the weedicides whereas BS9 exhibited highest sensitivity.(Plate 4)

Table 6. Sensitivity of *Bacillus* isolates to pesticides

CFU of <i>Bacillus</i> isolates ×10 ⁷															
Isolates	CFU in Control	Imidacloprid (0.4%)		Thiomethoxom (0.4%)		Carboxin+Thiram (0.3%)		Thiram (0.3%)		Carbendazim (0.1%)		Pendimethalin (0.7%)		Imazethypr (0.3%)	
		CFU	% reduction	CFU	% reduction	CFU	% reduction	CFU	% reduction	CFU	% reduction	CFU	% reduction	CFU	% reduction
Bs1	140	57.78	58.73	52.14	62.84	00	100	00	100	78.55	44.15	82.77	40.88	57.78	58.92
Bs2	140	57.59	58.87	50.44	64.05	00	100	00	100	75.57	46.27	77.59	44.58	57.59	59.06
Bs3	140	60.37	56.88	63.48	54.76	00	100	00	100	80.22	42.97	83.33	40.48	60.37	57.08
Bs4	140	55.44	60.40	49.14	64.98	00	100	00	100	72.25	48.63	74.81	46.56	55.66	60.43
Bs5	140	55.66	60.24	45.33	67.70	00	100	00	100	60.55	56.95	69.85	50.11	54.53	61.23
Bs6	140	54.53	61.05	36.44	74.03	00	100	00	100	70.55	49.84	71.10	49.21	55.44	60.58
Bs7	140	52.37	62.60	45.33	67.70	00	100	00	100	56.57	59.78	50.33	64.05	52.37	62.77
Bs8	140	50.62	63.84	44.00	68.65	00	100	00	100	56.31	59.97	49.64	40.48	50.62	64.01
Bs9	140	48.26	65.53	36.44	74.03	00	100	00	100	42.00	70.14	47.00	66.43	48.26	65.69
Bs10	140	52.99	62.15	40.14	71.39	00	100	00	100	57.67	59.00	65.33	53.34	52.78	62.33
Ftest		Sig	-	Sig	-	-	-	-	-	Sig	-	Sig	-	Sig	-
SE(m)±		0.26		0.15						0.30		0.24		0.26	
CD (P=0.01)		1.24		0.70						1.43		1.12		0.91	

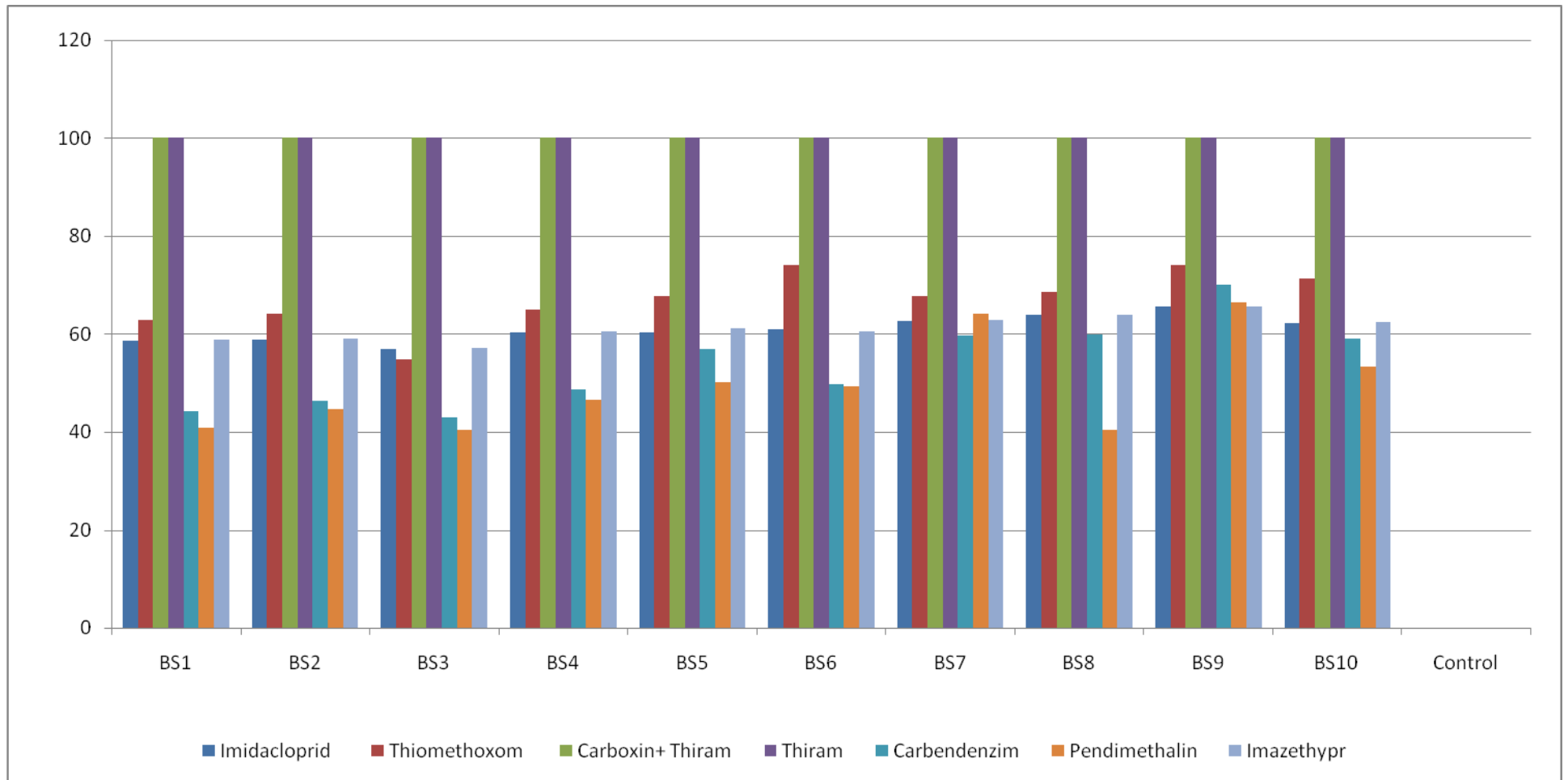


Fig. 2 Sensitivity of *Bacillus* isolates to pesticides



Thiram



Thiomethoxom



Carboxin + Thiram



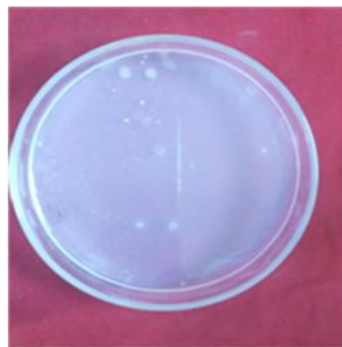
Imazethyper



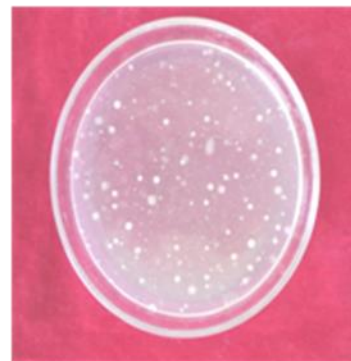
Carbendezim



Pendamethalin



Imidacloprid



Control

Plate 4. Sensitivity of *Bacillus* isolates to pesticides

In vitro studies clearly established that *Bacillus* spp can compatible with recommended dose of pesticides but in some cases i.e. in combined product of Carboxin + Thiram and Thiram alone the bioagent was sensitive, Shetti et al. (2014) studied the biodegradation ability of soil bacterium *Bacillus weihenstephanensis* to imidacloprid, they showed that *Bacillus* was able to degrade 78% imidacloprid within four weeks of incubation. Comparatively less toxic effect of insecticides and weedicides on *Bacillus* is also recorded in present study. Inhibitory effect of some fungicides on *B. subtilis* was also reported by Ahiladevi and Prakasam (2013) . Basmana and kulkarni (2017) also recorded more tolerance to carbendenzim by *B. subtilis* compared to other fungicides which favours the findings of present study. Compatability by carbendenzim with *B. subtilis* was also recorded by Harshita et al. (2019).

4.7 Growth promoting ability of *Bacillus subtilis* by Paper towel method

Isolates of *Bacillus subtilis* were treated to Pigeon pea for observing its growth promoting ability. The seedling vigour index was recorded and Presented in Table 8.

The data presented in Table 7 revealed that significantly highest germination percentage 75.00% was recorded due to application of BS3 @ 10 gm/ kg of seeds, which was at par with BS1 @ 10 gm/kg of seeds (74.55%) and BS2 @ 10gm/kg of seeds (74.04%). It was followed by BS4 @ 10gm/kg of seeds with 73.22%, BS5 @ 10gm/kg of seeds with 72.07%, BS6 @ 10gm/kg of seeds with 72.03%, BS10 @ 10gm/kg of seeds with 71.55%, BS8 @ 10gm/kg of seeds with 71.10%, BS7 @ 10gm/kg of seeds with 71.00% and BS9 @ 10gm/kg of seeds with 70.00 %.

Similarly root length, shoot length and seedling vigour index was also highest due to isolate BS3 i.e. 8.44 cm, 19.07 cm and 2082 respectively. Compared to 6.0 cm, 11.44 cm and 1046 root length, shoot length and seedling vigour index in control. Among all isolates, isolate BS9 proved inferior in promoting germination %, root length, shoot length and

there by seedling vigour index of pigeon pea where the parameters were recorded as 70%, 6.89 cm, 17.03 cm and 1687 respectively.(Plate 5)

Table 7. Growth promoting ability of *Bacillus subtilis* in Pigeon pea (Paper towel method)

Isolates	Applicat ⁿ rate gm/kg seeds	Germination %	Root length (cm)	Shoot length (cm)	Seedling vigour index
BS1	10	74.55	8.03	18.54	2004
BS2	10	74.04	8.13	18.44	1953
BS3	10	75.00	8.44	19.07	2082
BS4	10	73.22	8.31	18.14	1921
BS5	10	72.03	7.87	18.00	1873
BS6	10	72.07	8.03	18.00	1874
BS7	10	71.00	7.54	17.71	1808
BS8	10	71.10	7.07	17.33	1736
BS9	10	70.00	6.89	17.03	1687
BS10	10	71.55	7.44	17.88	1835
Control	-	60	6.0	11.44	1046
F test	-	Sig	Sig	Sig	-
SE (m)±	-	0.28	0.08	0.11	-
CD(P=0.01)	-	0.98	0.37	0.40	-

All isolates proved significantly superior over control in promoting the growth of pigeon pea. These findings are in conformity with Podile and Dube (1988) who observed enhancement in plant growth through seed bacterization with an antibiotic producing strain of *B. subtilis*. Sumitha and Gaikwad (1995) showed that antagonists *Bacillus subtilis* produced no adverse effects on pigeonpea seed germination, seed coated with antagonists germinated better than untreated seeds and produced longer roots and shoots. Jacob and Sivaprakasam (1993) and Dukare et al. (2020) also recorded same findings in the line of agreement of present.

Overall it was observed that native isolates of *Bacillus subtilis* having good antagonistic activity when tested against soil borne pathogens

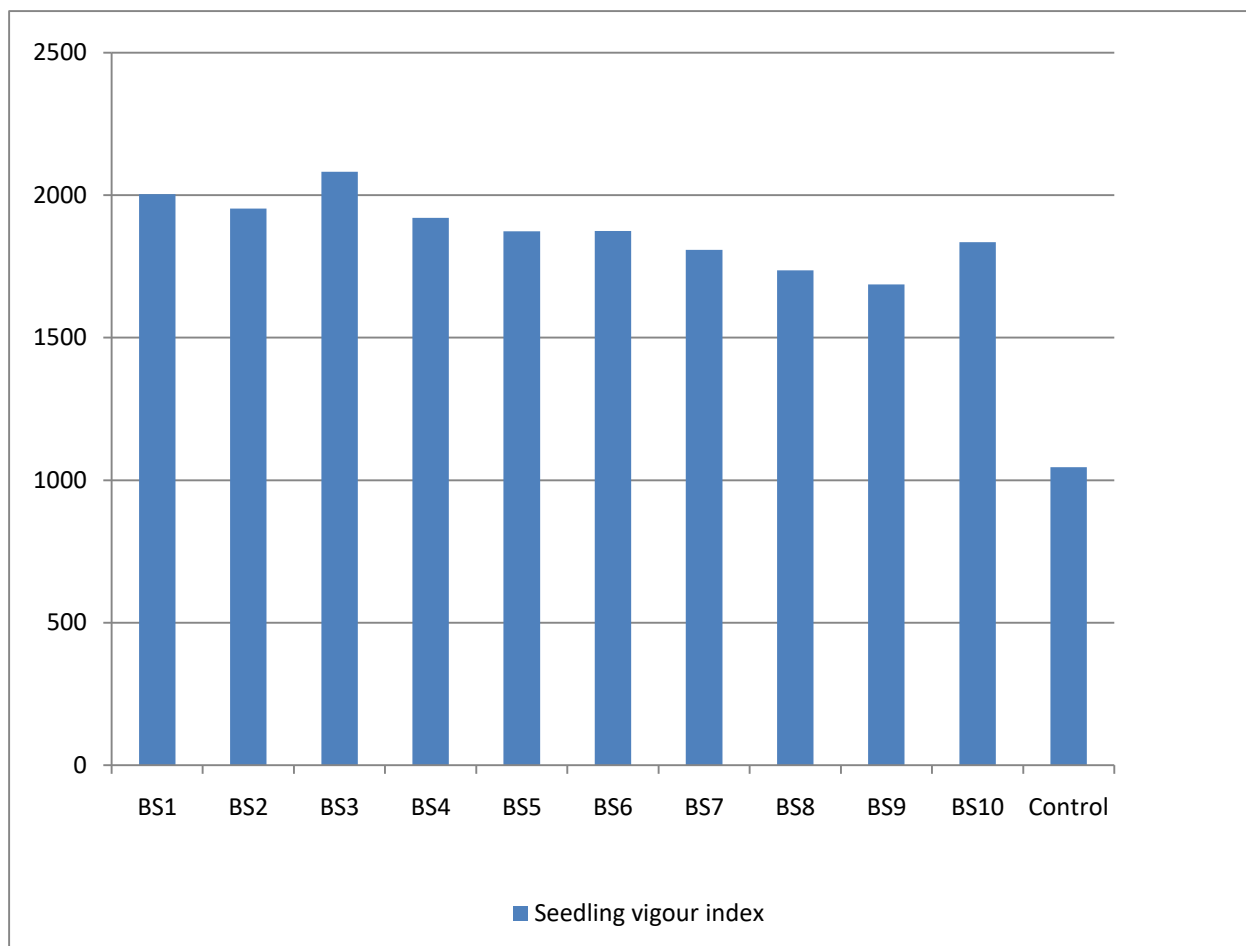


Fig. 3 Growth promoting ability (Seedling vigour index) of *Bacillus subtilis* in Pigeon pea (Paper towel method)



Treated



Control

Plate 5. Growth promoting ability (Germination) of *Bacillus subtilis* on pigeon pea by paper towel method

of pigeon pea i.e. *F. udum*, *R. bataticola* and *S. rolfsii* and growth promoting ability of pigeon pea.

Among all *Bacillus* isolates BS3 from Medshi proved best exhibiting maximum zone of solubilization on CCA media, highest antagonistic activity against *F. udum*, *R. bataticola* and *S. rolfsii*, least sensitivity to pesticide and enhanced seedling vigour index.

Biocontrol *Bacillus* is now a days becoming popular among the farmers. The present study can be consider as basis for promoting *Bacillus subtilis* also for management of soil borne pathogens of pigeon pea and also for providing highly efficient isolate of this bioagent to the end users.

CHAPTER V

SUMMARY AND CONCLUSIONS

The research work on “Antagonistic activity of *Bacillus subtilis* against *Fusarium udum*” was carried out during *Kharif* 2018-19 in Department of Plant Pathology, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola.

Fourty soil samples were collected i.e. 9 from Akola, 9 from Buldhana, 9 from Amravati, 9 from Washim, 2 from Nagpur, 1 from Chandrapur, 1 from Gadchiroli from different places of fields during June to September 2019. Ten soil samples contained *Bacillus* isolates by exhibiting zone of solubilization on CCA.

Hydrogen ion concentration and electrical conductivity of soil samples containing *B. subtilis* having chitin degrading ability on CCA was ranged from 8.1 to 8.6 and 0.21 to 0.52 dSm⁻¹ respectively.

The population of the *Bacillus subtilis* on its respective media also varied and seen to be fluctuated according to the ability of chitin degradation. Isolate BS3 show maximum population (54.33X10⁷ cfu/g) followed by BS1(50.66X10⁷ cfu/g) which were having maximum zone of solubilisation on CCA media i.e. 9mm. the least population of 34.10x10⁷ cfu/g of soil was recorded in BS9 followed by BS8 38.10x10⁷ cfu/g where the zone of solubilisation was also least i.e 5mm. this indicated that the population of the bioagent varied depending upon their aggressiveness or ability of having chitin degradation which on, although since logically true, needs more data for confirmation.

Biochemical tests were carried out to identified the *Bacillus subtilis* and results revealed that the strains collected from different regions were *B. subtilis* strains. Obtained from various test that all the isolates tested positive for Gram reaction, negative for KOH, positive for catalase test, gelatin liquification, growth in 5% NaCl, Arginine dehydrolase, H₂S production and negative for methyl test.

Tested bacterial antagonist were able to minimize growth of fungus causing disease in pigeon pea. Among ten isolates of *Bacillus* BS3

(Medshi) allowing only 6.92 mm mycelial growth with 92.31% proved best over all other isolates however was at par with BS1 i.e. Patur isolates. Both the isolates exhibited maximum zone of solubilization on CCA media also. Least inhibition of *Fusarium udum* was observed in isolate BS7 80.73%. all other isolates also inhibited the growth of *F. udum* significantly over control.

Similar trend of efficiency was achieved against *R. bataticola* due to *Bacillus* isolates. Maximum effect was noted due to BS3 with 9.57 mm growth of pathogen and 89.37% inhibition. Minimum ability was noted due to BS9 with 27.59% inhibition of test pathogen. Isolate BS3 and BS1 were at par with each other in inhibiting the growth of *R. bataticola*. Highest efficiency with 88.97% inhibition of *S. rolfsii* was also noted due to BS3. where the pathogen could grow only upto 9.92 mm as against 90 mm without antagonist followed by BS1 with 88.49% growth inhibition of pathogen.

Sensitivity of *Bacillus subtilis* to commonly used pesticides, Imidacloprid exhibited more than 50% inhibition almost all isolates of *B. subtilis* as compared to control indicating detrimental against *Bacillus*. Isolate BS3 recorded highest CFU i.e. 60.37×10^7 which was being lowest i.e. 48.26×10^7 in BS9. Thiomethoxom was also found non tolerable to isolates as less than 50% viable colonies in this chemical were observed. Isolate BS3 was least sensitive to Thiomethoxom with 54.76% inhibition. Isolate BS3 was found significantly superior over all others isolates in sustaining the effect of both the insecticides.

Combined product of Carboxin + Thiram and Thiram alone was completely toxic to all isolates of *Bacillus* while Carbendazim exhibited maximum inhibition of 70.14% in BS9. The least sensitive isolate to this fungicide was also exhibited by BS3 with 42.97% inhibition. Only which was significantly or superior over all other isolates.

Weedicides were found to be comparatively less hazardous to *Bacillus* isolates than insecticides and fungicides. Pendimethalin in most cases supported more than 50% growth of *Bacillus* isolates. The maximum inhibition in this weedicide was 66.43%, while in imazethypr it was 65.69%

in BS9 isolates. Among all the isolates, BS3 showed least sensitivity to the weedicides whereas BS9 exhibited highest sensitivity.

Root length, shoot length and seedling vigour index was also highest due to isolate BS3 i.e. 8.44 cm, 19.07 cm and 2082 respectively. Compared to 6.0 cm, 11.44 cm and 1046 root length, shoot length and seedling vigour index in control. Among all isolates, isolate BS9 proved inferior in promoting germination %, root length, shoot length and there by seedling vigour index of pigeon pea where the parameters were recorded as 70%, 6.89 cm, 17.03 cm and 1687 respectively.

CONCLUSIONS :

- Ten soil samples from forty showed chitin degrading ability on CCA.
- Janephal isolate with minimum zone of solubilization sample exhibited more pH of 8.5 and less EC of 0.29 dSm⁻¹.
- Isolate BS3 showed maximum population (54.33×10^7 cfu/g) followed by BS1 (50.66×10^7 cfu/g) which were having maximum zone of solubilisation on CCA media i.e. 9mm.
- All the isolates *B. subtilis* tested positive for Gram reaction, negative for KOH, positive for catalase test, gelatin liquification, growth in 5% NaCl, Arginine dehydrolase, H₂S production and negative for methyl test.
- Among ten isolates of *Bacillus* BS3 (Medshi) allowing only 6.92 mm mycelial growth of *F. udum* with 92.31% proved based over all other isolates however was at par with BS1 i.e. Patur isolates.
- Maximum inhibition of *R. bataticola* was noted due to BS3 with 9.57 mm growth of pathogen and 89.37% inhibition.
- Highest efficiency with 88.97% inhibition of *S. rolfsii* was also noted due to BS3. where the pathogen could grow only upto 9.92 mm as against 90 mm without antagonist followed by BS1 with 88.49% growth inhibition of pathogen.
- Imidacloprid exhibited more than 50% inhibition of almost all isolates of *B. subtilis* as compared to control indicating detrimental against *Bacillus*.

- Isolate BS3 was least sensitive to Thiomethoxom with 54.76% inhibition. Isolate BS3 was found significantly superior over all others isolates in sustaining the effect of both the insecticides.
- Combined product of Carboxin + Thiram and Thiram alone was completely toxic to all isolates of *Bacillus*.
- Pendimethalin in most cases supported more than 50% growth of *Bacillus* isolates. The maximum inhibition in this weedicide was 66.43%, while in imazethypr it was 65.69% in BS9 isolates.
- Among all the isolates, BS3 showed least sensitivity to the weedicides whereas BS9 exhibited highest sensitivity.
- Root length, shoot length and seedling vigour index was also highest due to isolate BS3 i.e. 8.44 cm, 19.07 cm and 2082 respectively, compared to 6.0 cm, 11.44 cm and 1046 root length, shoot length and seedling vigour index in control.

CHAPTER VI

LITERATURE CITED

- Aggarwal C, Paul S, Tripathi V, Paul B, and Khan MA (2017) Characterization of putative virulence factors of *Serratia marcescens* strain SEN for pathogenesis in *Spodoptera litura*. J Invertebr Pathol pp-143
- Agnihotru V (1953) Soil condition and root diseases rhizosphere microflora of some important crop plant of south India. Proc Indian Acad Sci 37 (8): 1-13
- Ahiladevi and Prakasan (2013) Effect of Azoxystrobin 8.3%w/w + Mancozeb 68.75% on Powdery and Downy mildew Pathogens under In vitro condition. Journal of Agriculture and Veterinary Science 6(4): 50-55
- Allen MB (1953) The Thermophilic aerobic sporeforming bacteria. Hopkins Marine Station of Stanford University, Pacific Grove, California. 17(2): 125
- Annamalai N, Thavasi R, Jayalakshmi S, and Balasubramanian T (2009) Thermostable and alkaline tolerant xylanase production by *Bacillus subtilis* isolated from marine environment. Indian Journal of Biotechnology 8: 291-297
- Arsia SK, Mishra SP, and Saxena M (2018) *In vitro* evaluation of bioagents and agrochemicals against *Fusarium udum*
- Avinash M, Nagaraju K, Muthuraju R, Asha N, Krishna Murthy R, and Vasantha R (2019) Pigeon pea *Fusarium* wilt: *In vitro* pathogenicity test of *Fusarium* isolates and its biological control
- Avsar C, Koyuncu H, and Aras ES (2017) Isolation and molecular characterization of *Bacillus* spp. isolated from soil for production of industrial enzymes. Biological and Chemical Research, 72-86
- Awais M, Pervez A, Yaqub A, and Shah MM (2010) Production of antimicrobial metabolites by *Bacillus subtilis* immobilized in polyacrylamide gel. Pakistan J Zool 42(3): 267-275
- Baki A., A. A. and J. D. Anderson. 1973. Vigour determination in soybean seed by multiple criteria. Crop Science. (13): 630-633.
- Banakar S N, Sanath Kumar VB, and Thejesha AG (2017) *In vitro* evaluation of bio-agents and fungicides against foot rot pathogen (*Sclerotium rolfsii* Sacc.) of tomato. Inter J Curr Microbio Appl Sci, 6 (3): 1591-1598
- Barman P, Amrita Banerjee, Bandyopadhyay P, Mondal KC, Das PK, Mohapatra (2011) Isolation, identification and molecular

characterization of potential probiotic bacterium, *Bacillus subtilis* PPP13 from *Penaeus monodon*. Biotechnol Bioinf Bioeng 1(4): 473-482

- Basamma H, and Kulkarani S (2017) Studies on Compatibility of *Bacillus subtilis* (Ehrenberg) Cohn with Chemical Fungicides. Int J Curr Microbiol App Sci 6(3): 578-586
- Berini F, Katz CN, Ruzdev G, Casartelli M, Tettamanti D, and Marinelli F (2018) Microbial and viral chitinases attractive biopesticides for integrated pest management. Biotechnol Adv 36: 818–838
- Brian B, and Gardener MC (2004) Ecology of *Bacillus* and *Paeni Bacillus* spp. in Agricultural System. The American Phytopathological Society, Vol 94 (9): pp- 1252-1258
- Bruce and Highley TL (1991) Control of growth of wood decay Basidiomycetes by *Trichoderma* spp. and other potentially antagonistic fungi. Forest Products Journal (41): 2
- Butler EJ (1906) The wilt disease of pigeonpea and pepper. Agricultural Journal of India, 1: 25-36
- Butler EJ (1910) The wilt disease of pigeonpea and parasitism of *Neocosmospora vasinifectum*. Department Agricultural Bulletin of India 2 : 1
- Chen HL, Wang CX, Gong GH, Wang P, and Yu ZL (2008) Isolation and characterization of lipopeptide antibiotics produced by *Bacillus subtilis*. Letters in Applied Microbiology, 47(3): 180-186
- Dukare A, Paul S, and Ambam A (2020) Isolation and efficacy of native chitinolytic rhizobacteria for biocontrol activities against *Fusarium* wilt and plant growth promotion in pigeon pea (*Cajanus cajan* L.).
- Fadhal AL, Khafaji DAA and Fadhal AA (2019) Effect of *Bacillus subtilis* and *Pseudomonas fluorescens* in reducing cucumber seed rot and seedlings death caused by *Rhizoctonia solani* and *Fusarium solani* in green house conditions. Kufa Journal For Agricultural Sciences 11(1): 12-25
- Farag MA, and Nusarie A, and Taghreed S (2014) Production, optimization, characterization and antifungal activity of chitinase produced by *Aspergillus terreus* Afr J Biotechnol 13(4): 1567-1578
- Foldes T, Banhegy I, Herpai Z, Varga L, and Szigeti J (2000) Isolation of *Bacillus* strains from the rhizosphere of cereals and in vitro screening for antagonism against phytopathogenic, food-borne pathogenic and spoilage micro-organism. Journal of Appl Microbiol 89: 840-846
- Gaur VK, and Sharma LC (1991) Microorganism Antagonistic to *Fusarium udum* Butler. Proc, Indian natn Sci Acad B57 pp 85-88

- Gaur D, Jain PK, Sisodia YS, and Bajapai V (2012) Estimation of extracellular lipolytic enzyme activity by thermophilic *Bacillus spp.* isolated from arid and semi-arid region of Rajasthan, India. *Journal of Microbiology, Biotechnology and Food Sciences*, 2(2): 619-633
- Ghante P H, Kanase KM, Gaikwad PA, Pawar SV, and Magar SJ (2019) The efficacy of bioagents against *Fusarium oxysporum f. sp. udum* causing wilt disease of Pigeonpea. *Journal of Pharmacognosy and Phytochemistry* 8(1): 1966-1971
- Goudar SB, and Kulkarni S (2000) Bioassay of Antagonists Against *Fusarium udum* – The Causal Agent of Pigeonpea Wilt. *Karnataka J Agric Sci* 13(1): 64-67
- Gurney TR, and Quesnel LB (1980) Thermal activation and dry-heat in activation of spores of *Bacillus subtilis* MD2 and *Bacillus subtilis* var. niger. *Journal of Applied Bacteriology* 48: 231-247
- Hang YW, Zhansheng, and Chun I (2017) Rhizospheric *Bacillus subtilis* Exhibits Biocontrol Effect against *Rhizoctonia solani* in Pepper (*Capsicum annuum*) Article ID 9397619
- Harman GE, Chet I, and Baker R (1980) *Trichoderma hamatum* effects on seed and seedlings disease induced in radish and pea by *Pythium spp.* Or *Rhizoctonia solani*. *Phytopathology* 70: 1167-1172
- Harshita, Ankita Sinha, Khan JB, Tripathi UK, Ratan V, Shubha Trivedi, Mishra A, and Verma A (2019). *In-vitro* evaluation of systemic fungicides against *Fusarium oxysporum f. sp. lycopersici* and their compatibility with bioagents 8(3): 3117-3123
- Jacob CK, and Sivaprakasum K (1993) Development of a cheap system for mass multiplication and delivery of *T. harzianum* and *T. viride*. *Crop diseases Innovative Techniques and Management: Ludhiana, Kalyani Publishers* pp 143-155
- Jadhav RR, Giri GK, and Belkar YK (2014) Characterization of *Bacillus Subtilis* and management of wilt of pigeonpea caused by *Fusarium udum*. *J PL Dis Sci* 9(2): 180-184
- Jalander V, and Gachande BD (2015) Effect of Culture Media on Growth and Pigmentation of *Fusarium oxysporum f. sp. udum* Butler Isolated from Different Varieties of Pigeon Pea [*Cajanus cajan* (L.) Millsp.]. *Int J Curr Res Biosci Plant Biol* 2(6): 50-53
- Jayalakshmi S K, Raju S, Rani U, Benagi VI, and Sreeramulu K (2009) *Trichoderma harzianum* L1 as a potential source for lytic enzymes and elicitor of defence responses in chickpea (*Cicer arietinum* L.) against wilt disease caused by *Fusarium oxysporum f. sp. ciceri*. *Australian Journal of Crop Science* 3(1): 44-52

- Jha Sujata S., Joshi S, and Geetha SJ (2016) Lipopeptide production by *Bacillus subtilis* R1 and its possible applications. *Brazilian Journal of Microbiology*, 47: 955-964
- Junges E, Toebe M, Santos RF, and Brião muniz GF (2013). Effect of priming and seed coating when associated with *Bacillus subtilis* in maize seeds. *Revista Ciencia Agronomica*, 44(3): 520-526
- Kannaiyan J, and Nene YL (1981) Influence of wilt at different growth stages on yield loss in pigeonpea. *Tropical Pest Management* 27(1): 141
- Karimi K, Amini J, Harighi B, and Bahramnejad B (2012) Evaluation of biocontrol potential of *Pseudomonas* and *Bacillus* spp. against *Fusarium* wilt of chickpea *AJCS*, 6(4): 695-703
- Kaswate NS, Shinde SS, and Rathod RR (2003) Effect of biological agents against different isolates of *Rhizoctonia bataticola* (Taub.) Butler in vitro. *Indian Phytopath* 17(2): 167-168
- Keote GA, Surya M, Reddy P, Ashwini Kumar and Wasnikar AR (2017) Bio-inoculants against chick pea wilt incited by *Fusarium oxysporum* f. sp. *ciceri* 8(4): 1590-1594
- Khan HA, Naseem, Lavarti Rupa and B. Pratibha (2011) Screening and potency evaluation of antifungal from soil isolates of *Bacillus subtilis* selected fungi. *Advanced Biotech* 10(7): 101-105
- Khan RA, Bhat TA, and Kumar K (2012) Management of Chickpea (*Cicer arietinum* L.) Dry root Rot Caused by *Rhizoctonia bataticola* (Taub.) Butler. *International Journal of Research in Pharmaceutical and Biomedical Sciences* ISSN: 2229-3701, Vol. 3
- Khusro A, Aarti C, Preetam JP, Raj and Panicker SG (2014) Study on production of extracellular amylase from *Bacillus subtilis* strain KPA under mild stress condition of certain antimicrobials. *International Journal of Pharmaceutical and Clinical Research*, 6(3): 270-275
- Kumar S, and Upadhyay JP (2013) Cultural, morphological and pathogenic variability in isolates of *Fusarium udum* causing wilt of pigeonpea. *J Mycol PI Path*, 43: 76-79
- Lestari P, Prihatiningsih N, and Djatmiko HA (2017) Partial biochemical characterization of crude extract extra cellular chitinase enzyme from *Bacillus subtilis* B 298. *IOP Conf. Series: Materials Science and Engineering*, 172
- Madhukeshwara SS, and Seshadri VS (2001) Variation and Management of *Fusarium* Wilt of Pigeon Pea [*Cajanus cajan* (L.) Millsp.]. *Tropical Agricultural Research* 13: 380-394

- Madhuri V, Arunodhayam K, and Eswara NP, Reddy (2013) A review on non target effect of herbicides and compatibility of herbicides with fungicides on soil borne plant pathogens- *Sclerotium rolfsii* Sacc., *Rhizoctonia solani* Kuhn. and *Fusarium udum* Butler. Current Biotica 7(1&2): 105-123
- Mandla R, Shahanaz and Kalia VK (2017) Biochemical and molecular characterization of *Bacillus* spp. isolated from insects. Journal of Entomology and Zoology Studies, 5(5): 581-588
- Mane SS, Yadav SM (2015) Efficacy of bioagents against chickpea wilt caused by *Fusarium oxysporum* f. sp. *ciceri*. Bioinfolet 12(4A): 857-858
- Mohammadi M, Ahmadzadeh M, Saleni GH, Behboudi K, Ahangaram A, Mousivand M and Rashimian (2009) Characterization of some bioforming *Bacillus subtilis* strains and evaluation of their biocontrol potential against *Fusarium culmorum*. Journal of Plant Pathology 93 (2): 373-382
- Mugiastuti E, Suprayogi, Prihatiningsih N, and Soesanto L (2020) Isolation and characterization of the endophytic bacteria, and their potential as maize diseases control Vol 21(5): 1809-1815
- Nandakumar R, Babu B, Raguchander T, and Samiyappan R (2007) Chitinolytic activity of Native *Pseudomonas fluorescens* Strains. J Agric Sci Technol 9: 61-68
- Narayanan K, Chopde N, Raj PV, Subrahmanyam VM, and Rao JV (2013) Fungal chitinase production and its application in biowaste management. Journal of Scientific and Industrial Research, 72: 393-399
- Nehra S, Gothwal RK, Shivani Chandra, Poonam Meena, Medicherla KM, and Trivedi PC (2012) *Pseudomonas fluorescens* : A Biological control Agent against wilt Fungus in *Cajanus cajan* (L.) Millsp. An International Journal of life Sciences 1(3): 175-185
- Nusarat SS, Kulkarni SV, and Mulani MS (2014) Biodegradation of Imidachloprid, New Generation Neurotoxic Insecticides. Int J Innovative Res in Sci Engg and Tech 3(9): 16301-16304
- Okiror MH, and Kimani PM (1997) Pathogenic variation of *Fusarium udum* of pigeonpea. Indian J. Genetics and Pl. Breeding 57(2): 186-192
- Patil BG, and Gokhale VP (1965) Survey of Tur wilt disease in Vidarbha region. Indian Phytopath. 18(2): 40-41
- Pandey KK, and Upadhyay JP (2000) Microbial population from rhizosphere and non rhizosphere soil of pigeonpea screening for resident antagonist and mode of mycoparasitism. J Mycol Pl Pathol 30(1): 7-10

- Panse VG, and Sukhatme PV (1967) Statistical methods for agricultural workers. ICAR New Delhi., 2nd Edn pp 381
- Podile AR, and Dube HC (1988) Plant growth promoting activity of *Bacillus subtilis* AF1. *Curr Sci* 57: 183-186
- Pretorius IS, Deknock MJ, Britz TJ, Potgieter HJ, and Lategon PM (1986) Numerical taxonomy of α -analysis producing *Bacillus* spp. *Journal of Applied Bacteriology*, 60: 351-360
- Raghavendra KS, and Gundappagol RC (2017) Effect of herbicides on soil microcosm, nodulation and yield in chickpea (*Cicer arietinum* L.). 6(5): 1649-1655
- Rajasekhar L, Sain SK, and Divya J (2016) Evaluation of microbial consortium for plant health management of pigeon pea. *IJPAJX-CAS-USA*, 6(2)
- Rana J, Tiwari TC, Jadon VS, and Gupta S (2014) Bacterial isolates for the bio-control of *Fusarium* wilt of Pigeonpea. *J Dyn Agric Res* 1(3): 14-22
- Sammer WM (2019) Compatibility of biological control agents with fungicides against root rot diseases of wheat Article 13, Vol44, Issue 2, Autumn 2019, Page 146-155
- Sathya SS, Lakshmi and Nakkeeran S (2016) combined effect of biopriming and polymer coating against chilli damping off vol 6(3): 45-54
- Shetti AA, Kaliwal RB, and Kaliwal BB (2014) Imidacloprid induced Intoxication and its Biodegradation by soil Isolate *Bacillus weihenstephanensis*. *British Biotech J* 4(9): 957-969
- Shifa H, Gopalakrishnan C, and Velazhahan R (2015) Efficacy of *Bacillus subtilis* G-1 in suppression of stem rot caused by *Sclerotium rolfsii* and growth promotion of groundnut. *International Journal of Agriculture, Environment and Biotechnology* 8(1): 111-118
- Shubakov AA, and Kucheryavykh PS (2004) Chitinolytic Activity of Filamentous Fungi. *Applied Biochemistry and Microbiology* 40(5): 445-447
- Siddiqui ZA, and Shakeel U (2009) Screening of *Bacillus* isolates for potential biological of the wilt Disease complex of pigeonpea (*Cajanus cajan*) under greenhouse and small scale field conditions. *Journal of Plant Pathology*, 89(2): 179-183
- Singh B, and Dube SC (2010) Bioagents based integrated Management of Phytophthora blight of pigeonpea. *Archives PI Pathol and PI Protect* 43(9): 922

- Singh G, Bhalla A, Bhatti JS, Chandel S, Rajput A, Abdullah A, Andrabi W, Kaur P (2013) Potential of Chitinase as a Biopesticide against Agricultural Harmful Fungi and Insects. RRJMB, VOL 3
- Singh N, Raina S, Singh D, Ghosh M, and Heflish AA (2017) Exploitation of promising native strains of *Bacillus subtilis* with antagonistic properties against fungal pathogens and their PGPR characteristics. Journal of Plant Pathology, 99 (1): 27-35
- Sumi K, Sharma NT, Chakruno MB, Sema P, Chishi T, and Kent N (2018) *In vitro* evaluation of native bio agents isolates against stem rot of pigeon pea caused by *Sclerotium rolfsii*. Journal of Food Legumes 31(3): 194-195
- Sumitha R, and Gaikwad SJ (1995) Checking *Fusarium* wilt of pigeonpea by biological means. J Soil and Crops 5(2): 163-165
- Sumitha R, and Gaikwad SJ (1995) Checking *Fusarium* wilt of pigeonpea by biological means. J. Soil and Crops. 5(2): 163-165
- Suryawanshi KK, Ingle ST, Shingote PR, and Chaudhari SR (2013) Chitinase production ability of *Trichoderma viride* mutants. Bioinfolet. 10(3B): 1048-1050
- Umashankar N, and Kumar NU (2013) Isolation and screening of bacterial isolates against *Fusarium* sp. Volume 4, International journal of retailing & rural business perspectives© Pezzottaite Journals. ISSN (Print): 2279-0934, (online): 2279-0942
- Vincent JM (1927) Distortion of fungal hyphae in presence of certain inhibitors. Nature. 159: 850.232-235
- Velusamy P, and Das J (2014) Identification and characterization of antifungal chitinase from *Bacillus subtilis* JD-09 and their role in inhibition of viable fungal growth. Int J Pharm Sci 6 Issue 7
- Willemsse MF, Tromp F. J and Huizinga T (1980) A simple and rapid computer assisted technique for the identification of some selected *Bacillus* species using biochemical test. Journal of Applied Bacteriology, 49(3): 385-393
- Zhang CX, Zhao X, Jing YX, Chida T, Chen H, and Shen SH (2008) Phenotypic and properties of two antagonist *Bacillus subtilis* strains. World J. Microbiol Biotech, 24: 2179-2181
- Zhenxiang LU, Weina GU, and Cheng LIU (2018) Isolation, identification and characterization of novel *Bacillus subtilis*. J Vet Med Sci 80(3): 427-433

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Appendix-I

Media used with their compositions

NA Media:

Beef extract	-	3g
Peptone	-	5g
Agar	-	20g
Distilled water	-	1000ml

PDA Media:

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