Plant Growth Regulation and Suppression of Sugarcane Fungal Diseases through Multi-stress tolerant Rhizobacteria

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Plant Growth Regulation and Suppression of Sugarcane Fungal Diseases through Multi-stress tolerant Rhizobacteria

A thesis is submitted in the partial fulfillment of requirements for the degree of

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In

Plant Microbe Interaction

By

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No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Plant Sciences, Quaid-I-Azam University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the field of Plant Sciences, Department of Plant Sciences of Quaid-I-Azam University.

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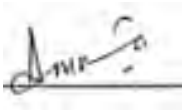
AUTHOR'S DECLARATION

The research work presented in this thesis was carried out by me in the Plant microbe interaction laboratory, Department of Plant Sciences, Quaid-i-Azam University, Islamabad. The findings and conclusions are of my own investigation with discussion of my supervisor, Dr. Hassan Javed Chaudhary. No part of this work has been presented for any other degree.



Amna

Dedication

To My Most Beloved Mother
&
Affectionate Father

Without the inspiration, drive and support they gave me, I might not be the person I am today

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cane weight, cane diameter, root length, chlorophyll a, chlorophyll b, carotenoids, Brix, POL, CSS)

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LIST OF ABRREVIATIONS

ABA	Abscisic acid
ABs	Agricultural biostimulants
ACC	1-*aminocyclopropane*-1-*carboxylic acid*
$AlPO_4$	Aluminum phosphate
ANI	Average Nucleotide Identity
ANOVA	Analysis of Variance
B	Boron
BLAST	Basic Local Alignment Search Tool
BP	Base pair
Ca	Calcium
$Ca_3(PO_4)_2$	Tricalcium phosphate
Ca_3PO_4	Calcium Phosphate
CAT	Catalase
°C	Celsius/Centigrade
CDRI	Crop Disease Research Institute
CFU g^{-1}	Colony forming units per gram
chl. cont	Chlorophyll content
CK	Cytokinins
cm	Centimeter
cm^2	Square Centimeter
Cu	Copper
cv.	Variety
cvv.	Varieties
Df	Dworkin and Foster medium
DI	Disease incidence
DMSO	Dimethyl sulfoxide
DS	Disease severity
EC	Electrical conductivity
EDTA	Ethylenediaminetetraacetic acid

EMBL	European Molecular Biology Laboratory
ET	Ethylene
F	Forward
Fe	Iron
$FeCl_3$	Ferric chloride
$FePO_4$	Iron phosphate
G	Gram
GA	Gibberellic acid
GI	Germination index
gl^{-1}	Gram per liter
$^{\bullet}OH$	Hydroxyl radical
H	Hour
H_2O_2	Hydrogen peroxide
H_3PO_4	Phosphoric Acid
Ha	Hectares
$HClO_4$	Perchloric acid
HCN	Hydrogen cyanide
$HgCl_2$	Mercury chloride
IAA	Indole-3-acetic acid
IAM	Indole-3-acetamide
IDM	Integrated management
ISR	Induced Systemic Resistance
JA	Jasmonic acid
K	Potassium
$K_2Cr_2O_7$	Potassium dichromate
K_2HPO_4	Dipotassium phosphate
KCS_2	3-ketoacyl-CoA synthase 2
LB	Luria-Bertani medium
LPs	*L*ipopeptide biosurfactants
LSD	Least Significance Test
MAMPs	Microbe-associated molecular patterns

MAS	Marker Assisted Selection
MES	2-(N-morpholino)ethanesulfonic acid
Mg	Magnesium
Min	Minutes
ml	Milliliter
mm	Millimeter
mM	Millimolar
Mn	Manganese
Na	Sodium
NARC	National Agriculture Research Centre
NB	Nutrient Broth
NCBI	National Center for Biotechnology Information
nm	Nanometer
NCP	National Centre for Physics
OD	Optical density
OM	Organic matter
P	Phosphorous
PAL	Phenylalanine ammonia-lyase
PAMPs	Pathogen-associated molecular patterns
PCA	Principal component analysis
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
PGPR	Plant Growth Promoting Rhizobacteria
PGRs	Plant Growth Regulators
POD	Peroxidase
Ppm	Parts per million
PPO	Polyphenol oxidase
PR	Pathogenesis-related
PRRs	Pattern Recognition Receptors
PSBs	Phosphate Solubilizing Bacteria’s
PVK	Pikovskayas broth medium

PVP	Polyvinylpyrrolidone
R	Reverse
ROS	Reactive oxygen species
rpm	Revolution per minute
rRNA	Ribosomal RNA
$^{\bullet}O^{-2}$	Superoxide radical
S	Second
S	Sulphur
SA	Salicylic acid
SAR	Systemic acquired resistance
SDW	Shoot dry weight
SFW	Shoot fresh weight
SH	Shoot height
SI	Solubilization index
SOD	Superoxide dismutase
sp.	Specie
spp.	Species
TCS	Tetra correlation search
TDS	Total dissolved solids
TSA	Tryptone soy agar
U	Unit enzyme activity
v/v	Volume by volume
w/v	Weight by volume
Zn	Zinc
µg	Microgram

PUBLISHED RESEARCH ARTICLES FROM THIS STUDY

S. No.	Titles	Impact Factor
I.	**Amna,** Xia Y, Farooq MA, Javed MT, Kamran MA, T Mukhtar, Ali J, Tabassum T, Rehman SU, Munis MFH, Sultan T, Chaudhary HJ. (2020) Multi-stress tolerant PGPR *Bacillus xiamenensis* PM14 activating sugarcane (*Saccharum officinarum* L.) red rot disease resistance. Plant Physiology and Biochemistry. 151. 640-649.	**3.72**
II.	**Amna,** Sarfraz S, Din B, Xia Y, Kamran MA, Javed MT, Sultan T, Chaudhary HJ (2019). Mechanistic elucidation of germination potential and growth of wheat inoculated with exopolysaccharide and ACC- deaminase producing *Bacillus* strains under induced salinity stress. Ecotoxicology and Environmental safety 183: 109466	**4.872**
III.	**Amna**, Tariq Mahmood, Umar Nawaz Khan, Babar Amin, Muhammad Tariq Javed, Shehzad Mehmood, Muhammad Asad Farooq, Tariq Sultan, Muhammad Farooq Hussain Munis, Hassan Javed Chaudhary (2020). Characterization of bio-fabricated silver nanoparticles for distinct anti-fungal activity against sugarcane phytopathogens. Microscopy Research and technique. (*accepted: In press*).	**2.11**
IV	**Amna**, Muhammad Tariq Javed, Nida Zainab, Saliha Ahmad, Muhammad Asad Farooq, Syed Waqas Hassan, Tariq Sultan, Muhammad Farooq Hussain Munis, Hassan Javed Chaudhary (2021). Elucidating the potential of bio-fabricated silver nanoparticles as a sustainable approach for augmentation of sugarcane growth and fungal disease control. (Plant physiology and Biochemistry). (Submitted Manuscript ID: PLAPHY-D-21-00363)	**3.72**
V	**Amna,** Hassan Javed Chaudhary (2021). Suppression of Sugarcane fungal diseases through Plant growth Promoting *Bacillus siamensis* PM13: a potential candidate (*In process*)	-

Abstract

Sustainability in agricultural development has emerged as one of the most significant concerns of the current era as 20–40% of the reduced crop yields and estimated losses of 40 billion dollars worldwide caused by phytopathogens consider the rising concern for food security. Among the possible disease control methods, biocontrol of phytopathogens by using plant growth promoting bacteria (PGPB) seems to be a good option for the development of eco-friendly, cost effective, and sustainable approaches. Keeping in mind this scenario the current study was designed and undertaken. In the study, 93 bacterial strains were finalized to evaluate their biocontrol activity along with plant growth promoting traits. Bacterial strains showed Indole acetic acid (IAA), phosphate solubilization, ammonia, siderophore, and hydrogen cyanide (HCN) production. Out of 93 strains, 10 strains that showed best plant growth promoting (PGP) traits and antagonistic activities were selected and noticed to be positive towards enzyme activities, and ACC deaminase and exopolysaccharide production (EPS) production. Of these, 9 bacterial strains belonged to the genus *Bacillus* whereas one was identified as *Bravibacterium frigoritolerans* (PM12). Multi-stress tolerance against heat, drought, salinity and heavy metals along with resistance to antibiotics was observed. Bacterial strain PM19 (*Bacillus methylotrophicus*) showed resistance against 14 out of 15 antibiotics. *Bacillus siamensis* (PM13) and *Bacillus xiamenensis* (PM14) showed prominent multi abiotic stress tolerance among others. All bacterial strains showed distinct inhibition coefficient values against fungal strains. In another experiment, characterization of biologically-produced silver nanoparticles (AgNPs) by Xray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), Diffused reflectance spectroscopy (DRS) and Scanning electron microscopy (SEM) confirmed the production of silver nanoparticles by bacterial supernatant, fungal supernatant and sugarcane husk solution. Among all, sugarcane husk based AgNPs showed significant antifungal properties as compared to others. In the second phase, a greenhouse study was performed to analyze plant growth promotion and control of fungal diseases *Colletotricum falcatum* (red rot) and *Fusarium monilliforme* (pokha boeng). All inoculated bacterial strains enhanced sugarcane plant growth and yield in sugarcane. Examination of growth parameter revealed that the application of bacterial strain *Bacillus siamensis* (PM13) significantly enhanced the plant growth parameters with increased percent values of 21.77, 33.66,23.38, 32.96, 16.9, 36.9 , 62.68, 62.27, 43.76, 38.73, 23.7 and, 40.9 for plant height, plant fresh weight, cane length, cane weight, cane diameter, root length, Chlorophyll a, Chlorophyll b, carotenoids, Brix, POL and CCS,

respectively, in comparison to the control plants without bacterial strains inoculation. Results of the greenhouse experiment against red rot disease of sugarcane showed that augmentation of *Bacillus siamensis* (PM13)*, Bacillus tequlensis* (PM17)*, Bacillus xiamenensis* (PM14) and *Bravebacterium frigoritolerance* (PM12) to *S. officinarum* suppressed the symptoms of fungal disease and its drastic effects on sugarcane growth (enhanced) and yield parameters. The bacterial strain, *Bacillus siamensis* (PM13), showed the highest values among all for growth enhancement and red rot disease suppression. Whereas, inoculation of *Bacillus siamensis* (PM13)*, B. tequlensis* (PM17)*, Bacillus gibsonii* (PM11) *and Bacillus sp.* (PM15) not only enhanced growth of the plants but also alleviated the pokha boeng disease. In this experiment, the highest plant growth parameters were noticed in sugarcane plants inoculated with *Bacillus tequilensis,* while the lowest disease severity index was observed in *Bacillus siamensis* (PM13) treated plants. Application of silver nanoparticle played a positive role to control both diseases with increased plant growth in two varieties of sugarcane (variety 1: CO1148, and variety 2: CSSG32). Sugarcane plants of variety 2 (CSSG32) responded better in both aspects (plant growth and disease control) after the application of silver nanoparticles. It appears that augmented production of antioxidative enzymes and proline content in the greenhouse studies may lead to the induced systemic resistance against red rot and pokha boeng disease of sugarcane. Role of *B. xiamenensis* (PM14) as plant growth promoting multi-stress tolerant antagonistic bacterium is perhaps a new finding in this study and has not been reported previously adding new knowledge to scientific information. *In vitro* and *in vivo* experiments revealed native microflora of sugarcane as the potential growth promoting and fungal disease control agents. The present study has the potential to be implemented in sustainable agricultural. Thus, the potential application of native bacteria with multi-stress tolerance and biocontrol abilities; along with biologically produced silver nanoparticles studied presently may pave an interesting avenue for food security by implementing ecofriendly strategy.

Chapter 1

General introduction and Review of Literature

Introduction

Sugarcane (*Saccharum officinarum* L.: Poaceae) is a semi perennial grass. Its stalk is roughly cylindrical with distinct nodes and internodes. It is the largest crop of the world in terms of production and is grown in 110 different countries of tropical and sub-tropical regions. Along with sugar production, this crop is also the most competitive and economical source of ethanol production. Other byproducts of this crop include bagasse used as fuel, molasses, press mud and important component of some items, such as fiber, insecticides, detergent, chemicals, plastics, paints and synthetics. Due to its commercial and economic importance, ways of improving productivity of sugarcane is the subject of investigation in many countries. According to the 2018 database, 26 million hectares of agricultural area was devoted to sugarcane worldwide with average sugarcane yield of up to 73 tons per hectare. In 2018, global total production was 1.91 billion tons, with 39% of the world total contribution by Brazil, followed by India (20%), and China and Thailand about 6% each. During the years 2001 and 2018, an increase in world sugar consumption was observed from 123.454 million tons to 172.441 million tons (FAO STAT, 2018)

1.2. Growth stages of sugarcane

Depending upon different morphological and physiological changes and ecological factors, like aeration, soil temperature and moisture, nutrient contents, the growth of sugarcane crop is divided into four phases. Under field conditions, the germination of sett starts within 7- 10 days of sowing that leads to the complete germination of buds after 30-35 days. Tillering stage is the development of underground branches repeatedly from the nodes of primary stem. This initiate after 40 days and continues till four months after sowing. Increased number of stalks by growth of secondary shoots helped to obtain a reasonably good crop yield. After the completion of tillering phase, grand growth starts proceeding up to nine months in a twelve-month sugarcane crop. During this phase, ripening and maturation of canes occur that leads to less vegetative growth with enhanced sugar build up in the stalks of sugarcanes (Li et al., 2009). It usually starts after grand growth and continues for about three months in a 12-month phase of crop growth. During the phase of ripening, simple monosaccharides i.e. fructose and glucose are transformed to disaccharides. Cane ripening starts from bottom that gradually proceed towards the top of the sugarcane stalk. That's why less sugar content is noticed in the upper part as compared to the bottom one. Different stages of sugarcane are provoked by the numerous biotic and abiotic factors, such as light, temperature, soil texture, its

moisture and agriculture practices like application of fertilizers, proper spacing in plants, irrigation practices, control of weeds and pathogen attack (Sanghera et al., 2019).

1.2.1. Status of Sugarcane industry in Pakistan

Pakistan is the 6th largest sugarcane producer of the world with an area of 1.3 million hectares under cultivation for this crop. The average sugarcane yield per ha in Pakistan (62 t/h) is much lower than the overall reported yield of the world (70 t/h) (Pakistan Bureau of Statistics) In Pakistan, sugarcane is categorized as a major "Kharif" crop with its cultivation from February to April and harvesting normally between the months of October and December. Most of the area under sugarcane cultivation in Pakistan is in the province of Punjab (65%), followed by Sindh province (25%) and KPK province (10%). Due to lack of resources and modern farming technology in Pakistan, sugarcane farming is still an extremely labor-intensive process. Along with the production of sugar from sugar cane, "Gur" also occupies a healthy share. Pakistan has 9th position in the world for the production of sugar from sugarcane, while only 1% is contributed by beet. There are currently around 89 functional sugar producing mills in the country with an average crushing capacity of less than 6,000 TCD. Due to numerous unavoidable factors, average sugar recovery in Pakistan is still lower than 10%. In the country, the highest recovery rate is provided by Sindh province as compared to other provinces. In 2017, 7 Mn tons of sugar was witnessed from a record production of 73.6 Mn tons of sugarcane. Currently, domestic sugar consumption in Pakistan has been standing at around 5.5 Mn tons (Economic survey of Pakistan, 2019).

1.2.2. Current scenario of sugarcane in Pakistan

In Pakistan, sugarcane is considered as high value cash crop. Annual production of sugarcane contributes 0.5% to the overall GDP and 2.9% to the agriculture's value. During 2018-2019, 19.4 percent loss in sugar cane crop production was observed:16.174 million tons compared to 83.333 million tonns production for the year 2017-2018. One of the major reasons for this decline in sugar cane production was reduction in cultivated land for sugarcane by 17.9% (from 1343 thousands of last year to 1102 thousand hectares) resulting from water shortage. Besides, the relatively low economic revenues discourage the sugarcane growers to bring more area under sugarcane cultivation. Disposal problem of the cane and payment difficulties also limit the acreage for sugarcane cultivation.

In addition, during the rather long developmental stages, sugarcane plants might face multiple biotic and abiotic stresses. Crops are significantly affected by plant pathogens, high temperature, drought, soil salinity, etc., and, the beneficial soil microbial communities are also distressed by the harsh environmental conditions. About, 20–40% of the reduced crop yield and estimated losses of 40 billion dollars worldwide are caused by phytopathogens (Rahman et al. 2018). Considering the rising concern for food security, worldwide food production by 2050 must be raised by 70% (Ingram, 2011).

1.3. Major sugarcane diseases

Various kinds of viral, bacterial, fungal, and nematodal diseases of sugarcane are stated worldwide. Among all and specifically amongst the fungal diseases caused by *C. falcatum* (red rot) is the most destructive disease prevailing mostly in Indo-Pakistan. However, pokha boeng is the emerging disease of sugarcane (Sharma and Tamta, 2015; Vishwakarma et al., 2013).

1.3.1. Red rot

Red rot is one of the major phytopathogen of sugarcane; results to cause irresistible drop in yield of sugarcane crop globally (Mohanraj et al., 2002). In 1983, disease was reported for the very first time as red smut in Java. However, in 1906 it was renamed as red rot by Bulter. The disease is caused by a pathogenic fungus *Colletotrichum falcatum* Went and can cause severe losses of sugarcane susceptible varieties to the fungus. Reduced cane weight (29%) and loss in sugar recovery (31%) have been observed in red rot infected sugarcane plants (Viswanathan and Samiyappan, 2001). After infection, *C. falcatum* produces an enzyme, invertase, which hydrolyzes the stored sucrose and breaks the sucrose into simple sugars (glucose and fructose). As a result, the quantity of sucrose in infected plants decreases directly effecting cane quality (Sharma and Tamta, 2015). It is difficult to recognize red rot infection during early stages of the crop. Initial signs of the red rot normally appear after rainy and humid season when vegetative growth stops, and formation of sucrose begins. In the begining, a slight discoloration and drooping of leaves occurs that results in the wilting/dryig of the whole tip and progress down towards the margins. With time, the pathogen effects the whole midrib of leaves by appearance of blood red lesions that which elongates rapidly, forming blood red lesions with darker margins. The reddening in the split open infected cane is more noticeable in the vascular bundles that advance in the direction of the cane pith (Viswanathan and Samiyappan, 2001).

3.2. Pokha boeng disease of sugarcane

The causal agent of pokha boeng fungal disease in sugarcane is *Fusarium moniliforme* first reported by Sheldon in 1929 (Vishwakarma et al., 2013). This disease has been reported from all sugarcane growing countries and the pathogen can spread via infected sugarcane cuttings, sugarcane stem borers and windblown rains. Although this disease and its symptoms were recognized for quite a long time, initially the disease severity was noted in a few varieties. Now, it has become a newly emerged devastating disease of sugarcane crop that results in reduced cane quality. It has been reported that commercial cultivars may be affected by this disease from 1%-90% (Vishwakarma et al., 2013). Initial symptom of *F. moniliforme* infectionis chlorosis at the base of new leaves; however, in acute cases the pathogen results in the cane distortion by cut like lesions in external and internal parts, destroying cane of the sugarcane. The base of leaves in infected plants are comparatively narrow as compared to healthy and non-infected leaves. Disease symptoms occur in four phases: i) chlorotic phase I, ii) chlorotic phase II, iii) top rot, and iv) knife cut phase. Curling, wrinkling and twisting of apical leaves may also occur depending upon the susceptibility of sugarcane variety. This disease has been notified as an emerging disease of sugarcane due to its increased incidence. Additionally, many sugarcane varieties are becoming susceptible to this pathogen as reported by Vishwakarma et al., (2013).

1.4. Management of sugarcane diseases

During the rather long developmental stages of sugarcane, the plants face numerous environmental challenges in the form of a variety of biotic and abiotic stresses, like high temperature, drought stress, salinity, heavy metal contamination as well as various pathogenic viruses, bacteria, fungi and nematodes. There are different strategies to manage sugarcane fungal diseases. Among these, the important ones are the agronomic and cultural practices, heat therapy of setts, usage of synthetic chemicals and cultivation of resistant varieties.

1.4.1. Agronomic and cultural measures

Cultivation of sugarcane via ratoon cropping must be discouraged. Fungal disease incidence can be diminished by crop rotation after two to three years. After harvesting, the crop residues must be collected and burnt. Hygienic cultivation and proper sanitation of sugarcane fields is one of the most

important means to stop the spreading of fungal diseases caused by *C. falcatum* and *F. moniliforme* (Leclerc et al., 2013).

1.4.2. Heat treatment of canes

Heat therapy is one of the important strategies practiced sterilizing the sugarcane setts before sowing. It is quite an old practice to destroy seed or soil-borne pathogens by using temperature high enough to kill the pathogens without harming the germination viability of the seed (Yuan and Wen, 2018). Seed material can also be treated with moist hot air as an effective strategy to eradicate *C. falcatum* spore contamination. For this, setts are treated at 52°C for 45 minutes and with hot air for 2 hours at 54°C (Sharma and Tamta, 2015).

1.4.3. Usage of resistant sugarcane varieties

Sowing of resistant varieties is one of the attractive and successful approaches that can be used in conditions of diverse growing regions having comparatively durable resistance towards pathogens. Plant disease control by resistant varieties is relatively inexpensive strategy as compared to the rather expensive pesticides; however, development of such varieties might take time for approval and consumer acceptance. Despite of the advantages, resistance varieties also become susceptible to pathogens, such as adaptation of the pathogens to become virulent, issues in uniformity of varietal genetics, with time decreased field resistance. Breakdown of resistance is quite common in different plants and varieties (Rahman et al., 2018).

1.4.4. Chemical control and constrains

The demand for food has been increasing with the growing population. To overcome the demand and fulfill the food requirements, high input of agrochemicals has been one of speedy pest management approaches. Numerous chemicals, especially fungicides, are used to control the red rot and pokha boeng disease in sugarcane. Dipping of sugarcane setts for 5-10 minutes in some organomercurial (0.25% Aretan and Agallol) for about 5-10 minutes helps in the eradication of surface fungal spores but is not effective against deep-seated mycelium. Use of chemical based antifungal sprays to inhibit the red rot and pokha boeng is usually restricted to the treatment of setts before sowing. However, frequent use of fungicides can result in development of resistance in pathogens against these chemical fungicides. Moreover, most of the fungicides has been restricted by various environmental protection agencies due to their toxic health and environmental hazards.

Nevertheless, the mentioned disease control measures have some limitations. Therefore, biological control is the eventual long-term solution to control fungal pathogens of sugarcane. Irrespective of disease control and pest management by using chemicals, development of resistance by pathogens to fungicides is one of the common problems. Due to this constrain, higher costs as well as higher doses of new and old chemicals are required in the field for the protection of crops and plants. Consequently, a lot of side effects, i.e., environmental pollution, contaminated food, its dispersal and higher costs for food production are faced (Manyi-Loh, 2018). The fungicides and pesticides also confer negative effects on beneficial microbes. Growth inhibition of certain mycorrhizal fungi was also noticed in the fields practiced with the use of Oryzalin and Trifluralin. Furthermore, some pesticides may inhibit the beneficial ammonia- oxidizing bacteria and some diazotrophs that aid in nitrogen fixation. Also, due to the toxic and persistent nature of these chemicals, alternative environment friendly methods are desirable for sustainable agriculture and proper control of pathogens (Chattopadhyay, 2017).

1.4.5. Biological control

Soil and sett-borne nature of sugarcane red rot and pokha boeng favors growth of pathogens as epidemics during favorable environmental conditions. Utilization of fungicides to control these diseases is limited under field conditions as the spores of these fungal strains are deep seated. Under such limitations, management of sugarcane fungal diseases through biocontrol agents could be a substitutive strategy to overcome the crop losses (Kohl et al., 2019).

Biological control is a sustainable and ecofriendly strategy that refers to control diseases or pests through effective beneficial microbes and other available natural components. Biocontrol strategy comprises of pests, such as bacteria, fungi, insects or nematodes by exploitation of pathogenic organisms, living predators, competitive microbes or parasites and decomposing biological materials that can minimizes the population of pathogens in crop fields. Biocontrol is usually categorized in two distinct approaches: i) classical biocontrol and augmented biocontrol. In classical biocontrol, generally, a non-native biological agent is used to regulate and conquer a specific pathogen, whereas augmentation involves the utilization of native microbial population whose population can be enhanced through massive mass culture (Landis and Orr, 2020).

1.5. Biocontrol agents

Biocontrol agents are the microbes that are proficient enough to restrict the lifecycle of phytopathogens by adversely prompting their proper growth and eventually the survival. Besides, employment of wide range of microorganisms as biocontrol agents, potentials are those that can pose dual effects of disease suppression and plant growth promotion and include both mycorrhizal fungi and rhizospheric bacteria. These beneficial microbes increase the plant growth directly by production of phytohormones, mobilization of insoluble nutrients and enhanced water uptake in addition to their biocontrol activity. Use of biocontrol agents to manage plant diseases is an economical, ecofriendly and sustainable approach that is much safer as it is assumed to be non-pollutant as compared to chemical pesticides. An ideal biocontrol possesses the following traits: i). show the high survival rate in the soil for prolonged period: ii). high probability to contact targeted pathogen: iii) are not health hazardous, iv). having massive easy and economical multiplication potential, and v), having climatic adaptability and short handling time.

1.5.1. Sustainable mechanisms adapted by biocontrol agents

1.5.2. Parasitism

One of the common interaction/phenomena among various microbes is parasitism. Growth and development of numerous bacteria and fungi was noticed by feeding on other fungi. However, their mode of action varies like *Trichoderm*a species paralyses the host fungus by the formation of coiled hyphal structures and its penetration into the host cell wall by production of cell wall hydrolytic enzymes (Zeilinger et al., 1999). Same mechanism of mycopagy is shown by some antagonistic bacteria (Fritsche et al., 2006). *Paenibacillus sp* isolated from *Laccaria bicolor* was found to be physiologically active bacteria against fungal pathogens (de Boer et al., 2005). This bacterium also inhibits the pathogen *Fusarium oxysporum* of *Sorghum bicolor* (Budi et al., 2000). *Pseudomonas* and *Bacilli* are two commonly reported genera for their role in suppression of fungal pathogens. Various fungicidal compounds/ metabolites released by antagonistic bacteria are normally stated as suppressive agents of phytopathogens.

1.5.3. Competition

Biocontrol agents also implement the mechanism of competition to survive in the rival environments. Biocontrol agent in the rhizosphere compete in an environment by utilization of a specific compound as its energy source, that remaining microbes are unable to utilize; thus, facilitate it a competitive

advantage (Lugtenberg et al., 2001). Use of organic acids by biocontrol agents offers them a viable benefit to survive in the rhizosphere (Goddard et al., 2001). Moreover, struggle to exploit inorganic compounds as energy source is one of the important aspects to determine the success of a potential biocontrol agents to conquer the phytopathogens. Iron is an essential nutrient for growth and development of fungal pathogens. Many siderophore producing rhizobacteria are introduced in soils infected with fungal pathogens to release iron chelating compounds; siderophores and suppress the fungal strains by depleting the iron readily available for their poliferation (Raaijmakers et al., 1995; Whipps, 2001).

1.5.4. Hydrolytic enzymes production by antagonistic bacteria

Release of enzymes involved in the degradation of pathogen cell wall is a potential mode of action reported previously in biocontrol agent. Lytic enzymes released by microbes degrade many polymeric compounds like chitin, cellulose and hemicellulose which are main components of fungal cell wall (Chin-A-Wing et al., 2003). Release of specific hydrolytic enzymes by various microorganims have been linked with their antagonistic potential (Grevesse et al., 2003; Harman et al., 2004; Howell, 2003). Indirect pathogen conquest is also noticed as activity of these enzymes may induce plant host defences by activation of various genes (Benhamou, 2004).

1.5.5. HCN production

Cyanide, a poison released by some bacterial to suppress the phytopathogens and inhibit the metabolism by hidering the activity of enzyme cytochrome C oxidase as well as many other enzymes. Term cyanogenesis revealed the production of cyanide and this phenomenon is observed both in bacteria and plants (Voisard et al., 1994). strains producing HCN Suppression of wheat pathogens by HCN producing recombinant *P. putida* BK8661 strain was reported previously (Flaishman et al., 1996).

1.5.6. Systemic Acquired Resistance

Induced systemic resistance (ISR) is considered as a plant defense mechanism stimulated by nonpathogenic host friendly microbes. Growth promoting bacteria potentiate this as a resistant mechanism for pathogen suppression of both soils borne and aerial phytopathogens (Kloepper et al., 2004). Normally ethylene and jasmonate are involved in the regulation of ISR pathways (Pangesti et al., 2016). Factors influencing systemic resistance in host plants include the production of 2,4

diacetylphloroglucinol by *Pseudomonas fluorescence* that induced resistance in Arabidopsis (Weller et al., 2004); moreover, the lipo-polysaccharides and siderophores produced by *P. putida* provoke systemic resistance in Arabidopsis, tomato and bean (Meziane et al., 2005). *Bacillus amyloliquefaciens* and *Bacillus subtilis* have also been found to be involved in the same mechanism in Arabidopsis plants (Ryu et al., 2004). An important role was also shown by salicylic acid production that triggered ISR and also activate plant growth promotion. This resistance is also called systemic acquired resistance (SAR). This phenomenon is normally observed in plants exposed to phytopathogens, however, it can also be stimulated when inoculated/treated with nonpathogenic organisms. Systemic acquired resistance usually involves the acclimatization of pathogenesis related (PR) proteins that directs to the induction of a hypersensitive response by plants towards pathogens.

1.5.7. Plant growth promoting bacteria as biocontrol agents

Useful free-living bacteria in soil are usually known as plant growth-promoting rhizobacteria (PGPR) (Hayat et al., 2010). PGPRs either inhabit the rhizospheric soil, the rhizoplane, or the root itself independent of vegetative growth promotion mechanisms (Gray and Smith, 2005). Only few bacteria (1 to 2%) of rhizospheric bacteria promote plant; among these *Bacillus* and *Pseudomonas* genera are predominant (Podile and Kishore, 2006). Microbes that provide a front-line defense against various phytopathogens are ideal to be use as biocontrol agents. Growth promoting bacteria may affect plant indirectly or directly. The direct plant growth promotion mechanism involves the production of certain compounds, enzymes, phytohormones or by facilitation of the uptake of nutrients from the surroundings (Afridi et al., 2019). Role of antagonistic PGPR is well known to suppress the deleterious effects of phytopathogens that directly or indirectly enhance the growth of associated plants. These bacteria play a role by inducing disease resistance in plants or via production of antagonistic elements (Glick, 1995).

Active PGPR acting as biocontrol agents compete with other microbes to colonize plant roots by active utilization of root exudates and root mucilage and mediate biocontrol activity by the synthesis of allelochemicals, production of siderophores, antibiotics, hydrolysis or lytic enzymes, and degradation and detoxification of virulence factors. Furthermore, they indirectly enhance plant growth by inducing the systemic resistance in plants (Compant et al., 2005).

1.5.8. Silver nanoparticles in agriculture: an important tool for plant disease management

After experiencing, the negative effects of first green revolution, there is a dire need for the second one that should have an economic, environment friendly and comparatively sustainable approach. Consequently, current agricultural practices adopt an advanced technology known as nanotechnology to manage various harsh environmental factors (Nair et al., 2010; Ghormade et al., 2011; Wang et al., 2013). In general, the agricultural production is largely affected by huge economic losses due to various kinds of phytopathogens. In the current scenario, the concept of biopesticides has emerged to introduce advanced pathogen control techniques and limit the indiscriminate use of chemical pesticides as well. Among various nanoparticles, silver nanoparticles (AgNPs) are the most prevalent metallic nanoparticles that have been implemented in every field due to their inimitable properties. including their antimicrobial property. AgNPs are mostly used to manage plant diseases (Park et al., 2006; Jo et al., 2009; Kim et al., 2012; Mishra et al., 2014). Previously, antimicrobial activity of AgNPs have been reported against vast range of phytopathogens. As an example, biologically synthesized endophytic bacterium isolated from garlic, had shown a great potential to defend wheat plants from *Fusarium graminearum* infection (Ibrahim et al., 2019).

1.6. Objectives

Current research was planned to sort out the alternative use of chemical fertilizers, like biocontrol agents and application of biologically produced nanoparticles, by analysis of various mechanism involved in disease control and enhanced plant growth. Further study was aimed:

- To do the isolation, evaluation, and characterization of plant growth promoting bacteria (PGPB) antagonistic to *Colletotrichum falcatum* and *Fusarium moniliforme*
- To analyze the build-in advantages of antagonistic PGPB as multi-stress tolerant
- To ensure the biogenic production of silver nanoparticles and their characterization
- To conduct green house studies to evaluate the efficacy of antagonistic bacteria and nanoparticles to disease tolerance in sugarcane for enhancing plant growth.

Chapter. 2

Isolation, Screening and Characterization of Antagonistic Plant Growth Promoting Bacteria from Sugarcane (*Saccharum officinale* L.)

2.1. Introduction

Sugarcane is significant cash crops grown all over the world both in tropical and subtropical areas. Among all 110 sugarcane cultivated countries of the world the main purpose of growing sugarcane is to fulfill the human requirements of sugar. Two major fungal diseases, red rot and pokha boeng, drastically affect the growth and yield of sugarcane.

These pathogens normally reside on the decaying or decayed parts of the host plant as active saprophytes or in the soil as dormant spores. Under favorable conditions, these spores germinate and infect the healthy canes of plant. Their management in the field is hard as the genetics of these fungi changes continuously. These fungal diseases can be managed by using resistant varieties; (2) Use of effective fungicides; and (3) biological control through potential antagonistic PGPR. Although the usage of resistant varieties is an important approach to manage phytopathogens, these resistant varieties also become susceptible due to the frequent emergence of new fungal variants (Nayyar et al., 2017). Moreover, the frequent use of synthetic fungicides and pesticides results in their harmful residual effects on plants and agricultural soils. They may also exert undesirable effects on the advantageous microbes. In addition, phytopathogens may adapt and overcome to build resistance against these fungicides (Bardin et al., 2015)

Due to these constrains, there is a growing demand for biological fertilizers (Bhardwaj et al., 2014). Biological control of plant pathogens comprises of numerous mechanisms to slow down or inhibit the growth of pathogens (O'Brien, 2017). Sugarcane roots-associated native microflora may have the potential to control *Colletotricum falcatum* and *Fusarium moniliforme.* Furthermore, they can play important role in disease protection and growth enhancement of sugarcane crop by producing IAA, siderophores, ammonia, phosphate solubilization, production of various extracellular enzymes and EPS (Mehnaz et al., 2011). Buiding of an operative biocontrol strategy against *C. falcatum* and *F. moniliforme* entails isolation, screening and assessment of potential native antagonistic PGPR capable to reduce the symptoms of red rot and pokha boeng under *in vitro* conditions based on which these can be further tested at pilot and field scale. Previously, numerous bacterial genera, such as *Bacillus*, *Pseudomonas*, *Enterobacter*, and *Burkholderia* have been noticed to be allied with sugarcane rhizosphere and capable to conquer the fungal pathogens (Hassan et al., 2014; Katiyar et al., 2017). Inoculation of bacterial isolates with ability to control the red rot and pokha boeng diseases along with the boosted sugarcane growth is a desirable option to reduce the heavy cost of fertilizers and fungicides for sustainable agriculture. In this respect,

exertions have been directed towards the isolation, screening and characterization of native sugarcane associated PGPB for the biological control of *C. falcatum and F. moniliforme.*

2.2. Materials and methods

2.2.1. Site selection and sampling of diseased and healthy plant material

For sample collection, sugarcane growing areas of Punjab; Faisalabad (31.4504° N, 73.1350° E), and Mianwali (32.5839° N, 71.5370° E) were selected. Sugarcane plants were taken with intact roots along with adhered soil particles. Rhizospheric soil was also taken. Collected samples were preserved in sterile plastic bags and preserved at 4^{o}C for further processing.

2.2.2. Isolation, identification and preservation of pathogenic fungi

2.2.2.1. Isolation of sugarcane fungal pathogens (Red rot and Pokkha boeng)

For isolation of fungal strains, susceptible sugarcane variety CO1148 infected with red rot and another diseased sugarcane plant (variety CSSG32) infected with *F. moniliforme* were collected. Infected parts of diseased sugarcane plants were separated. Selecetd sugarcane stalks were washed and cut into small pieces to separate the red rot and pokha boeng infected tissues. Minuscule pieces approximately of size 2-3 mm were vigorously washed with distilled water. Further, surface sterilization was done by initial washing with mercuric chloride (0.1%) for 30 seconds followed by disinfection with 70% ethanol. Disinfected plant material was thoroughly washed with autoclaved distilled water and dried by slightly pressing under blotting paper. Sterilized plant tissues were then transferred to the petri plates containing potato dextrose agar (PDA). Plates were properly sealed with parafilm and incubated for five days at 28±2°C. Following the same procedure of disinfection and drying, plants tissues inefected with *F. monilliforme* were transferred to Malt extract media (MEA) with malt extract (20g), peptone (1g), dextrose (20g), agar (20g) for 1L distilled water (Skaar and Skenwig et al., 1996). Fungal growth from infected tissues was noted after 5-7 days of incubation. Both fungal strains were tentatively identified on the base of general appearance of mycelia and its color. Morphology of respective spores was observed under microscope using standard mycological literature. Both isolated phytopathogens were further purified by consequent sub-culturing and also by single spore isolation technique. A seven-day old culture was scrapped from fresh fungal culture slants and suspended in autoclaved distilled water. A 100µl suspension suspension was dispersed on petri plates containing respective growth media and incubated at

28±2°C for seven days. A single spore was picked with sterilized cork borer by marking under microscope and transferred to respective media slants (PDA and MEA) to observe monoconidial culture growth (Kumar et al., 2007).

2.2.2.2. Pathogenicity test

To verify the virulence of isolated fungal strains *C. falcatum* and *F. moniliforme* to susceptible sugarcane varieties and meanwhile to fulfill the Koch's postulate rule; a pathogenicity test was directed in the sugarcane fields of National Agriculture Research Centre (NARC), Islamabad. Symptoms of red rot and pokha boeng disease on susceptible varieties of sugarcane CO1148 and CSSG32 confirms the virulence of inoculated strains. Sugarcane plants (variety YTTH-55) showed no symptoms or appreance of both diseases at NARC, Islamabad that showed its resistance.

2.2.3. Isolation of bacterial strains from sugarcane rhizosphere and its plant parts

2.2.3.1 Isolation of rhizospheric bacteria

The bacterial strains were isolated from the rhizospheric soil collected from various sugarcane growing areas of Punjab by following serial dilution method (Ahmad et al., 2008). Samples were arranged by separating l g soil sticked to the roots of sugarcane plants. For serial dilution, 9 screw capped McCartney bottles with 9 ml distilled water were autoclaved and numbered. All the bottles were marked in a sequence from 1 to 9; the stored 1 gram of rhizosphere soil sample was added to bottle No.1 and was mixed vigorously using the vortex mixer to allow proper mixing of soil samples and water, then 1 ml of the solution from test tube no. 1 was taken using a micro pipette and poured in into bottle No. 2, then the test tube No.2 was shaken vigorously and 1ml solution from this bottle was added to bottle No.3. Similar procedure was repeated for the remaining bottles to give 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, $10^{-8}$and 10^{-9} serial dilutions for use in the study. 100 µL was taken form each dilution and spread on the Luria Bertani (LB) agar plates. The plates were placed in an incubator for 48 hours at 28±2°C.

2.2.3.2. Isolation of endophytic bacteria

To isolate endophytic bacteria healthy sugarcane plants were prudently removed from the soil. Collected plants were separated into roots, stems and leaves; thoroughly washed and further cut into small pieces. Plant parts were surface disinfected by following the standard procedure (rinsed in 70% alcohol (1 min), followed by sodium hypochlorite (4 min), ethanol (30 seconds), at the end washed

3 times with sterile distilled water). Plant parts were then macerated along with 6ml autoclaved distilled water separately in sterile pestle and morter. Macerated extract was incubated at 28 ºC for 3 hours to make sure the complete discharge of endophytes from plant tissue. For endophytic bacterial isolation, plant tissue extracts were subsequnetly diluted in aqueous solution. From each dilution, 100 µL was spread on the LB agar plates. Inoculated plates were placed in an incubator for 2-5 days at 28±2°C (Thomas et al., 2015).

2.2.3.3. Screening and purification of isolated bacterial colonies

Each petri plate was thoughly analyzed and bacterial colonies were evaluated and selected according to their appearance, growth, and morphology (size, color, shape). Single colonies of bacteria with different morphology were selected and picked with sterile loop and further streaked on fresh LB agar to obtain pure colonies. Bacterial strains were then stored in LB slants at 4°C.

2.2.3.4. Morphological and physiological characterization of isolated bacteria

Representative colony of each bacterial isolate was picked by sterile loop incubated culture plates/slant and streaked on a LB agar plates then further inoculated on petri plates with specific medium for each test.

2.2.3.4.1. Biochemical identification

The representative bacterial strains were also characterized via biochemical tests by using QTS-24 miniaturized system. Bacterial cultures were grown overnight in 0.85 % NaCl saline solution before inoculation to QTS kits (Mufti et al., 2015).

2.2.4 Plant growth promoting traits in isolated bacteria

2.2.4.1. Production of IAA

To analyze PGP traits, 24 hrs old bacterial culture with 10^6 CFU/mL was used. For IAA production, *B. xiamenensis* was grown in LB broth amended with L-tryptophan. To prepare broth 5g NaCl, 10g tryptone, 5g yeast extract, and 1g tryptophan was added in 1000 ml distilled water and autoclaved in glass test tubes. Bacteria were inoculated and test tubes were kept in shaker incubator at 120 rpm for 3-4 days at 28ºC. Kovac's reagent (5 drops) was added in each tube. Formation of cherry-red ring confirmed the production of IAA (Hussain et al., 2019).

2.2.4.2. Phosphate solubilization

Pikovskaya's agar plates (10g glucose, 5 g tricalcium phosphate (Ca_3PO_4), 0.5 g ammonium sulphate $(NH_4)_2$ SO_4, 0.1 g magnesium sulphate ($MgSO_4$. $7H_2O$), 0.2 g sodium chloride (NaCl), 0.2 g potassium chloride (KCl), 0.5 g yeast extract, 0.3g calcium carbonate ($CaCO_3$), manganese sulphate ($MnSO_4$) and iron sulphate ($FeSO_4$) in trace,15 g agar in 1 litre distill water, pH was adjusted to 7) were used. Bacteria was spot inoculated at the middle of each plate. Inoculated plates were then incubated at 28 ºC for 7 d and observed for the formation of clear halo zone round bacterial colony. Halo zone formed by bacterial strain was measured after 7 days of incubation (Mukhtar et al., 2020).

2.2.4.3. Siderophore production

For siderophore production, Chrome azurol S agar media was primed by mixing 60.5 mg Chrome azurol S in water (50 ml) and iron (III) solution (1 mM $FeCl_3$. $6H_2O$, 10 mM HCl) (10 ml) to analyze the siderophore production. This solution was mixed slowly with Hexadecyl trimethyl ammonium dissolved in water (40 ml). Medium was then autoclaved. Spot inoculation was done at the center of CAS media plates and incubated at 28ºC for 48-72 hours. Formation of yellow to the orange zone around bacterial colonies indicated the siderophores production (Amna et al., 2019).

2.2.4.4. Hydrogen cyanide production

LB agar plates amended with glycine L^{-1} (4.4 g) were used for hydrogen cyanide (HCN) production. Whatman filter paper 1 was soaked in 0.5% picric acid and 2 % sodium carbonate (Na_2Co_3) solution and placed on the upper lid of petri plates. Bacterial streaked petriplates were wrapped with parafilm and incubated at 28 ºC for 5 days. Change in color of filter paper from yellow to orangish brown showed positive results for HCN production (Amna et al., 2019).

2.2.4.5. Ammonia production

24 hr old bacterial culture were inoculated in ten ml of peptone water (15 g peptone water in 1000 ml) in each tube and incubated in a shaker at 28 ºC for 2-3 d. After incubation each tube was amended with 0.5 ml of Nessler's reagent. Change in the color of media from brown to yellow showed positive response of bacteria towards ammonia production (Mukhtar et al., 2020).

2.2.5. Production of extracellular enzymes

2.2.5.1. Protease production

Production of protease enzyme was estimated by spot inoculation of each bacterial strain on skimmed milk agar medium (SMA). Inoculated plates were incubated for 72 hours at 30°C. Appearance of halozone around the colonies by hydrolyzation of skimmed milk was considered as positive result (Amna et al., 2019).

2.2.5.2. Catalase production

Fresh bacterial colony was picked from petri plate and placed on glass slide and mixed with few drops of H_2O_2 (3%) (Amna et al., 2019). Formation of oxygen bubbles indicated the catalase activity.

2.2.5.3. Amylase production

For agar starch media bacterial strains were spot inoculated on starch agar medium containing yeast extract (10 g), $MgSO_4.7H_2O$ (0.1 g), KH_2PO_4 (2 g), (K_2HPO_4 (7 g), NaCl (5 g), $NH_4)_2SO_4$ (1 g), starch (5g), agar (15 g) with pH adjusted to 7. Inoculated plates were incubated at 28 °C for 48 h. Formation of clear zone around the bacterial colony when treated with iodine solution showed the positive results for amylase production (Afridi et al., 2019).

2.2.5.4. Pectinase production

Pectin agar media containing yeast extract (1 g), $(NH_4)_2SO_4$ (2 g), Na_2HPO_4 (6 g), KH_2PO_4 (3 g), pectin (5 g) and agar 20 g was prepared and poured in petriplates. Bacterial strains were spot inoculated on these plates and incubated at 32 °C for 48 h. After incubation plates were flooded with iodine solution (50mM). Occurance of halo zone represents positive results for pectinase production (Afridi et al., 2019).

2.2.5.5. Cellulase production

All strains were screened by using cellulose agar media (cellulose 2 g, $MgSO_4$ 0.25 g, KH_2PO_4 0.5 g, agar 15 g, and gelatin 2 g; 1 litre water with pH 6.8–7.2). Congo-Red was used as an indicator to monitor the cellulase degradation. Bacterial strains showing clear halo zone on cellulose medium indicated positive results to produce cellulose (Sethi et al., 2013).

2.2.5.6. Chitinase production

Colloidal chitin was prepared from practical grade crab shell chitin as described by Hjort et al. (2014). Ten g of chitin taken from crab shell powder. One hundred and fifty ml HCl was added to the chitin incubated for onehour in a shaker incubator. After incubation, chilled water (500ml) was added in the chitin. After few minutes, it was mixed well and then filtered with wattman filter paper. After filtration chilled water was added again. This step was repeated at least four times. After washing and filtration chitin was stored at 4^{o}C temperature for further use. Chitinase production was evaluated following the method of Gohel et al. (2004). Each rhizobacteria was spot inoculated on chitin agar plates (Colloidal chitin 10 g, Yeast extract 5 g, $MgSO_4$ 0.5 g , Sodium nitrate 2 g, KC1 0.5 g, FeSO4 trace, K2HPO4 1 g, Agar, 20 g Final pH (using 1N NaOH) (6.0±0.2) and incubated for 7 d at 30°C and observed for the development of zone around the colonies.

2.2.5.7. Detection and quantification of ACC-deaminase production

For qualitative production of ACC- deaminase, cell pellet grown in Tryptic Soy Broth (TS) was collected and washed twice with 0.1 M Tris-HCl. Pellet was again re-suspended in sterile 0.1 M tris-HCl and spot inoculated on DF salt minimal medium agar plates amended with 3mM ACC (Afridi et al., 2019). DF medium plates with and without ammonium sulphate were taken as positive and negative control, respectively.

2.2.6. Exopolysaccharide (EPS) production

The potential for exopolysaccharide production was determined on ATCC medium no.14 according to Afridi et al., (2019). To check the production of exopolysaccharide bacterial strains were streaked on ATCC medium no.14 (Sucrose 20g, yeast extract 0.5g, $FeCl_3$2mg, $CaSO_4.2H_2O$ 0.1g, K_2HPO_4 0.8g, KH_2PO_4 0.2g, MgSO4.7H2O, $Na_2MoO.2H_2O$ pH 7.2) incubated for three days at 28˚C under hygienic atmosphere. Formation of slimy layer around the bacterial colonies confirmed the production of exopolysaccharide.

2.2.7. *In-vitro* antagonistic activity of isolated bacteria against sugarcane fungal diseases

Bacteria were analyzed for *in vitro* antagonistic activity against virulent phytopathogens. A dual culture assay was performed as described by Al-Hinai et al., (2010) with little modifications. A 5 mm disk of freshly grown fungi was placed on the center of plate containing potato dextrose agar (PDA), Malt Extract Agar (MEA) according to fungus. Bacterial strains were streaked on the other

opposite side of fungus plug. Petri plates with only fungal plug were taken as control. Plantes were incubated for 7 d at 28±2 °C. The percentage inhibition of phytopathogen was calculated by following equation:

$$\text{Percentage inhibition} = \frac{C-T}{C} \times 100$$

Where, "C" represents the radial growth of fungus in control and "T" is the radial growth of fungal pathogen in the presence of bacterial strain.

2.2.8. DNA extraction

DNA of all strains was extracted by phenol-chloroform method. Cell pellet was suspended in 450 µl TE buffer (Appendix 2). The cells were then incubated for an hour at 37°C after adding 45 µl sodium dodecyl sulphate and 5 µl of Proteinase K (20mg/ml). Following incubation, 600µl phenol-choloroform (1:1) was added to samples and mixed thoroughly. The samples were centrifuged at 10,000 rpm for 20 mins to separate upper aqueous phase. The process was repeated twice by reducing time of centrifugation to 5 mins. Upper aqueous phase was transferred to new eppendorf and samples were mixed until formation of DNA precipitates after adding 50 µl sodium acetate and 300 µl isopropanol. To remove liquid phase, samples were centrifuged for 5 mins at 10,000 rpm, and washed with 70% chilled ethanol. Precipitated DNA was stored in 70 µl TE buffer at -20ºC.

Gene amplification for 16S rRNA genes

Bacterial DNA was amplified by using primers for 16S rRNA genes

27F (5-AGAGTTTGATC AC TGGCTCAG-3)

1492R (5-CGG CTTACCTTGTTACGACTT-3).

PCR products were analyzed from Macrogen, Korea for commercial sequencing.

Agarose gel Electrophoresis

The PCR products were observed in 2% agarose gel made in 1X TAE buffer. Samples were run at 85V for 35 minutes in a horizontal electrophoresis unit and were visualized in a gel doc system.

2.3. Results

2.3.1. Isolation of sugarcane fungal pathogens

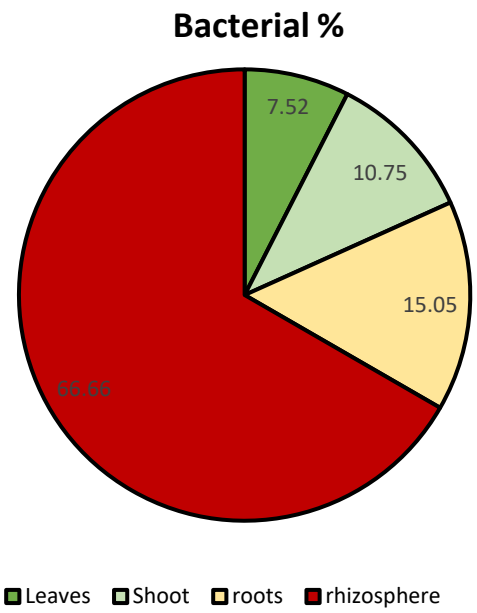
Both fungal strains were isolated from diseased sugarcane plants. Verification of fungal isolates was done in CDRI, NARC, Islamabad. Isolates were purified and recognized as *C. falcatum* and *F. monilliforme* based on the general appearance, color of mycelia in petri plates and by confirming spore morphology.

2.3.2. Pathogenicity test of isolated fungal strains under field conditions

Three different varieties of sugarcane were selected to analyze the pathogenicity of isolated fungal strains. Sugarcane variety YTTH-55 was observed to be resistant against both fungal strains *C. falcatum* and *F. moniliforme* while sugarcane variety CO1148 and sugarcane variety CSSG32 were susceptible against *C. falcatum* and *F. monilliforme* respectively. Appearance of disease symptoms after two months confirmed the virulence of isolated fungal pathogens.

2.3.3. Isolation of endophytes and rhizobacteria from sugarcane

About 102 bacteria were isolated from rhizospheric soil and different parts of sugarcane. Total 26 strains were endophytic, isolated from different plant parts i.e. 9 bacteria were isolated from roots of sugarcane, 7 from leaves, 10 from shoots and rest from rhizospheric soils. Evaluation of finalized 93 strains was done based on similar and dissimilar morphology. All bacterial strains were preserved in glycerol at -20°C for further characterization and screening.



2.3.4. Characterization of isolated bacterial strains from leaves, shoot, root, and rhizosphere for plant growth promoting activities

Screening of isolated strains was done against various plant growth promoting assays (Table 2.1)

2.3.4.1. Production of Indole acetic acid (IAA)

Out of 93 bacterial strains 34 were positive for indole acetic acid (IAA) production. Formation of cherry red ring on the top of tube confirms the production of IAA. While green coloration exhibited negative results of the test (Table 2.1).

2.3.4.2. Phosphate solubilization

Out of 93, fifty-two bacterial strains solubilized inorganic phosphates $Ca_3(PO4)_3$. Differential response of bacterial strains was noted for solubilization of the inorganic phosphate (Table 2.1). Level of phosphate solubilization by each bacterium was portrayed by the formation of halo zones around the bacterial colonies (Fig. 1.) Strain PM17 exhibited maximum efficacy forphosphate solubilization followed by bacteria PM14 and PM16. Among all, comparatively low activity was noted in PM15.

2.3.4.5. Siderophores production

Siderophore production by bacteria was noticed by the change of CAS media color from blue to orange. Fourteen strains showed positive results towards siderophore production. Though some

bacteria showed good growth, but no color change was observed in CAS medium around the colony. Hence, these were taken negative for siderophore production.

2.3.4.6. HCN production

A change in the colour of filter paper from yellow to orange brown indicated the production of cyanide. Among the tested isolates, 15 bacteria (FSS3, FSS7, FSR3, JRZ2, JR26, JRZ16, FRZ5, FRZ7, FRZ8, FRZ9, FRZ14, FRZ23, PM7 and PM18) produced HCN.

2.3.4.7. Estimation of ammonia production

Most of the strains showed the production of ammonia (Table 2.1). 70 out of 93 bacteria were observed positive towards ammonia production. Yellow color of media indicated moderate production of ammonia while a high production was observed in tubes showed dark brown color of media.

Table 2.1. Initial screening of isolated strains for *in vitro* PGP activities

Sr.no.	Bacterial codes	Indole acetic acid	Phosphate solubilization	Siderophore	HCN	Ammonia
1.	FSL1	-	+	-	-	-
2.	FSL2	-	+	-	-	-
3.	FSL3	-	+	+	-	+
4.	FSL4	-	-	-	-	+
5.	FSL5	+	+	-	-	-
6.	FSL6	+	+	-	-	-
7.	FSL7	-	+	-	-	++
8.	FSS1	-	+	-	-	-

9.	FSS2	-	-	-	-	++
10.	FSS3	-	-	-	+	+
11.	FSS4	-	+	+	-	-
12.	FSS5	-	+	-	-	-
13.	FSS6	-	+	-	-	+++
14.	FSS7	+	+	-	+	-
15.	FSS8	-	-	-	-	-
16.	FSS9	+	+	+	-	++
17.	FSS10	-	+	-	-	+++
18.	FSR1	++	+	-	-	+
19.	FSR2	-	+	-	-	++
20.	FSR3	-	+	+	+	+
21.	FSR4	-	+	+	-	+++
22.	FSR5	++	-	-	-	+++
23.	FSR6	+	+	-	-	+
24.	FSR7	++	+	-	-	+
25.	FSR8	-	+	-	-	++
26.	FSR9	-	+	-	-	+

27.	FSR10	-	-	+	-	+++
28.	FSR11	-	+	-	-	+++
29.	FSR12	-	-	-	-	++
30.	FSR13	++	+	+	-	-
31.	FSR14	++	+	+	-	-
32.	JRZ1	-	+	-	-	+
33.	JRZ2	-	-	-	+	+
34.	JRZ3	-	+	-	-	+
35.	JRZ4	-	-	-	-	+
36.	JRZ5	-	-	-	-	-
37.	JRZ6	+	+		+	-
38.	JRZ7	-	+	+	-	+++
39.	JRZ8	-	-	-	-	+
40.	JRZ9	-	+	-	-	+
41.	JRZ10	+	+	-	-	+
42.	JRZ11	-	-	-	-	-
43.	JRZ12	-	-	-	-	-
44.	JRZ13	-	-	-	-	-

45.	JRZ14	-	+	-	-	++
46.	JRZ15	+	-	-	-	-
47.	JRZ16	-	+	-	+	++
48.	JRZ17	-	-	-	-	+
49.	FRZ1	-	-	-	-	++
50.	FRZ2	-	-	-	-	+
51.	FRZ3	+	-	-	-	-
52.	FRZ4	-	-	-	-	+
53.	FRZ5	-	-	-	+	+
54.	FRZ6	+	+	-	-	+
55.	FRZ7	-	+	-	+	++
56.	FRZ8	-	-	-	+	+
57.	FRZ9	-	+	-	+	+
58.	FRZ10	++	+	-	-	++
59.	FRZ11	+	-	-	-	-
60.	FRZ12	++	+	-	-	+
61.	FRZ13	++	+	-	-	+
62.	FRZ14	-	-	-	+	+

63.	FRZ15	++	-	+	-	+++
64.	FRZ16	-	-	-	-	+
65.	FRZ17	-	-	-	-	++
66.	FRZ18	++	-	-	-	+
67.	FRZ19	-	-	-	-	+
68.	FRZ20	-	+	-	-	+
69.	FRZ21	-	-	-	-	+++
70.	FRZ22	-	-	+	-	+++
71.	FRZ23	-	-	-	+	-
72.	FRZ24	-	-	+	-	-
73.	FRZ25	++	+	-	-	++
74.	PM1A	-	+	-	-	++
75.	PM2A	++	+	-	-	++
76.	PM3A	-	+	-	-	+
77.	PM4A	-	-	+	-	++
78.	PM5A	+	-	-	-	-
79.	PM6A	-	-	-	-	-
80.	PM7A	+	+	-	+	++

81.	PM8A	-	-	-	-	-
82.	PM9A	-	+	-	+	++
83.	PM10A	+	-	+	-	+++
84.	PM11	+	+	+	-	+++
85.	PM12	+	+	+	-	+++
86.	PM13	+	+	+	-	+++
87.	PM14	+	+	+	-	+++
88.	PM15	+	+	+	-	+++
89.	PM16	+	+	+	-	+++
90.	PM17	+	+	+	-	+++
91.	PM18	+	+	+	+	+++
92.	PM19	+	+	+		+++
93.	PM20	+	+	-	-	+++
94.	Control	-	-	-	-	-

HCN: Hydrogen cyanide; (-): negative; (+++) strongly positive; (++) moderately positive; (+) slightly positive.

2.3.5. Screening of antagonistic bacteria

All bacteria were examined for *in vitro* antagonistic activity against fungal pathogens *C. falcatum* and *F. monilliforme* on potato dextrose agar (PDA) and Malt extract agar (MEA) respectively. Twenty-nine bacteria were proficient to suppress *C. falcatum*. However, 32 isolates antagonize *F. monilliforme* by making an inhibition zone around the inoculated colony of bacteria. These isolates

were scored based on inhibition zone. Remaining isolates showed no antagonistic activity against both fungal strains. It was also noticed that most of the antagonistic bacterial strains were rhizospheric in nature while only 4 endophytic strains depicted their potential to suppress the growth of fungal strains. Only one strain isolated from the leaf of sugarcane plant reduce the growth of *C. falcatum* and one endophytic bacterium from the leaf and two from the stem of sugarcane plant antagonized the *F. monilliforme* (Table 2.2).

Table 2.2. %age inhibitionof phytopathogens by antagonistic PGP bacteria

Sr.no.	Strain code	Inhibition assay of *C. falcatum*		Inhibition assay of *F. monilliforme*	
		Mycelial growth (mm)	Mean% (±SD) inhibition	Mycelial growth (mm)	% inhibition
1.	FSL5	70	17.64±1.1	-	-
2.	FSS4	-	-	63	21.25±3.2
3.	FSS7	-	-	48	40±1.1
4.	FSR1	-	-	57	28.75±1.5
5.	FSR5	64	24.7±1.2	47	41.25 ±2.5
6.	FSR6	59	30.58±5.1	53	33.75±4.5
7.	FSR8	67	21.17±2.3	-	-
8.	FSR13	61	28.23±1.1	52	35±3.6
9.	FSR14	59	30.58±2.0	49	59±2.1
10.	JRZ6	65	23.52±1.5	-	-
11.	JRZ10	53	37.64±2.5	-	-
12.	FRZ6	60	29.41±1.4	53	26.25±4.3
13.	FRZ9	68	20±1.8	-	-
14.	FRZ10	48	43.52±1.0	-	-
15.	FRZ11	57	32.94±1.2	54	32.5±2.4
16.	FRZ12	57	32.94±2.0	44	45±3.0
17.	FRZ13	68	20±1.3	-	-
18.	FRZ15	53	37.64±2.4	41	48.75±3.6

19.	FRZ18	50	41.17±1.7	53	33.75±2.1
20.	FRZ20	53	37.64±3.5	-	-
21.	FRZ22	-	-	53	33.75±2.1
22.	FRZ23	71	16.47±2.5	57	28.75±1.1
23.	FRZ24	-	-	53	33.75±1.0
24.	FRZ25	56	34.11±1.6	46	42.5±1.5
25.	JRZ2	-	-	49	38.75±1.5
26.	JRZ3	-	-	43	46.25±2.0
27.	JRZ5	-	-	53	33.75±2.4
28.	JRZ7	-	-	51	25±1.6
29.	PM7A	-	-	45	43.75±1.3
30.	PM10A	-	-	52	35±2.4
31.	PM11	27	68.23±1.0	24	70±3.4
32.	PM12	28.5	66.47	37.5	53.51±2.1
33.	PM13	23	72.94±4.5	27	66.25±5.0
34.	PM14	32.70	52.43±4.6	27.53	65.869±1.5
35.	PM15	28	67.05±2.8	34	57.5±2.8
36.	PM16	35	58.82±4.5	26	67.5±4.5
37.	PM17	26	69.41±2.5	22.5	71.875±5.0
38.	PM18	44	40±1.0	41	48.75
39.	PM19	45.6	34.11	50.5	36.87±4.2
40.	PM20	47	29.41	45	43.75±1.3
41.	Control	85	0±0.0	80	0±0.0

(-): negative; (+) positive

2.3.6. Growth suppression of sugarcane phytopathogens by the antagonistic PGP bacteria

Ten bacterial strains with maximum PGP ability and antagonistic potential were selected for further tests and experimentation.

2.3.7. Extracellular enzyme production

2.3.7.1. Catalase activity

All 10 bacteria were tested for catalase production. Formation of bubbles was observed on addition of H_2O_2 (few drops) on slide having bacterial colony (Table 2.3).

2.3.7.2. Amylase activity

After incubation, iodine was flooded on bacterial colony grown on specific media. Formation of clear zone around the colony showed hydrolysis of starch by amalyse production. All tested bacterial isolateswere positive for amylase activity except PM19 (Table 2.3).

2.3.7.3. Pectinase test

For pectinase activity all isolates were tested. Bacterial strain PM18 observed negative however strains from PM10 to PM17, PM 19 and PM 20 were positive for pectin degradation (Table. 2.3).

2.3.7.4. Protease production

All bactetia showed protease except PM20 (Table 2.3), showing the role of protease in antagonistic activity against phytopathogens.

2.3.7.5. Cellulase production

Bacterial strains showed clear zone around the bacterial colony in cellulose agar media amended with Congo red showed positive results for cellulase production. PM11, PM12, PM13, PM14, PM15, PM16, PM17, PM18 were positive however, PM19 and PM20 showed negative results as there was no clear zone around the colonies of both bacteria (Table 2.3).

2.3.7.6. Chitinase production

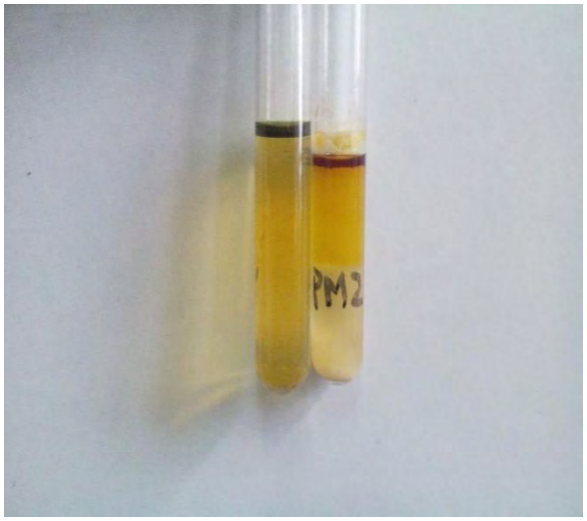
Strain PM18-PM20 showed no results for chitinase production whereas all other tested microbes were positive.

2.3.7.8. ACC-deaminase

All tested strains PM10-PM20 were evidenced to produce enzyme ACC- deaminase (Table 2.3) by using ACC as sole source of nitrogen.

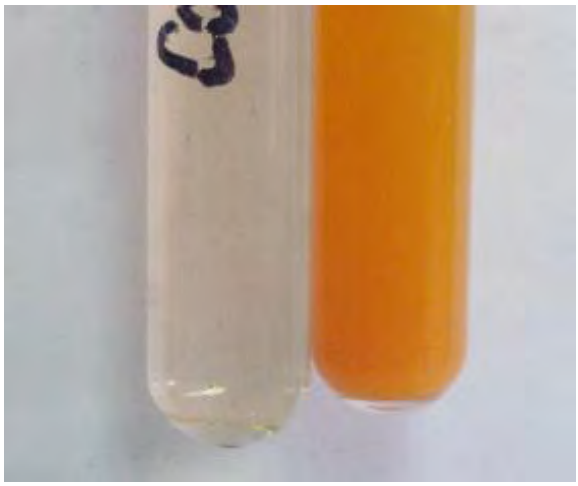
2.3.8. Exopolysaccharide production

Production/ appearance of slimy layer around the streaked colonies on specific ATCC medium no.14 showed the presence or production of exopolysaccharides by the bacteria. All strains were noticed to have the production of slime around the bacterial colonies (Table 2.3).

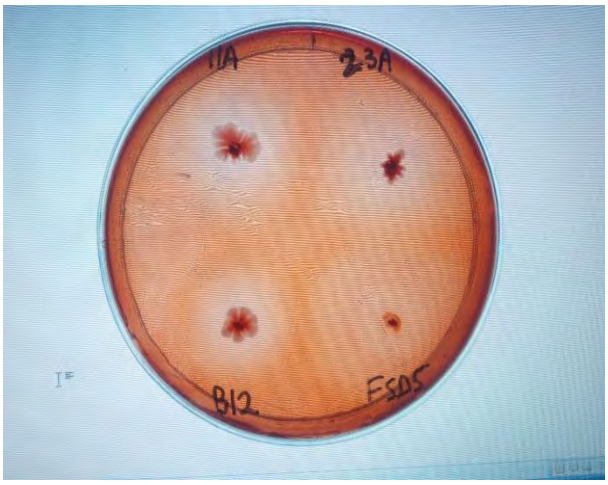


Indole acetic acid test

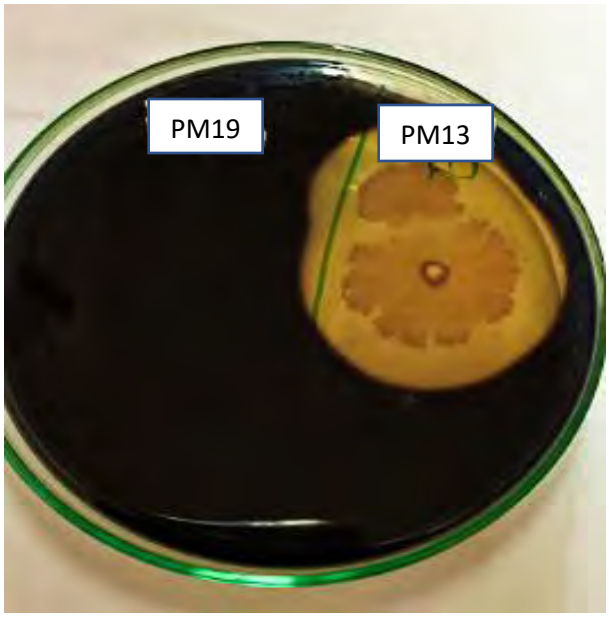
HCN test



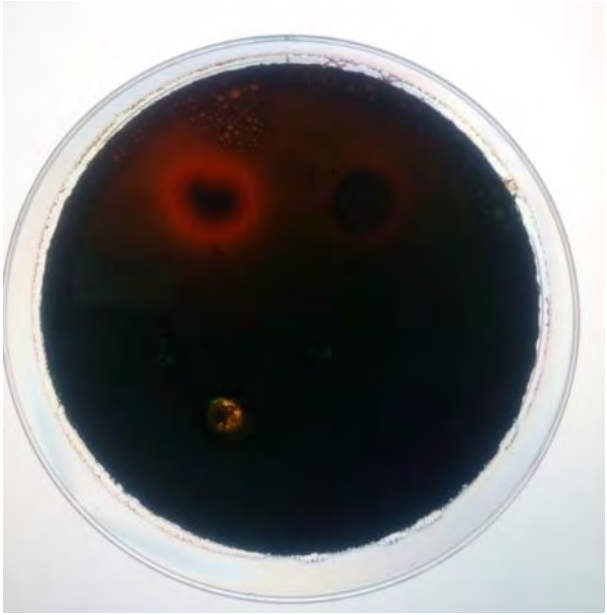
Ammonia test



Siderophore

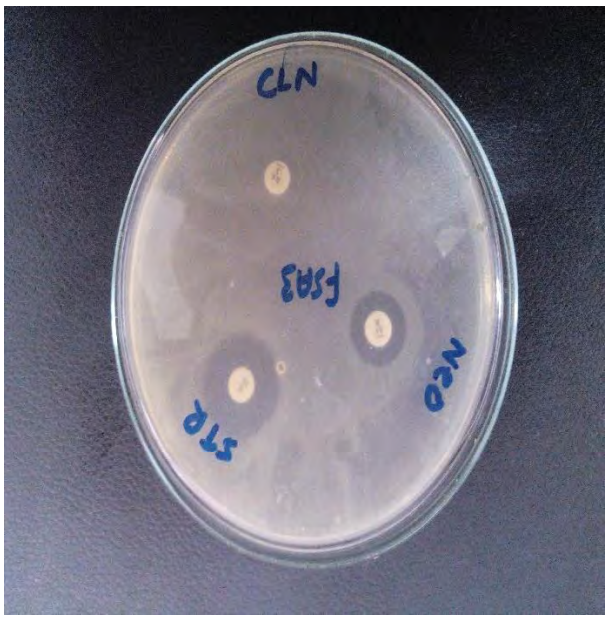


Pectinase test

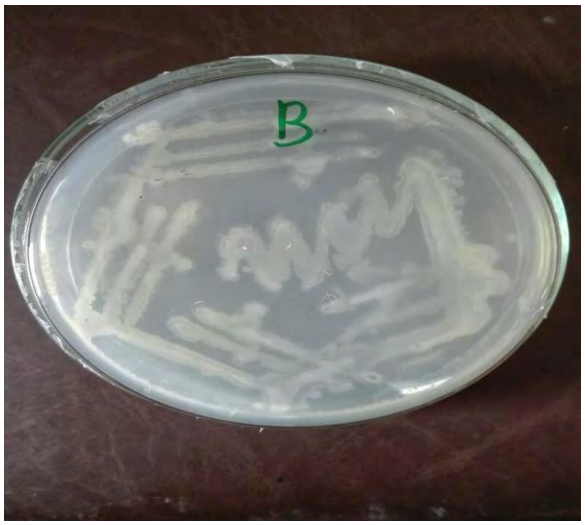


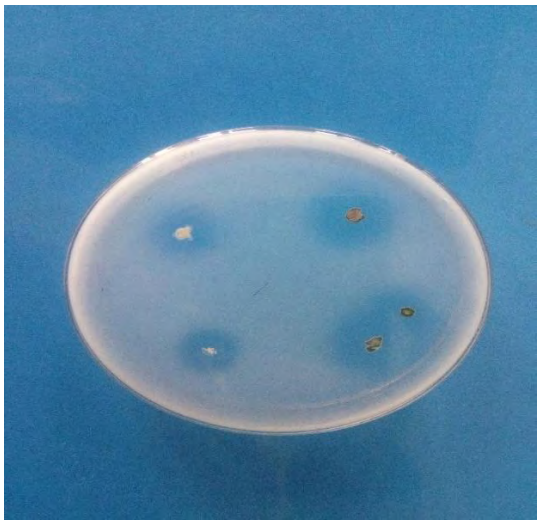
Siderophore test

Catalase test

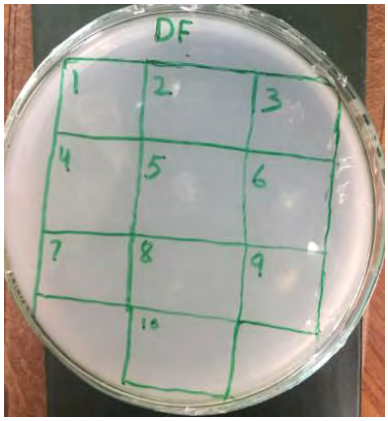


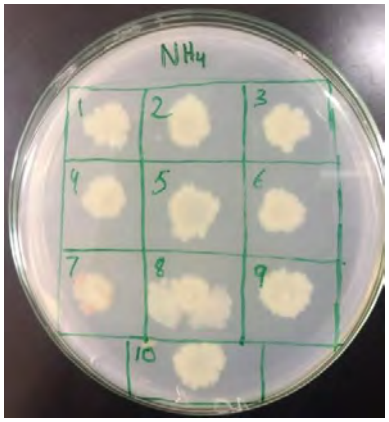
Antibiotic resistance test

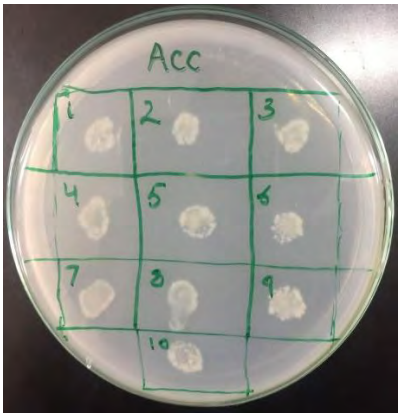




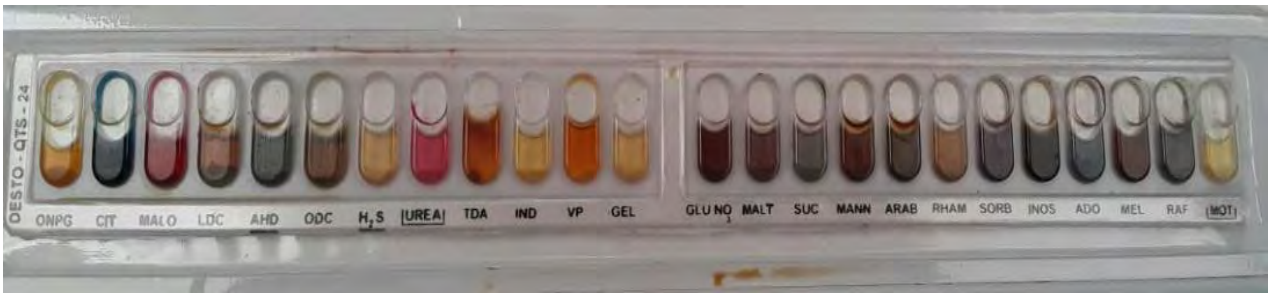
Exopolysaccharide production **Phosphate solubilization**







Negative Control **Positive Control** **ACC deaminase activity**



QTS test analysis

Table 2.3. Extracellular enzyme activity of selected bacteria

Enzyme activity	PM11	PM12	PM13	PM14	PM15	PM16	PM17	PM18	PM19	PM20
Catalase	+	+	+	+	+	+	+	+	+	+
Amylase	+	+	+	+	+	+	+	+	-	+
Pectinase	+	+	+	+	+	+	+	-	+	+
Protease	+	+-	+	+	+	+	+	+	+	-
Cellulase	+	+	+	+	+	+	+	+	-	-
Chitinase	+	+	+	+	+	+	+	-	-	-
ACC-deaminase	+	+	+	+	+	+	+	+	+	+
Exopolysaccharide production	+	+	+	+	+	+	+	+	+	+

(-): negative; (+) positive.

2.3.9. Morphology and biochemical characterization

All 10 finalized antagonistic PGPR were noted different by appearance and colony morphology. Color of bacterial colonies were whitish, some are creamy white, while few were light yellow to light orange. These strains were also categorized by on the base of Gram staining and biochemical screening by using QTS-24 miniaturized system (DESTO Laboratories Karachi, Pakistan).

The overnight grown bacterial cultures were suspended in 0.85 % NaCl (saline solution) before their inoculation to QTS kits. Tests like CIT, ONPG, IND, VP, ODC, H_2S, URE, TDA, GEL, MALO, LDC, ADH, GLU, MAL, SUC, MAN, ARA and RHA were performed (Table 2.4).

Table 2.4: Biochemical characterization (QTS-24) of isolated bacteria

QTS Test	PM11	PM12	PM13	PM14	PM15	PM16	PM17	PM18	PM19	PM20
ONPG	+	+	+	+	+	+	+	+	+	+
CIT	+	+	-	+	+	-	-	-	+	+
MALO	+	+	+	+	+	+	+	+	+	+
LDC	-	-	-	-	-	-	-	+	-	-
ADH	-	+	-	-	-	-	-	-	-	-
ODC	-	-	-	-	-	-	-	-	-	-
H_2S	-	-	-	-	-	+	+	-	-	-
UREA	+	+	-	+	+	+	+	-	-	+
TDA	+	+	+	+	-	+	+	+	+	+
IND	-	-	+	-	-	-	-	-	-	-
VP	+	-	-	+	+	+	+	+	+	+
GEL	+	-	-	-	-	-	-	-	-	-
GLU	+	+	+	+	+	+	+	+	+	+
MALT	+	+	+	+	+	+	+	+	+	+
SUC	+	-	+	+	+	+	+	+	-	+
MANN	+	+	+	+	+	+	+	+	+	+
ARAB	+	+	+	+	+	-	-	-	-	+
RHAM	+	+	+	+	+	+	+	+	+	+
SORB	+	-	+	+	+	-	+	+	+	+
INOS	+	-	+	+	-	-	-	+	-	+
ADO	+	-	-	-	-	+	+	-	+	+
MEL	+	+	+	+	+	+	+	+	+	+
RAF		+	-	-	+	+ +	+	+	-	+

ONPG=orhto nitro phenyl β-D-galactopyranoside; CIT=sodium citrate; VP=(vogesproskauer)= Acetion; GELl=Gelatin hydrolysis; MALO=sodium malonate;LDC=Lysine decarboxylase; ADH=Arginine dihydrolase;ODC=orthinine decarboxylase; H2S= H2S production; URE=urea hydrolysis;TDA=tryptophan deaminase; Indole; GLU= Acidic from glucose; MAL= Acid from maltose; SuC= Acid from sucrose; INO=Acid from inositol; MAN= Acid from mannitol; ARA=Acid from arabainose; RHA= Acid from Rhamnose; SOR=Acid from sorbitol; ADO=Acid from adonitol; MEL=Acid from Melibiose; RAE=Acid from raffino

2.3.10. Bacterial strain identification and Accession Number

All strains were identified and submitted to NCBI for accession number. All identified strains belong to the genus, *Bacillus*, except for PM12 which is known as *Bravibacterium frigoritolerans*. Belonging of most strains to *Bacillus* is because of the reason that mostly antagonistic mechanism related to the genera, *Bacillus* and *Pseudomonas,* has been reported in earlier studies (Table 2.5).

Table 2.5. Identified bacterial strains including strain code and accession number

Sr. no.	Strain code	Scientific name	Accession number
1.	PM11	*Bacillus gibsonii*	MK045646
2.	PM12	*Bravibacterium frigoritolerans*	MK049916
3.	PM13	*Bacillus siamensis*	MN240927
4.	PM14	*Bacillus xiamenesis*	MK358984
5.	PM15	*Bacillus sp.*	MN241465
6.	PM16	*Bacillus sp.*	MT978398
7.	PM17	*Bacillus tequilensis*	MT975274
8.	PM18	*Bacillus velezensis*	MK350360
9.	PM19	*B. methylotrophicus*	MK351221
10.	PM20	*B. licheniformis*	MK351222

2.4. Discussion

Sugarcane diseases caused by *C. falcatum* and *F. monilliforme* are the most disastrous diseases and a big threat to both farmers and sugar industry (Patel et al., 2019). Development of resistant fungal variants, various health hazards and environmental pollution associated with extensive use of agrochemicals lead to the adoption of biological control measures using native plants-associated rhizobacteria as a supplemental strategy to minimize pesticide usage (Peterson et al., 2016). Certain strains of plant growth promoting rhizobacteria (PGPR) have been used as versatile weapon to protect plants from various soil- borne pathogens along with enhancement of plant growth. These bioantagonists adopt single or multiple mechanisms of action to suppress the phyto-pathogens (Huang et *al.*, 2004), production of iron chelators, secretion of hydrolytic enzymes, synthesis of hydrogen cyanide (Beneduzi et al., 2012) and induced systemic resistance (Siddiqui and Shaukat, 2002). Thus, application of antagonistic PGPB or their metabolic products can play a vital role in disease control that is both effective and sustainable while reducing or eliminating the need for synthetic chemical pesticides and fungicides. In recent years, isolation and characterization of bacterial strains from the rhizosphere of different crops has widely been studied and the existence of all PGP traits in selected isolated strains has been reported. *In vitro* inhibition of various phytopathogens by plant growth promoting bacteria can be used as sustainable alternative/substitutes for the heavy input of agrochemicals (Zuluaga et al., 2020).

In present research, bacteria from the sugarcane plant parts and rhizosphere were isolated and screened for their antagonistic activity along with their plant growth promoting traits. *In vitro* prescreening of bacterial strains showed noticeable antagonistic activity of some isolates against *C. falcatum* and *F. monilliforme* with a variable range of percentage inhibition. These results support the fact that, virtually, all agricultural soils possess some suppressive effects on soil-borne phytopathogens because of the existence of microbes possessing antagonistic activities. This spectacle is also known as “general antagonism” or “general suppression” (Weller et al., 2002).

Most of the isolated strains from rhizosphere of sugarcane are found to be the best solubilizers of inorganic phosphates via release of organic acids that result in the formation of halo zone around the bacterial colony (Hussain et al., 2019). Plant growth promoting rhizobacteria as P-solubilizers show an effective role in its solubilization and conversion to readily available form for plants for uptake and utilize for their growth promotion. Most of the strains showed positive results for IAA production. Growth hormones, like IAA produced by PGPR, regulate a various biological function,

together with the behavior of plants towards various biotic and abiotic pressures. Though the role of IAA to inhibit *in vitro* fungal growth is not evident but its exogenous application abridged disease severity and crop yield loss triggered by phytopathogens (Afridi et al., 2019). EPS play important role in soil aggregation, thereby, improving soil water holding capacity and fertility (Paul et al., 2014). Current research showed that native PGPR play a vital role in improving soil texture, nutrients acquisition and assimilation. Moreover, secretion and modulation of extracellular productions i.e. hormones, various antibiotics, various signalling molecules and and some secondary metabolites leads to improvement of plant growth and biomass. The exploitation of the multiple antagonistic mechanisms adopted by plant growth promoting rhizobacteria (PGPR) has opened new horizons and facilitated the researchers to design different strategies of getting maximum benefit by improving the efficacy of biocontrol agents (Backer et al., 2018). By unrevealing significant antagonistic mechanisms, i.e. production of hydrolytic enzymes and HCN, antibiosis, can be discovered to isolate capable biocontrol agents (Zala *et al*., 1999). In current investigation, finalized antagonistic PGPR revealed multiple mechanisms to suppress isolated funal strains. Bacterial strains were noted positive to produce more than one metabolite and various hydrolytic enzymes production. (Table 2.3). Production of these hydrolytic enzymes by PGPR degrade the cell wall components of phytopathogens by breaking glycosidic linkages between the polysaccharide compounds of fungal cell wall. This mechanism plays a significant role in sustainable plant disease management (Vishnoi et al., 2020).

The antagonistic strains produced siderophores which have been implicated in biocontrol mechanisms for fungal and bacterial plant pathogens. Normally, these siderophores are produced in iron-limited environments that deprive the phytopathogens of available iron by sequestering the available iron from the surroundings and thereby, which ultimately leads to its inhibition. On the other hand, make it available for plant to enhance its growth (Shanmugaiah et al., 2015). Production of HCN by various bacteria act as secondary metabolite. HCN disrupts the cytochrome oxidase that directly alters the ATP synthesis in fungal strains (Olanrewaju et al., 2017). Bacterial strains with cyanide production ability might be considered as comparatively better biocontrol agents as cyanide is reported previously to induce resistance in plants against pathogens (Suryadi et al., 2019).

Antimicrobial substances like chitinase, cellulase, HCN, cellulase, various antibiotics, siderophore production along with nutrients competition play an important role to inhibit fungal growth (Chung et al., 2008; Singh et al., 2008; Hu et al., 2008). The production of the extracellular enzymes by the above reported bacteria supports the results of percentage inhibition of both fungal strains by antagonistic PGPR. The secondary metabolites produced by antagonistic PGPR are also reported to bring about certain physiological changes in entire plants, thereby inducing resistance in them against broad range of pathogens. This phenomenon of indirect suppression of pathogens is termed as induced systemic resistance (ISR) and has been well studied as well as demonstrated in several plants by numerous growth promoting rhizobacteria. Biochemical test kits (QTS-24) were utilized to analyse bacteria that showed positive response towards various compounds. That revealed the potential of these bacteria to utilize different sugars and substrates from surroundings in the rhizosphere while competing for resources in the rhizosphere.

Among all isolated strains, it was noticed that *Bacillus* species are predominant and reported to be more tolerant to different environmental conditions as compared to other genera. The reason might be the formation of extremely resistant dormant forms of endospores by *Bacillus spp.* that withstand the negative ecological circumstances (Nicholson et al., 2000). In stress-affected soils, the dominance of gram-positive bacteria might be explained by their higher resistance to stresses (Naylor et al. 2017).

2.5. Conclusion

The present study results suggest that out of 93 finalized strains most exhibited plant growth promoting activities by producing siderophores, IAA, HCN and catalase, solubilized insoluble phosphorus and 29 out of 93 isolates were capable to suppress the *C. falcatum*. Whereas, 32 isolates inhibit the growth of *F. monilliforme* by persuading a zone around the inoculated bacteria. In the present study, presence of plant growth promoting traits in isolated strains offered an attractive strategy to use these microbial inoculants further for *invitro* pot experiments**.** The PGP activity and the fungicidal effects of bacteria could lead to an effective and long-term strategy to reduce the growth of phytopathogens in comparison to chemical fungicides that exert stronger selective pressure on phytopathogens for better plant growth and induced resistance.

Chapter 3

Antibiotic resistance and multi-stress tolerance of antagonistic PGPR isolated from sugarcane

Introduction

Crops are significantly affected by certain biotic and abiotic stresses. About 20–40% of the reduced crop yield and estimated losses of nearly40 billion dollars worldwide are caused by phytopathogens (Rahman et al., 2018). Considering the rising concern for food security, worldwide food production needs to be increased by 70% till 2050 (Ingram, 2011). Among the alternatives of disease control methods, biocontrol of plant diseases appears to be a good option for the development of eco-friendly, cost-effective, and sustainable approaches (Rahman et al. 2018). The established niche of active rhizospheric microbes, initiate the disease resistance either by direct suppression of pathogens, stimulation of host plant defenses, and/or by outcompeting the nutrient/niche with the plant pathogens (Abdullah et al., 2017). Other mechanisms involve the secretion of toxins, volatile organic compounds, diffusible antibiotics, assembly of cell wall degrading enzymes, such as pectinase, β-1,3-glucanase, chitinase, beta- xylosidase, and 1-aminocyclopropane-1-carboxylate deaminase (*AcdS*) production (Shoda 2000; Tiwari et al., 2017).

A broad range of PGPB has been exploited for the crop health management and their resistance against different plant pathogens. Various genera, such a*s Pseudomonas and Bacillus,* have been extensively studied for their capability to increase resistance against different stress conditions, biocontrol potential, solubilize nutrients, and PGP traits in a range of crop production systems (Rahman et al., 2018; Ferreira et al., 2019). However, a main predicament in the rainfed agro-ecosystems is the predominance of environmental stresses like salinity, drought stress, and high temperature where the endurance of bio-inoculants is a serious concern (Etesami and Beattie 2018). Rhizobial bio-augmentation is needed every year to maintain the process of nodulation in Madhya Pradesh and Chhattisgarh, India, due to decline of free living rhizobial population as high soil temperature prevailed (Kumar et al., 2014). These problems can be overcome by the screening of proficient multi stress tolerant PGPRs for their effective usage both under laboratory and field conditions (Tiwari et al., 2019). The goal of the study is to develop sustainable agriculture by the practical application of multiple stress tolerant beneficial strains to enhance the health and yield of sugarcane and other crops in various ecological regimes.

3.2. Materials and methods

3.2.1. Selection of additional fungal strains

Initially screened bacterial strains showed significant antagonistic results against two fungal strains, *C. falcatum* and *F. moniliforme*. In order to conduct additional evaluation of bacterial strains for their broad antagonistic spectrum, more fungal strains were selected for *in vitro* experiment. Selected fungal strains *F. oxysporum, Rhizoctonia solani, Macrophomina phaseolina,* and *Pythium splendens* were provided by Crop Development and Research Institute (CDRI), NARC, Islamabad (33.6701° N, 73.1261° E), Pakistan.

3.2.2. Intrinsic resistance against different antibiotics

Disk diffusion method was followed to screen the bacterial resistance against various antibiotics (Amna et al., 2019). Strain was inoculated in broth and incubated for 24 h. Freshly grown bacterial cells were swabbed (100 µL) on LB agar plates and the antibiotic discs were placed in the same plates. Plates were then incubated overnight at 37 ºC and the results were recorded by measuring the diameter of the inhibition zone around the antibiotic disks.

3.2.3. Abiotic stress tolerance

The potential of PGP strains isolated from sugarcane rhizosphere to restrict water supply was evaluated in Trypticase Soy Broth amended with the final concentration of PEG-6000 (0%, 10%, 20%, 30%). PEG-6000 was added in the medium to trigger osmotic stress (Marulanda et al., 2009). One percent overnight bacterial cultures grown in TSB were added as initial inoculums. Each concentration was incubated at 28 ºC for 48 h shaking at 120 rpm for 48 h and absorbance (600 nm) was recorded using a spectrophotometer (Agilent 8453 UV-visible Spectroscopy System). All strains were also grown in LB broth amended with different concentrations of NaCl (1 M, 2 M, 3 M). Growth of bacterial strains at different salinity stress levels were noted by recording the optical density (OD) at 600 nm (Ramadoss et al., 2013). At particular stress level, bacterial cells with OD 0.1 were considered as tolerant (Audrain et al. 2015). To analyze the tolerance of bacterial strains at different temperatures ranges, i.e., 30 °C to 50 °C, their growth was monitored for 48 h. The optical density of more than 0.2 (600 nm) at 45 ºC was considered as thermo- tolerant (Ali et al., 2009). Nutrient agar accompanied with different toxicity levels of heavy metals,

such as Cu, Ni, Cr, and Cd with concentration starting from 50 mg/l up to 1000 ppm was used to evaluate the tolerance of all strains against the heavy metals stress Uddin et al., 2019).

3.2.4. Inhibition co-efficient of selected PGPB against different fungi

Fungal growth rates and inhibition co-efficient values were determined for all fungal strains (Cray et al., 2015). Radial dimensions of all fungal colonies on the adjacent side of antagonistic was noted over time and the maximum growth rate of fungus was calculated (A). Rate of the radial extension (%) of fungal pathogens in the control cultures was also expressed (B). The radial dimensions of fungal strains on the side adjacent to the bio-control agent were used to calculate the percentage of the distance between the sites of inoculation of fungus and bio-control agent (C). Measurements of radial extensions were made after the fungus contacted with the bio-control agent to determine the fungal growth rates in the zone of mixed culture (D). This value "D" was also expressed as a percentage of the rate of radial extension of fungus in the control cultures (E).
Inhibition coefficient was quantified by using the equation given below: Inhibition coefficient:
[(100 - B) x 0.4] + [(100 - C) x 0.4] + [(100 - E) x 0.2]

3.3. Results

3.3.1. Intrinsic resistance of bacteria against different antibiotics

All the selected isolates were tested for antibiotic resistance using disk method. Some of them showed resistance whereas some were intermediate, and some were stated susceptible towards antibiotics.

Fifteen antibiotics were used for the test. Bacteria characterized by a hollow zone around the disk were considered susceptible. Ability for antibiotic resistance is shown in Table 1. From the data it is observed that PM19 showed the highest tolerance against antibiotics. It showed resistance against 14 antibiotics while PM14 showed resistance against 12, PM18 and PM12 against 10 antibiotics respectively. Other bacteria showed resistance in descending order as follows: PM20>PM15>PM13>PM17>PM11>PM16 (Table 3.1)

Table 3.1. Intrinsic resistance of antagonistic PGPR against antibiotics

Sr.no	Bacteria name	Antibiotics	Given concentration	Zone of inhibition(mm)	Zone diameter interpretation		
					Resistant	*Intermediate*	*Susceptible*
1.	**PM11**	Kanamycin	30 µg	20	-	-	+
		Erythromycin	15 µg	32	-	-	+
		Gentamycin	10 µg	19	-	-	+
		Chloramphenicol	30 µg	31	-	-	+
		Tetracycline	30 µg	37	-	-	+
		Fosfomycine	50 µg	13	-	+	-
		Ampicillin	10 µg	18	-	-	+
		Spectinomycin	25 µg	0*	+	-	-
		Lincomycin	15 µg	0	+	-	-
		Rifampicin	5 µg	12**	-	+	-
		Penicillin	1 µg	0	+	-	-
		Cephalothin	30 µg	38	-	-	+
		Neomycin	10 µg	13	-	+	-
		Streptomycin	10 µg	0	+	-	-
		Clindamycin	2 µg	10	+	-	-
2.	**PM12**	Kanamycin	30 µg	0	+	-	-
		Erythromycin	15 µg	5	+	-	-
		Gentamycin	10 µg	10	+	-	-
		Chloramphenicol	30 µg	28	-	-	+
		Tetracycline	30 µg	31	-	-	+
		Fosfomycine	50 µg	0	+	-	-
		Ampicillin	10 µg	9	+	-	-

		Spectinomycin	25 µg	0	+	-	-
		Lincomycin	15 µg	13	-	+	-
		Rifampicin	5 µg	12	-	+	-
		Penicillin	1 µg	0	+	-	-
		Cephalothin	30 µg	36	-	-	+
		Neomycin	10 µg	0	+	-	-
		Streptomycin	10 µg	0	+	-	-
		Clindamycin	2 µg	0	+	-	-
3.	**PM13**	Kanamycin	30 µg	0	+	-	-
		Erythromycin	15 µg	6	-	+	-
		Gentamycin	10 µg	0	+	-	-
		Chloramphenicol	30 µg	24	-	-	+
		Tetracycline	30 µg	15	-	+	-
		Fosfomycine	50 µg	0	+	-	-
		Ampicillin	10 µg	0	+	-	-
		Spectinomycin	25 µg	4	+	-	-
		Lincomycin	15 µg	14	-	+	-
		Rifampicin	5 µg	12	-	+	-
		Penicillin	1 µg	0	+	-	-
		Cephalothin	30 µg	5	+	-	-
		Neomycin	10 µg	0	+	-	-
		Streptomycin	10 µg	19	-	-	+
4.	**PM14**	Kanamycin	30 µg	1	+	-	-
		Erythromycin	15 µg	0	+	-	-
		Gentamycin	10 µg	4	+	-	-
		Chloramphenicol	30 µg	0	+	-	-
		Tetracycline	30 µg	22	-	-	+

		Fosfomycine	50 µg	0	+	-	-
		Ampicillin	10 µg	0	+	-	-
		Spectinomycin	25 µg	0	+	-	-
		Lincomycin	15 µg	0	+	-	-
		Rifampicin	5 µg	6	+	-	-
		Penicillin	1 µg	0	+	-	-
		Cephalothin	30 µg	24	-	-	+
		Neomycin	10 µg	15	-	+	-
		Streptomycin	10 µg	0	+	-	-
		Clindamycin	2 µg	3	+	-	-
5.	**PM15**	Kanamycin	30 µg	0	+	-	-
		Erythromycin	15 µg	14	-	+	-
		Gentamycin	10 µg	17	-	-	+
		Chloramphenicol	30 µg	17	-	-	+
		Tetracycline	30 µg	18	-	-	+
		Fosfomycine	50 µg	20	-	-	+
		Ampicillin	10 µg	0	+	-	-
		Spectinomycin	25 µg	0	+	-	-
		Lincomycin	15 µg	0	+	-	-
		Rifampicin	5 µg	6	+	-	-
		Penicillin	1 µg	0	+	-	-
		Cephalothin	30 µg	18	-	-	+
		Neomycin	10 µg	4	+	-	-
		Streptomycin	10 µg	2	+	-	-
		Clindamycin	2 µg	6	+	-	-
6.	**PM16**	Kanamycin	30 µg	19	-	-	+
		Erythromycin	15 µg	23	-	-	+

		Gentamycin	10 µg	15	-	+	-
		Chloramphenicol	30 µg	18	-	-	+
		Tetracycline	30 µg	27	-	-	+
		Fosfomycine	50 µg	0	+	-	-
		Ampicillin	10 µg	3	+	-	-
		Spectinomycin	25 µg	4	+	-	-
		Lincomycin	15 µg	14	-	+	-
		Rifampicin	5 µg	12	-	+	-
		Penicillin	1 µg	2	+	-	-
		Cephalothin	30 µg	23	-	-	+
		Neomycin	10 µg	16	-	-	+
		Streptomycin	10 µg	0	+	-	-
		Clindamycin	2 µg	18	-	-	+
7.	**PM17**	Kanamycin	30 µg	21	-	-	+
		Erythromycin	15 µg	0	+	-	-
		Gentamycin	10 µg	25	-	-	+
		Chloramphenicol	30 µg	32	-	-	+
		Tetracycline	30 µg	27	-	-	+
		Fosfomycine	50 µg	30	-	-	+
		Ampicillin	10 µg	6	+	-	-
		Spectinomycin	25 µg	14	-	+	-
		Lincomycin	15 µg	0	+	-	-
		Rifampicin	5 µg	0	+	-	-
		Penicillin	1 µg	0	+	-	-
		Cephalothin	30 µg	21	-	-	+
		Neomycin	10 µg	11	-	+	-
		Streptomycin	10 µg	2	+	-	-
		Clindamycin	2 µg	33	-	-	+

8.	**PM18**	Kanamycin	30 µg	23	-	-	+
		Erythromycin	15 µg	0	+	-	-
		Gentamycin	10 µg	19	-	-	+
		Chloramphenicol	30 µg	0	+	-	-
		Tetracycline	30 µg	17	-	-	+
		Fosfomycine	50 µg	0	+	-	-
		Ampicillin	10 µg	0	+	-	-
		Spectinomycin	25 µg	0	+	-	-
		Lincomycin	15 µg	0	+	-	-
		Rifampicin	5 µg	0	+	-	-
		Penicillin	1 µg	0	+	-	-
		Cephalothin	30 µg	0	+	-	-
		Neomycin	10 µg	15	-	+	-
		Streptomycin	10 µg	11	-	+	-
		Clindamycin	2 µg	0	+	-	-
9.	**PM19**	Kanamycin	30 µg	13	-	+	-
		Erythromycin	15 µg	0	+	-	-
		Gentamycin	10 µg	1	+	-	-
		Chloramphenicol	30 µg	9	+	-	-
		Tetracycline	30 µg	0	+	-	-
		Fosfomycine	50 µg	0	+	-	-
		Ampicillin	10 µg	0	+	-	-
		Spectinomycin	25 µg	3	+	-	-
		Lincomycin	15 µg	0	+	-	-
		Rifampicin	5 µg	8	+	-	-
		Penicillin	1 µg	0	+	-	-
		Cephalothin	30 µg	0	+	-	-

		Neomycin	10 µg	0	+	-	-
		Streptomycin	10 µg	0	+	-	-
		Clindamycin	2 µg	0	+	-	-
10.	**PM20**	Kanamycin	30 µg	20	-	-	+
		Erythromycin	15 µg	0	+	-	-
		Gentamycin	10 µg	20	-	-	+
		Chloramphenicol	30 µg	8	+	-	-
		Tetracycline	30 µg	28	-	-	+
		Fosfomycine	50 µg	32	-	-	+
		Ampicillin	10 µg	0	+	-	-
		Spectinomycin	25 µg	0	+	-	-
		Lincomycin	15 µg	0	+	-	-
		Rifampicin	5 µg	7	+	-	-
		Penicillin	1 µg	0	+	-	-
		Cephalothin	30 µg	0	+	-	-
		Neomycin	10 µg	13	-	+	-
		Streptomycin	10 µg	19	-	+	-
		Clindamycin	2 µg	0	+	-	-

(+): positive; (-): negative

3.3.2. Tolerance of selected bacteria against abiotic stresses

3.3.2.1. Drought tolerance

Bacterial strains PM11, PM12, PM13, PM14, PM15, PM16, PM17 and PM19 were found prolific in growth at all concentrations of PEG-6000 after 48 h of incubation, indicating their osmo-adaptive ability. The highest growth rate was observed in PM12, PM13, PM14, PM15 even at highest applied drought stress (30% PEG-6000) as compared to others. However, a decline in growth with the gradual increase in stress was observed.

3.3.2.2. Salinity tolerance

Bacteria, PM12, PM13, PM15, and PM19, were quite resistant to salinity stress while a gradual decline in growth was observed when the salt stress was increased up to 2 M and 3M NaCl. PM12, PM13, PM15, PM19 showed bacterial growth of 0.5227, 0.53, 0.67 and 0.508, respectively, at 3M NaCl concentration that revealed the tolerance of these strains for salinity stress.

3.3.2.3. Resistance against high temperature

All bacteria exhibited normal growth on LB agar plates at 30 ^{o}C after 24-36 h of incubation; however, PM18, PM19 and PM20 showed better growth after 48 h. Growth of bacteria was observed at different temperature regimes to analyze their maximum tolerance level for high temperatures. Bacterial strain PM11 and PM17 showed highest tolerance to temperature up to 60^{o}C. PM13 and PM14 can grow up to 50^{o}C. PM12, PM15 and PM16 at 45 ^{o}C and PM 18 at 40 ^{o}C, respectively. Bacterial strains PM19 and PM20 showed the lowest tolerance level for high temperature and showed proper growth only up to 35 ^{o}C.

3.3.2.4. Heavy metal tolerance

Bacteria PM13 and PM14 showed growth and tolerance up to 1000 mg/L concentration for Cr and Cd while these bacteria could tolerate 150 mg/L of Ni and 100 mg/L of Cu, respectively. However, the lowest tolerance for heavy metals was showed by PM18 and PM20 (Table 3.2). Most of the bacteria showed resistance against chromium and cadmium while bacteria were less tolerant to copper and nickel.

Table 3.2. Bacterial tolerance against abiotic stresses

Bacterial strains	Drought stress (PEG-600) (%)	Salinity Stress (M)	High temperature stress (C^{o})	Heavy metal stress (ppm)
PM11	30%	1 M	60	Cr 1000, Cd 700 Cu 50, Ni 150
PM12	30%	3 M	45	Cr 700, Cd 500 Cu 50, Ni 150
PM13	30%	3 M	50	Cr 1000, Cd 1000 Cu 100, 100, Ni 150
PM14	30%	2 M	50	Cr 1000, Cd 1000 Cu 100, Ni 150
PM15	30%	3 M	45	Cr 700, Cd 1000 Cu 0, Ni 150,
PM16	30%	1 M	45	Cr 500, Cd 500 Cu 100, Ni 150,
PM17	30%	0 M	60	Cr 700, Cd 500 Cu 50, Ni 150
PM18	10%	0 M	40	Cr 700, Cd 500 Cu 0, Ni 0
PM19	20%	3 M	40	Cr 50, Cd 50 Cu 100, Ni 150,
PM20	20%	0 M	35	Cr 150, Cd 150 Cu 0, Ni 0,

PEG: Polyethylene glycol; M: Molar; ppm: parts per million; Cr: Chromium; Cd: Cadmium; Cr: Chromium; Ni: Nickel.

3.3.3. Inhibition co-efficient of antagonistic PGPR against different fungi

Inhibition co-efficient of antagonistic PGPR was plotted against the subsequent decrease in area of fungal colonies (15-days of incubation period). Based on various observations and calculations, highest inhibition coefficient response was showed by *B. siamensis, B. tequilensis, B. gibsonii, B. xiamenensis, respectively,* as shown in Table 3.3-3.9. All bacterial strains were competent to sustain a zone of inhibition for all fungi while for some fungi restricted growth was observed in the form of diminutive colony. Moreover, weed-like behavior and traits were observed for *Bacillus gibsonii* PM11*, Bravibacterium frigoritolerans* PM12*, Bacillus siamensis* PM13*, Bacillus xiamenesis* PM14*, Bacillus sp.* PM15*, B. tequilensis* PM16, and *Bacillus sp.* PM17.

Table 3.3. Inhibition coefficient of fungal strains against *B. gibsonii* PM11 (values are mean±SE).

Plant pathogens	Fungal growth rate (mm day-1); [A]	Growth-rate A as a percentage of control; [B]	Growth of fungal as a percentage of the distance between sites of inoculation; [C]	Time until contact between Fungal and biocontrol agent (days)	Fungal growth rate in zone of mixed culture (mm day_1); [D]	Growth-rate D as a percentage of control; [E]	Inhibition Coefficient
P. splendens	0.775 ±0.052c	27.28 ±0.877d	38. 61 ±0.796d	6.33 ±0.333a	0	0	73.64 ±0.851a
R. solani	0.122 ±0.011d	4.24 ±0.277e	6 ±0.50e	2.33 ±0.333c	0	0	95.9 ±0.91a
F. monilliforme	1.17 ±0.098a	41.34 ±0.744a	58.50 ±0.456a	0 ±0d	0	0	60. 66 ±0.556d
F. oxysporum	1.08 ±0.06ab	38.4 ±0.808b	54.35 ±0.779b	4 ±0.577b	0	0	62.9 ±0.692d
C. falcatum	0.892 ±0.09bc	31.54 ±0.392c	44.64 ±0.825c	6 ±0.577a	0	0	69.52 ±0.879d
M. phaseolina	0.937 ±0.034bc	33.14 ±0.65c	46.84 ±1.06c	5.33 ±0.333a	0	0	67. 99 ±0.749c

All the means sharing common letter (s) are insignificantly different at $p < 0.05$ level by LSD

Table 3.4. Inhibition coefficient of fungal strains against *Bravibacterium frigoritolerans* PM12 (values are mean±SE).

Plant pathogens	Fungal growth rate (mm day-1); [A]	Growth-rate A as a percentage of control; [B]	Growth of fungal as a percentage of the distance between sites of inoculation; [C]	Time until contact between Fungal and biocontrol agent (days)	Fungal growth rate in zone of mixed culture (mm day_1); [D]	Growth-rate D as a percentage of control; [E]	Inhibition Coefficient
P. splendens	0.587 ±0.040c	20.75 ±0.617d	29.37 ±0.583d	2.33 ±0.333c	0	0	79.95 ±0.688a
R. solani	0.771 ±0.047c	32.43 ±0.825c	38.59 ±0.917c	6 ±0.577a	0	0	71.59 ±0.919b
F. monilliforme	1.17 ±0.135b	41.34 ±0.727b	58.5 ±0.866b	6.66 ±0.333a	0	0	60.06 ±0.516d
F. oxysporum	1.15 ±0.127b	41.02 ±0.588b	57.5 ±0.617b	4.33 ±0.333b	0	0	59.57 ±0.54d
C. falcatum	0.8051 ±0.06c	40.63 ±0.87b	40.25 ±0.73c	6.33 ±0.333a	0	0	67.64 ±0.662c
M. phaseolina	1.825 ±0.137a	64.48 ±0.733a	91.25 ±0.646a	0 ±0d	0	0	37.70 ±0.47e

All the means sharing common letter (s) are insignificantly different at $p < 0.05$ level by LSD

Table 3.5. Inhibition coefficient of fungal strains against *B. siamensis* PM13 (values are mean±SE)

Plant pathogens	Fungal growth rate (mm day-1); [A]	Growth-rate A as a percentage of control; [B]	Growth of fungal as a percentage of the distance between sites of inoculation; [C]	Time until contact between Fungal and biocontrol agent (days)	Fungal growth rate in zone of mixed culture (mm day_1); [D]	Growth-rate D as a percentage of control; [E]	Inhibition Coefficient
P. splendens	0.517±0.01d	1.7±0.118e	2.5±0.23e	1±0c	0	0	98.32±0.704a
R. solani	0.84±0.04c	29.32±0.704d	41.4±0.721d	1.33±0.33c	0	0	71.69±0.86b
F. monilliforme	1.33±0.19ab	46.990±0.97b	66.5±0.49bc	0±0d	0	0	54.60±0.871d
F. oxysporum	1.33±0.132ab	46.990±0.64b	66.167±0.34b	1.66±0.33c	0	0	54.60±0.75d
C. falcatum	1.066±0.07bc	37.66±1.09c	53.3±1.66c	5.33±0.33a	0	0	63.61±0.58c
M. phaseolina	1.46±0.10a	51.59±0.86a	73±0.57a	4.33±0.33b	0	0	50.16±0.55e

All the means sharing common letter (s) are insignificantly different at $p < 0.05$ level by LSD

Table 3.6. Inhibition coefficient of fungal strains against *B. xiamenensis* PM14 (values are mean±SE).

Plant pathogens	Fungal growth rate (mm day-1); [A]	Growth-rate A as a percentage of control; [B]	Growth of fungal as a percentage of the distance between sites of inoculation; [C]	Time until contact between Fungal and biocontrol agent (days)	Fungal growth rate in zone of mixed culture (mm day_1); [D]	Growth-rate D as a percentage of control; [E]	Inhibition Coefficient
P. splendens	0.66±0.05cd	23.046±1.78bc	33±2.59d	2.33±0bc	0	0	77.58±1.62ab
R. solani	0.83±0.03d	28.99±1.21c	41.6±1.73cd	3±0.33c	0	0	71.62±1.17a
F. monilliforme	1±0.11bc	34.12±3.94b	50±5.77bc	3.33±0.33b	0	0	69.70±5.13ab
F. oxysporum	1.066±0.06b	34.37±0.25b	53.33±0.25b	3±0bc	0	0	68.24±1.89b
C. falcatum	0.96±0.03bc	32.90±1.09b	48.33±1.66bc	4.66±0.33a	0	0	67.50±1.10b
M. phaseolina	1.46±0.08a	50.05±3.01a	73.33±4.40a	4.67±0.33a	0	0	53.81±2.14c

All the means sharing common letter (s) are insignificantly different at $p < 0.05$ level by LSD

Table 3.7. Inhibition coefficient of fungal strains against *Bacillus sp.* PM15 (values are mean±SE).

Plant pathogens	Fungal growth rate (mm day-1); [A]	Growth-rate A as a percentage of control; [B]	Growth of fungal as a percentage of the distance between sites of inoculation; [C]	Time until contact between Fungal and biocontrol agent (days)	Fungal growth rate in zone of mixed culture (mm day_1); [D]	Growth-rate D as a percentage of control; [E]	Inhibition Coefficient
P. splendens	0.759 ±0.513c	26.81 ±0.756e	37.95±1.01d	4.66 ±0.333b	0	0	74.09 ±0.65a
R. solani	0.705 ±0.059c	29.92 ±1.18d	35.27±0.67e	2.66 ±0.333c	0	0	73.92 ±0.586a
F. monilliforme	1.23 ±0.132b	43.46 ±0.842b	61.54±0.612b	6.66 ±0.333a	0	0	58. 01 ±0.58c
F. oxysporum	1.08 ±0.08b	38.16 ±0.496c	54±0.323c	4.33 ±0.333b	0	0	62.95 ±0.627b
C. falcatum	0.7260 ±0.022c	25.65 0.571e	36.3±0.346de	6.66 ±0.333a	0	0	75.22 ±0.606a
M. phaseolina	1.6391 ±0.079a	59.24 ±1.28a	84.57±0.381a	7.33 ±0.333a	0	0	41.03 ±0.845d

All the means sharing common letter (s) are insignificantly different at $p < 0.05$ level by LSD

Table 3.8. Inhibition coefficient of fungal strains against *Bacillus sp.* PM16 (values are mean±SE).

Plant pathogens	Fungal growth rate (mm day-1); [A]	Growth-rate A as a percentage of control; [B]	Growth of fungal as a percentage of the distance between sites of inoculation; [C]	Time until contact between Fungal and biocontrol agent (days)	Fungal growth rate in zone of mixed culture (mm day_1); [D]	Growth-rate D as a percentage of control; [E]	Inhibition Coefficient
P. splendens	0.785 ±0.076b	22.75 ±0.819d	39.27 ±0.65c	4.33 ±0.333b	0	0	73.19 ±0.68b
R. solani	0.006 ±0.0001c	0.212 ±0.008e	0.3 ±0.017d	1.33 ±0.333c	0	0	99.79 ±0.72a
F. monilliforme	0.70 ±0.051b	27.91 ±0.773c	39.5 ±0.617c	3.66 ±0.333b	0	0	72.7 ±0.431b
F. oxysporum	1.11 ±0.086a	39.55 ±1.25a	55.95 ±1.05a	3.33 ±0.333b	0	0	61.8 ±0.5d
C. falcatum	1.02 ±0.074ab	36.29 ±0.54b	51.35 ±0.77b	6.33 ±0.333a	0	0	64.94 ±0.54c
M. phaseolina	1.15 ±0.14a	40.74 ±0.65a	57.5 ±0.98a	3.66 ±0.333b	0	n 0	60.70 ±0.48d

All the means sharing common letter (s) are insignificantly different at $p < 0.05$ level by LSD

Table 3.9. Inhibition coefficient of fungal strains against B. *tequilensis* PM17 (values are mean±SE).

Plant pathogens	Fungal growth rate (mm day-1); [A]	Growth-rate A as a percentage of control; [B]	Growth of fungal as a percentage of the distance between sites of inoculation; [C]	Time until contact between Fungal and biocontrol agent (days)	Fungal growth rate in zone of mixed culture (mm day_1); [D]	Growth-rate D as a percentage of control; [E]	Inhibition Coefficient
P. splendens	0.719 ±0.051c	25.4 ±0.923e	35.95 ±0.721e	2.33 ±0.333d	0	0	75.46 ±0.66a
R. solani	1.07 ±0.045b	38.12 ±0.65c	53.95 ±0.95c	3.66 ±0.333bc	0	0	63.16 ±0.727c
F. monilliforme	1.18 ±0.086b	41.69 ±0.917b	59 ±0.981b	4.66 ±0.333b	0	0	59.72±0.648d
F. oxysporum	0.97 ±0.080bc	34.27 ±0.750d	48.5 ±0.69d	3.33 ±0.333cd	0	0	66.89 ±0.919b
C. falcatum	1.06 ±0.060b	37.63 ±1.056c	53.25 ±0.202c	6.33 ±0.333a	0	0	63.64 ±0.431c
M. phaseolina	1.91 ±0.174a	67.49 ±0.975a	95.6 ±1.44a	4.33 ±0.333bc	0	0	34.63 ±0.901e

All the means sharing common letter (s) are insignificantly different at $p < 0.05$ level by LSD

3.4. Discussion

In the rhizosphere, the competing environment among native microflora may limit the introduction of new strains. One way of reducing the competition might be the utilization of antibiotic resistance by PGPR used as inoculum. In our study, *Bacillus* strains (PM11, PM13, PM14, PM15, PM16, PM17, PM18, PM19, PM20) and *Bravibacterium frigoritolerans* showed resistance against 15 antibiotics. As already reported, the antibiotic resistance provides the benefit to certain bacteria to form niche and enhance the colonization of bacteria with plants as compared to the other (Beneduzi et al. 2012). Proposed mechanism of antibiotic resistance is the presence of antibiotic resistance genes (ARGs). These genes are either present on plasmids or on other chromosomes. Antibiotic resistance is due to the presence of multi-functional proteins which may also involve in other functions like efflux transporters of other molecules or metals. This resistance is required by the bacteria used as inoculants in green house experiments or field as in soil, antibiotics produced by other microbes may limit their growth (Ramakrishna et al., 2019). By employing the intrinsic resistance mechanism these microbes maintain their growth and survival in competing environments as noticed in current study. Antibiotic producing *Pseudomonas putida* M17 also had an advantage to significantly enhance the nodulation of beans in greenhouse and field trials reported previously (Pérez-Montaño et al., 2014).

In the current experiment, isolated PGPR showed significant potential against drought stress as all strains resist up to 30% PEG-6000 except PM18, PM19 and PM20 that showed resistance against drought stress up to 10% and 20%, respectively. To endure drought stress, soil bacteria employ various physiological mechanisms like accumulation of compatible solutes, exopolysaccharide (EPS) production as observed during the earlier screening of microbes (Table 2.1), and the production of spores by the bacteria belongs to the genera *Bacillus* (Table 2.5) (Sayyed et al., 2019).

Analysis of bacterial strains reveled that PM12, PM13, PM15, PM19 could survive salinity stress up to highest concentration applied (3M), PM14 (2M), PM16 and PM11 (1M), while bacteria PM17, PM18 and PM20 showed zero tolerance to salt stress. It was noticed that salt tolerant bacteria overcome the salinity stress by accumulation of compatible solutes for osmoregulation, ACC deaminase, EPS and extracellular proteases production (Table 2.3). Moreover, the role of activated Na^+/H^+ antiporters in bacteria is also reported previously (Ngumbi and Klopper, 2016). PM11 and PM17 tolerate 60 ^oC while PM13 and PM14 can survive 50^oC. Though the mechanism

of heat tolerance >60ºC is partially known however, variation of temperature tolerance in bacterial strain might be due to the presence of proteins with abundance of disulfides and presence of covalent bonds between sulfur atoms that enhance membrane stability and heat-tolerance in bacteria. Heavy metal tolerance of bacteria against the different concentration of Cd, Cr, Cu and Ni was noticed. Maximum tolerance was shown by *B. xiamenensis and B. siamensis*. Along with EPS and ACC deaminase production these microorganisms may possess various mechanisms to avoid heavy metal stress that includes biosorption and bioaccumulation of heavy metals to the cell walls, transport of metals across the cytoplasmic membrane, metal detoxification via oxidation–reduction, entrapment and accumulation of heavy metals (Tiwari and Lata, 2018).

Our study on the antifungal activity assays revealed that the bacterial strains PM11-PM 17 had a broad antifungal spectrum, which can inhibit the growth of *C. falcatum*, *F. monilliforme, P. splendens, R. solani, F. oxysporum* and *M. phaesolina,* (Table 3.3-Table 3.9). The production of various extracellular antimicrobial compounds as diffusible and/or volatile molecules may act on phytopathogenic fungi by exerting fungicidal effects, such as the inhibition of germination or the lysis of fungal mycelia (Haidar et al., 2016). Furthermore, these strains were identified as dominant among various fungal species by showing vigorous and competitive behavior and broad antibiotic resistant spectrum, which might help it to be dominant in the respective communities in open microbial habitats (Cray et al., 2013). The calculated values for the inhibition-co-efficient derived during current research are consistent with the previous literature that documented the inhibitory activity of *B. subtilis* and *Pantoea* strains against diverse fungal species and considerable reductions in their growth rates and metabolisms (Wharton and Kirk 2014; Cray et al., 2016).

This study with the evaluation of antagonistic activity and abiotic stress tolerance of above-mentioned strains confirmed that these bacteria can be effectively deployed in extreme and fluctuating environments.

3.5. Conclusion

Alleviating ecological problems using eco-friendly approach is of vital importance. From this study it was noted that selected strains showed significant tolerance against 15 different antibiotics. This antibiotic profiling will help in the selection of markers that can be used in the reisolation of original microbial inoculants applied for *invitro* experiments/ greenhouse studies. Based on multi stress

tolerance against high salinity stress, drought, heavy metal toxicity and high temperature along with strong antagonistic potential against fungal strains provide a base for further evaluation of these bacteria at pilot or field scale.

Chapter. 4

Biologically fabricated silver nanoparticles and their characterization

4.1. Introduction

From few years, nanotechnology has been prominent because of its broad range applicablity in food, agriculture, medicine and electronics. It is noted that molecules and materials in their nano form have various physical, chemical and biological characteristics (Jeevanandam et al., 2018). Among different nanoparticles, silver nanoparticles (AgNPs) have received the most attention because of their prominent effects and biological activities. It has been reported that some pathogenic microbes rapidly developed resistance to various antibiotics; but failed to do this against AgNPs because targeted microbes need to develop resistance and advance mechanisms to defend them from these silver ions. Various methods are available to produce nano particles. Among these, chemical processes include sol gel, andcondensation and chemical reduction, whereas physical processes involve flame pyrolysis, laser ablation, flame pyrolysis and ultrasonication (Bratovcic, 2020). However, these processes are not preferable due to non-biodegradability, toxicity and high cost (Song al., 2009). Alternative to physical and chemical methods is green synthesis of nanoparticles using microbes and plants. Biological methods employ the principle of ecofriendly reduction of metals to nanoparticles. Green synthesis is simple and low-cost method with short production time (Mie et al., 2014). Microbes and plants have various bioactive compounds. These bioactive compounds act as bio-capping agents that help in the reduction of metals to nanoparticles. Comparatively, AgNPs synthesis form plant extracts are adventitious than microbial based production because of its faster reduction rate and reduced biosafety issues. Furthermore, plant sources for nanoparticle production are nontoxic, relatively low-cost and apposite for scale up *in vitro* a well as *in vivo* activities. Moreover, plant extracts have an additional advantage of stabilized nanoparticles as plant biomolecules play dual role of reduction and capping of biosynthesized nanoparticles (Kuppusamy et al., 2014). AgNPs inhibit bacterial growth by intercalating with DNA, enzymes, proteins and cell wall of pathogens that gradually damage the metabolic pathways by induction of reactive oxygen species responsible for antibacterial effects of AgNPs.

Usage of silver nanoparticles as disinfectants/antimicrobial agents has become more known in previous years because of their economical production through biotechnology advances. Management of plant diseases is one of the potential applications of nanoparticles. Due to multiple inhibitory actions, AgNPs can control plant pathogens in an environment friendly way as compared to synthetic pesticides or fungicides. Until recently there have been limited evidences reporting the applicability of AgNPs to control plant diseases. Efficacy of disease suppression by silver nanoparticles can be increased by cumulative information about their antimicrobial activities to

various pathogens and development of better application strategies. Plant-mediated biological synthesis of nanoparticles is drawing attention now-a-days due to its ecofriendly and simple process. Agricultural sector is facing huge economic losses due to overabundance of threats provoked by various phytopathogens. Thus, innovative technologies have been introduced to minimize these losses in modern agriculture. Among such technical and scientific advances, nanotechnology is under debate due to its exclusive physical, chemical and biological properties and application in agriculture. Production and applicabilty of biologically produced AgNPs result in the opening of novel ways in agronomic research due to consistent, ecological, non-toxic, broad antimicrobial range and effective means of suppressing plant diseases. Now a day's interest has been paved to utilize the potential of nanoparticles to manage plant diseases. Previous reports have promoted the toxicity and inhibitory effects of AgNPs on distortion of fungal hyphae and its conidial development for many phytopathogens. This provides a considerable base to explore potential of AgNPs in controlling fungal pathogens under *in vitro* circumstances. Moreover, by keeping in mind the significant potential of agricultural important bacteria and plant extracts and their use to synthesize AgNPs with robust antifungal attributes can offer an alternative way to protect plants against phytopathogens. Consequently, the main aim of current research was to use plant growth promoting rhizobacteria (PGPR), *F. oxysporum* and sugarcane husk to synthesize silver nanoparticles and assess their disease control potential under *in vitro* conditions. In this regard, consortium of newly isolated strains of *Bacillus siamensis* (PM13), *Bacillus xiamenensis* (PM14), and *Bacillus sp*. (PM15) from sugarcane rhizosphere, consortium of previously isolated *F. oxysporum* from agricultural soil and aqueous solution of sugarcane husk that showed potential to synthesize silver nanoparticles with strong antimicrobial potential against pathogens of sugarcane *Colletotricum falcatum* (red rot) and *Fusarium moniliforme* (pokha boeng) were explored in the present study with an overall objective to analyse the competence of biologically produced AgNPs in suppression of red rot infection in sugarcane.

4.2. Material and methods

4.2.1. Chemicals and plant material

Analytical grade chemicals and reagents were used as received without any modification. Fresh plant material (*Saccharum officinarum*) was taken from sugarcane growing area of Faisalabad (31.4504° N, 73.1350° E), Pakistan, for experimentation

4.2.2. Source of microbes for nanoparticle production

Previously isolated bacterial strains *Bacillus siamensis* PM13, *Bacillus xiamenensis* PM14, and *Bacillus sp.* PM15 with accession numbers (MN240927), (MK358984) and (MN241465), respectively, were selected for the preparation of bacterial consortium. *Fusarium oxysporum* was provided by Crop Development and Research Institute (CDRI), NARC, Islamabad, Pakistan.

4.2.3. Preparations of plant extract and microbe supernatant

Fresh husk of sugarcane plant was thoroughly washed with deionized water to remove dust and adherents. Sample was then finely ground. Five g of sample was soaked in 100 mL distilled water. Sample was placed in the dark for 3 days. Solution was separated by decantation and allowed to settle. Further, Whatman No. 1 filter paper was used for filtration. Sugarcane husk extract as aqueous solution was stored at 0–4 °C till further use (Alves et al., 2020). *F. oxysporum* was grown in potato dextrose broth. Inoculated media was kept in the incubator shaker at 180 rpm at 25°C for 7 days. After incubation, samples were centrifuged at 6000 rpm and supernatant was separated (Marikani et al., 2016). Consortia of three PGP bacterial strains *B. siamensis*, *B. xiamenensis*, and *Bacillus sp.* was prepared by inoculation in LB broth (Iravani et al., 2014).

4.2.4. Silver nanoparticles synthesis

Aqueous solution and supernatant attained after the removal of husk, fungal suspension and bacterial cells from their respective solutions were separately mixed with of silver nitrate (1mM $AgNO_3$) solution (100 mL). Solutions were then incubated in a shaker at 180 rpm in dark conditions at 25°C. Positive control of all samples with only deionized water were also maintained under same conditions. Mixture in the flasks were placed in an oven at 100°C for overnight to remove the water content. Remaining residue was further placed for calcination in furnace for 5h at 500°C. After calcination the nanoparticles were obtained in powder form. Calcinated powder of nanoparticles was grounded into very fine particles by using pastel and mortar (Sabir et al., 2014).

4.2.5. Characterization of biologically synthesized nanoparticles

Characterization of bio-fabricated nanoparticles was done in Nanoscience and Catalysis Division, National Centre for Physics, Islamabad, Pakistan. The UV- diffused reflectance spectroscopy (UV-DRS) spectra of sample solutions were recorded from 200 to 1000 nm (DRS-Perkin Elmer UV/Vis/NIR Spectrophotometer Lambda 950). Deionized water was used as the blank. To check the purity and phase formation of the samples, X-ray powder diffraction (XRD) technique was used to analyze XRD patterns (X-Diffraction model: D8 ADVANCE BRUKER X-Source Copper/(anode) (Mehta et al., 2017). For functional group analysis, Fourier transform infrared spectroscopy (FTIR) (FT/IR-6600, Jasco) was done for all samples in the Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan (Jayappa et al., 2020). AgNPs were also observed under scanning electron microscopy (SEM) (JSM 6409A, JEOL, Japan) (Jayappa et al., 2020).

4.2.6. *In vitro* anti-microbial activity

Silver produced from sugarcane husk, fungal and bacterial supernatant were examined for their potent antifungal activity against sugarcane pathogenic fungus *C. falcatum* (red rot) and *F. moniliforme* (pokha boeng). *C. falcatum* was cultured in potato dextrose agar (PDA) medium while Malt Extract Agar (MEA) to determine the antifungal activity of biosynthesized AgNPs, well diffusion method was used. Different concentrations of nanoparticles (5mg, 10mg, and 15 mg) were prepared and loaded in respected wells of PDA medium. An empty well was maintained as control. After inoculation of fungal strain and loading of nanoparticles, plates were incubated for 7 days at 28ºC. Zone of inhibition was measured at time interval of of 24 hrs (Krishnaraj et al., 2012).

4.3. Results

4.3.1. Properties of bio-fabricated silver nanoparticles

4.3.1.1. Visual monitoring

In the present investigation, three different samples were taken one from aqueous filtrate of sugarcane husk, second from fungal supernatant of *F. oxysporum* and third from bacterial supernatant for steady production of silver nanoparticles. To verify their synthesis, flasks containing just filtrate of respective samples were taken for further production. Filtrate without amendment of silver nitrate solution was taken as positive control while only silver nitrate solution without any addition of

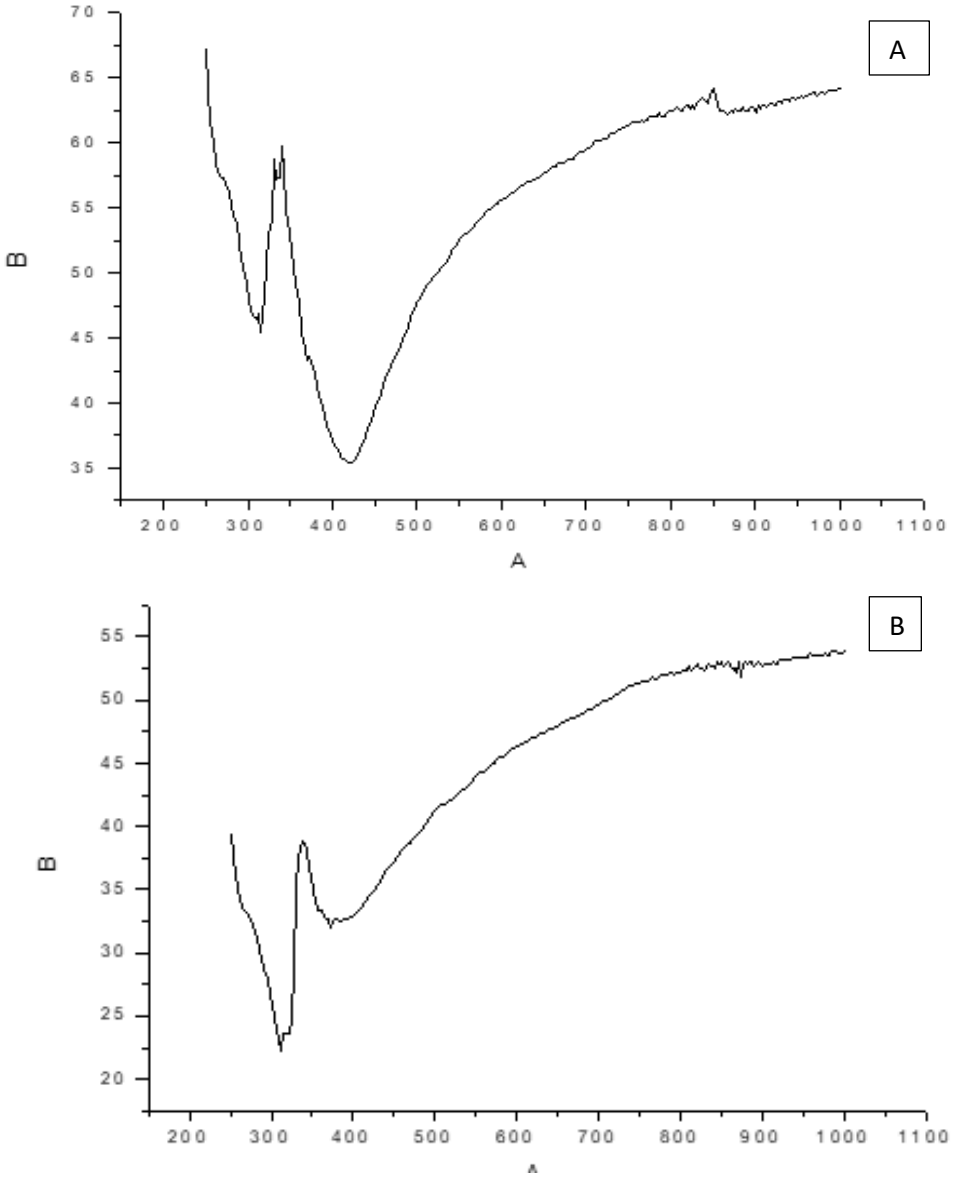
aqueous filtrate of samples were considered as negative control. For all the set samples, flasks were monitored visually.

Figure 4.1. Flasks containing bacterial consortium supernatant, fungal supernatant, sugarcane husk extract and distilled water without $AgNO_3$ solution (A). Flasks containing bacterial consortium supernatant, fungal supernatant, sugarcane husk extract and distilled water with $AgNO_3$ solution (B).

Silver nitrate solution without any amendment (negative control), showed no color change when treated with distilled water. However, when silver nitrate was treated with three different samples of aqueous filtrates and supernatant of sugarcane bacteria and fungus, respectively, color of the solution changed to dark brown after 3 days due to the deposition of AgNPs (Fig 4.1).

4.3.1.2. Diffused reflectance spectroscopy analysis of nanoparticles

The optical properties of biologically produced silver nanoparticles were characterized by DRS as shown in Fig. 4.2 (A-C). Primary peak of surface plasmon resonance band was noticed around 339.782 nm, 336. 735 nm and 338.258 nm for bacterial, fungal and sugarcane husk- produced AgNPs, respectively, which indicates the cluster formation and their dendritic nature. Peak positions and their sharp intensity of the absorption spectrum in all three samples revealed the presence of Ag-nanodendrites. Moreover, the absorption curve proposes that bio-fabricated nanoparticles were spherical in shape and well dispersed.



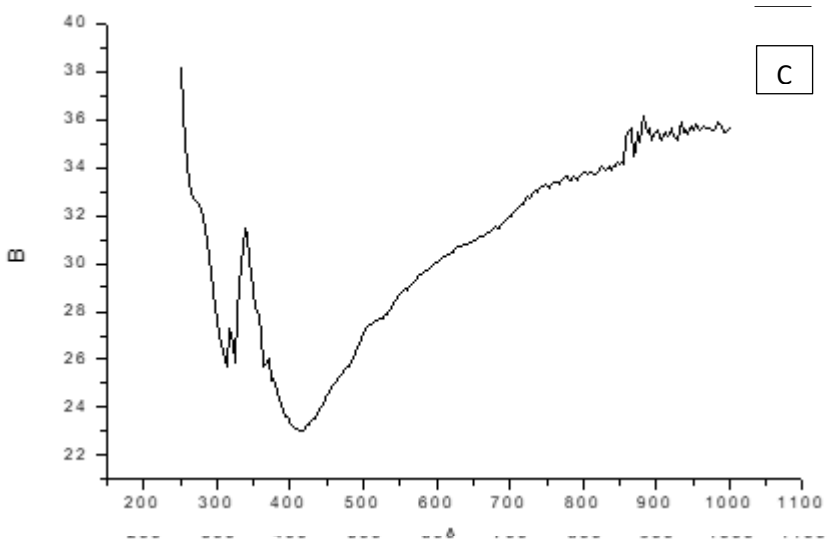
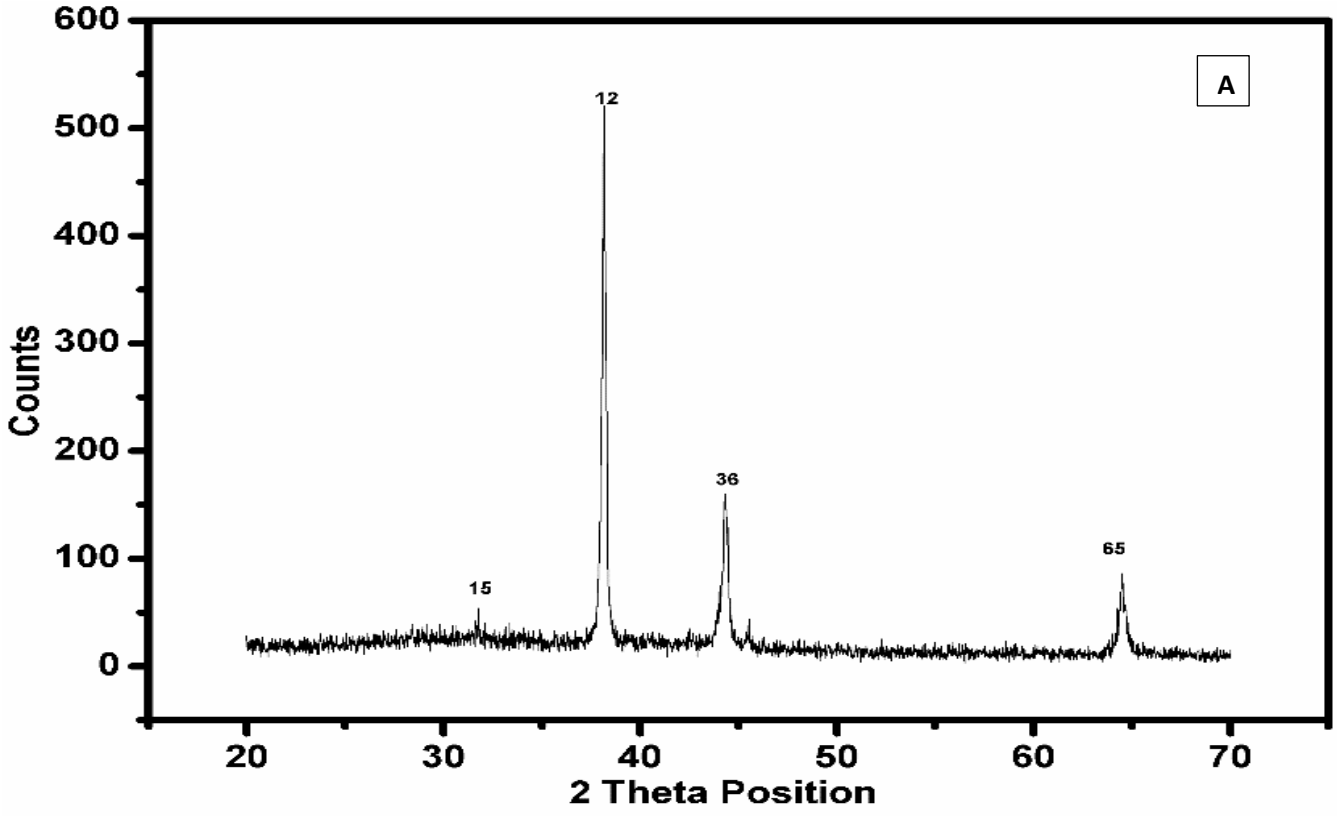


Figure 4.2. UV-DRS spectrum of silver nanoparticles formed after incubation of the aqueous filtrate of bacterial consortia (A), *F. oxysporum* (B), Sugarcane husk (C) treated with 1 mM silver nitrate solution and calcinated.

4.3.1.3. X-ray diffraction analysis

Pattern of X-ray diffraction revealed the crystalline structure of AgNPs produced by bacterial supernatant (Fig. 4.3A), Fungal supernatant (Fig 4.3B) and sugarcane husk extract (Fig 4.3C). It was indicated that structures of all biologically produced nanoparticles were crystalline cubic with nano size of 45.41 nm (JCPDS code: 00-003-0921), 49.06 nm (JCPDS code: 00-004-783), 42.75 nm (JCPDS code: 00-003-0921) for bacterial, fungal and sugarcane husk-based nanoparticles as calculated by Debye-Scherrer equation using XRD. For bacterial nanoparticles, the presence of peaks at 2theta values of 31.726, 38.163, 45.462 and 66.319 correspond to (1 5), (1 2), (3 8) and (6 5) planes of silver, respectively. For fungal nanoparticles, the presence of peaks at 2theta values of 66.278, 64.464, 56.503, 45.475, 44.365, 42.938, 38.163, 34.310 correspond to (8 3), (4 1), (4 6), (4 4), (4 3), (5 7), (2 9) and (6 5) planes of silver, respectively. For sugarcane husk nano particles, the presence of peaks at 2theta values of 32.287, 38.140, 44.139 and 64.467 correspond to (1 7), (8 4), (43) and (3 2) planes of silver, respectively. The XRD spectrum confirms
the presence of nanoparticles.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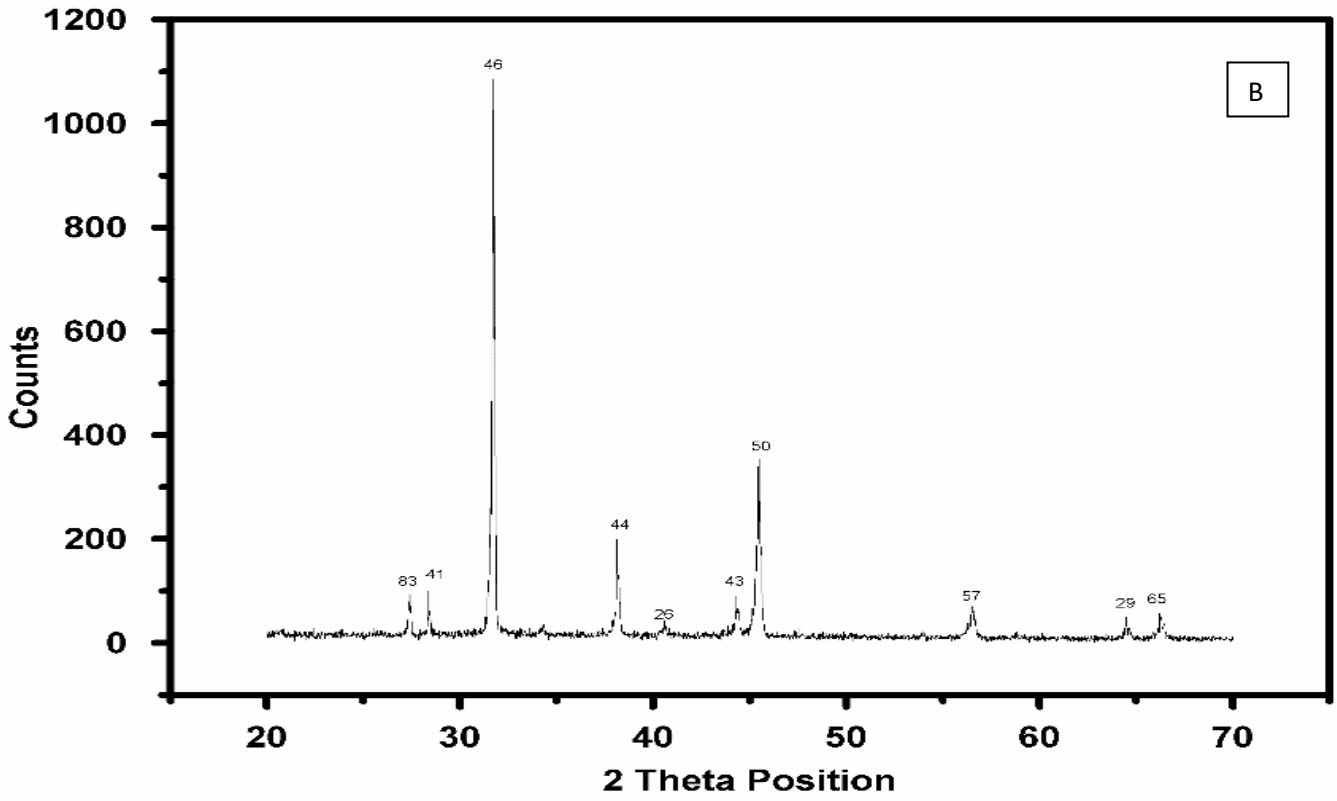
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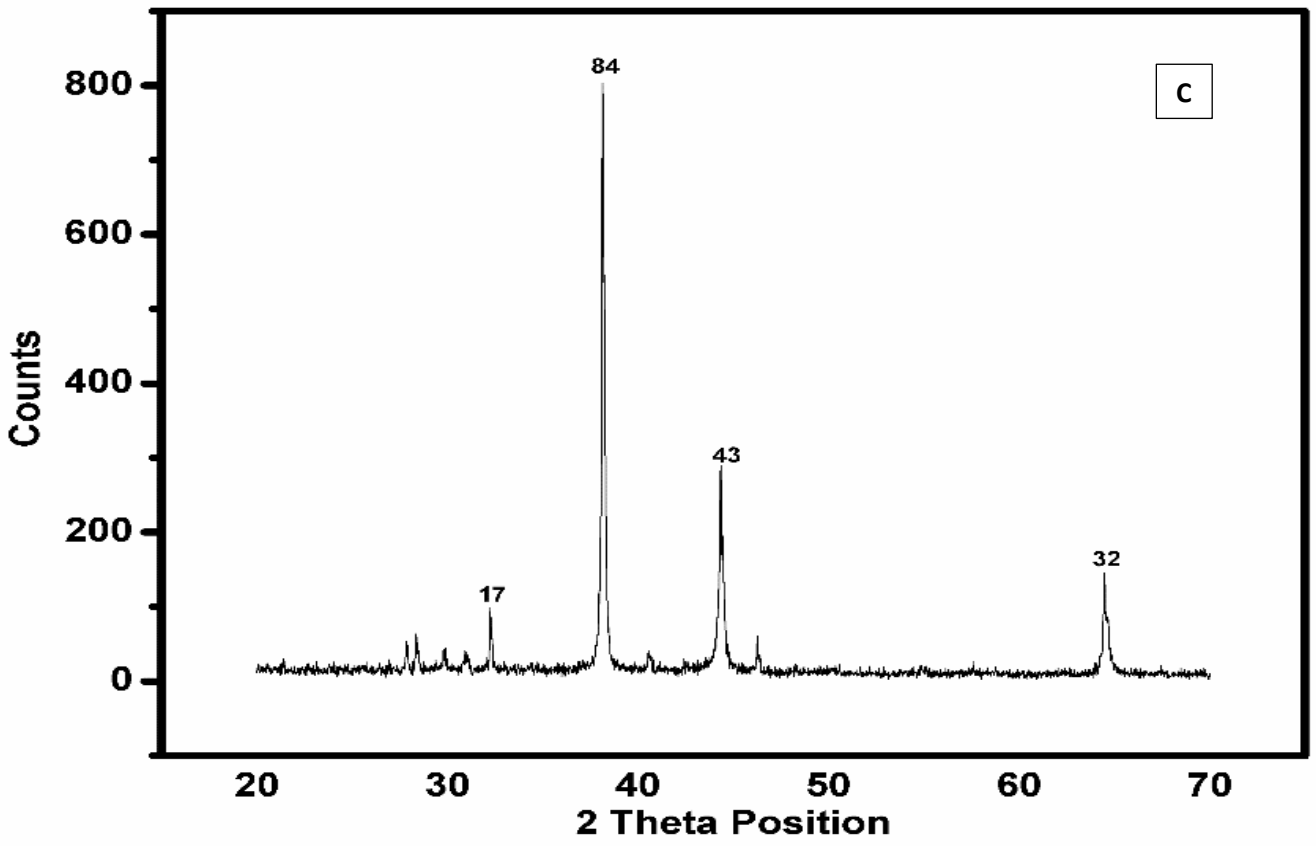
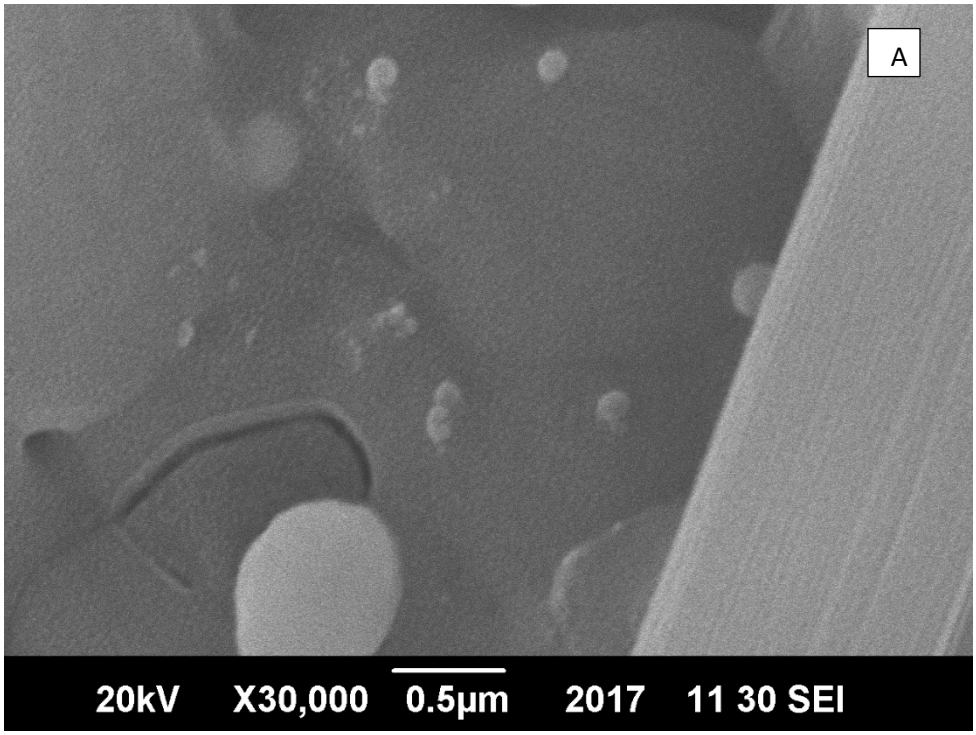


Figure 4.3. XRD spectra of aqueous filtrate of bacterial consortia (A), *F. oxysporum* (B), and Sugarcane husk (C) treated with 1 mM silver nitrate solution

4.3.1.4. Scanning Electron Microscopy (SEM)

The surface deposition of silver nanoparticles can be clearly observed in scanning electron micrographs of the silver nanoparticles prepared for different samples (Fig 4.5).



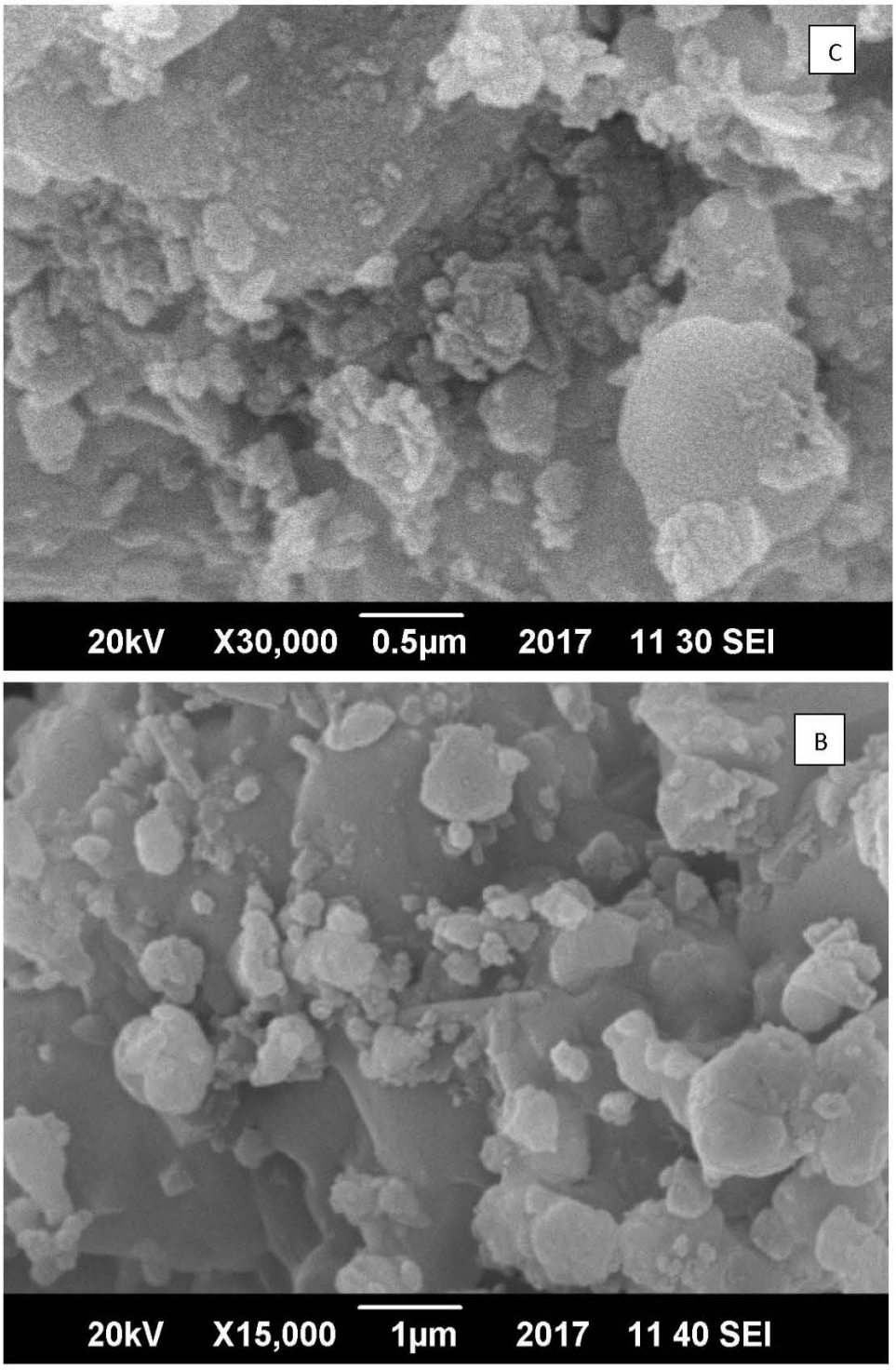
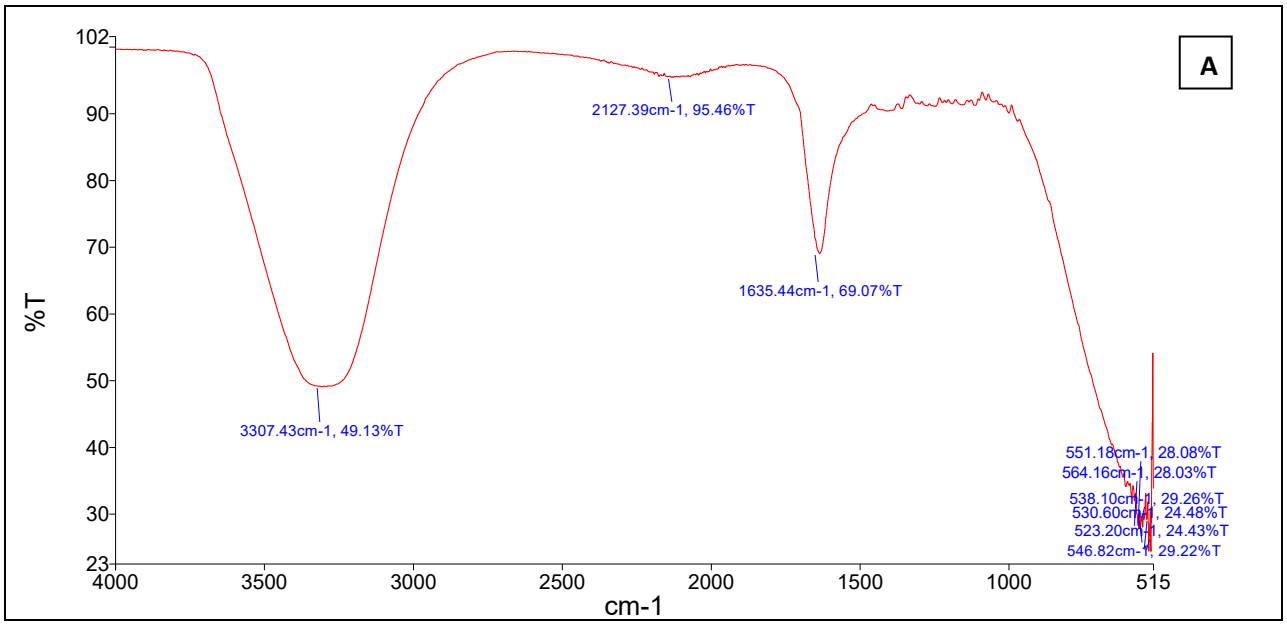
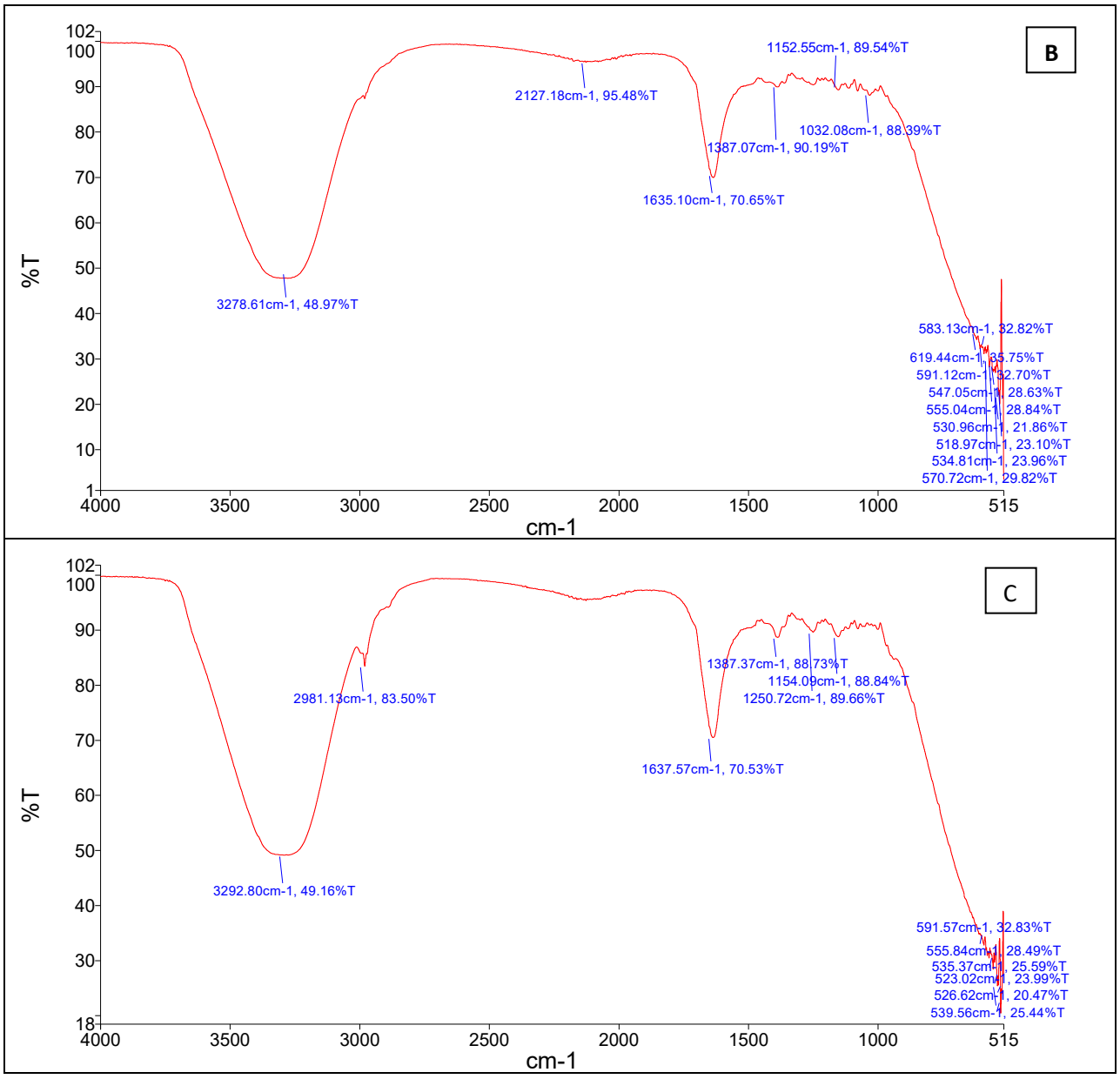


Figure 4.4. SEM images of silver nanoparticles formed after incubation of the aqueous filtrate of bacterial consortia (A), *F. oxysporum* (B), and Sugarcane husk (C) treated with 1 mM silver nitrate solution and calcinated.

4.3.1.5. FTIR analysis of samples

The analysis of data obtained from Fig. 4.6. presents the FTIR spectrum of AgNPs (dried powder) formed after incubation along with the fungal supernatant. For the FTIR spectrum of sample C the bands present between 1250 Cm1 showed stretching vibrations for acids while bands between 3278.6 Cm1 and 3292.8 Cm1 have OH band of alcohol in the sample of AgNPs formed after incubation of the aqueous filtrate of fugal and plant's aqueous solution respectively. Presence of benzene was noted by absorbance in the region of 1635.44, 1635.10 and 1637.57 Cm1 in sample A, B and C respectively. Moreover, the presence of two bands at 3307 cm1 and 1635 cm1 in sample A, 3278.6 Cm1 and 1635.10 Cm1 in sample B, 3292.8 and 1637.57 in sample C can be assigned to the N-H stretching vibrations of both aliphatic and aromatic amines, respectively. Presence of proteins were observed in all three samples of AgNPs. Moreover, presence of ether was noted in both fungal and plant-based silver nanoparticles while the presence of alkenes was noted only in fungal based nanoparticles.





4.3.2. Anti-fungal activity of biologically produced nanoparticles

The highest antifungal activity was notied by nanoparticles produced by aqoueus solution of sugarcane husk, followed by bacterial-based nanoparticles and fungal-based nanoparticles, respectively. The reason might be the reduced size of nanoparticles (Table 4.1).

Table 4.1. Zone of inhibition of nanoparticles against sugarcane fungal strains

Concentration of silver nanoparticles/ 10µL	*Colletotricum falcatum*	*Fusarium moniliforme*
Bacterial-based nanoparticles		
5mg	10mm	12mm
10mg	15mm	17mm
15mg	18mm	21mm
Fungal-based nanoparticles		
5mg	8mm	10mm
10mg	14mm	16mm
15mg	19mm	20mm
Sugarcane husk-based nanoparticles		
5mg	13mm	16mm
10mg	19mm	20mm
15mg	21mm	24mm

4.4. Discussion

Biologically produced nanoparticles have been reported as beneficial molecules with significant antimicrobial activity. Though various nanoparticles have been synthesized successfully by using aqueous extracts of microorganisms (bacteria, fungi) and plants, however, a lot of research is still going on to explore new nanoparticles with distinct the physical, chemical and biological features (Li et al., 2011). As the bacterial supernatant, fungal supernatant and sugarcane husk extract were thoroughly mixed with silver nitrate solution, change in color of solution occurs indicates the formation of AgNPs due to reduction of silver ions. Excitation of surface plasmon vibrations in AgNPs was represented by the appearance of yellowish-brown color in aqueous solution (Ponarulselvam et al., 2012). UV- diffused reflectance spectroscopy is used to study the variation in the absorption of electromagnetic radiations of nanoparticles. In DRS analysis, detection of surface plasmon resonance band indicates the dispersal and shape of the nanoparticles as observed

in the samples. Surface plasmon resonance band detected for a primary peak indicates the cluster formation and their dendritic nature in all samples of AgNPs. In a previous study, DRS analysis showed the primary peak in surface plasmon resonance band which indicated the cluster formation and a secondary peak was observed at 339 nm that confirmed the dendritic nature of AgNPs (Zhang et al., 2016).

The XRD analysis showed the crystalline structure and particle size of the nanoparticles. Furthermore, XRD is also used to analyze the crystalline phases and phase changes while analyzing a sample. Small-sized nanoparticles (<5) themself influence the XRD patterns interference fringes might cause these effects however these cannot be taken into account while analyzing whole XRD patterns. (Hassellov et al., 2008). Peaks observed after FTIR analysis confirmed the presence of molecules that previously have been reported to proceed the bioreduction of silver nitrate ions to AgNPs. Reduction of nanoparticles is due to the presence of capping material in all the three experimental samples, i.e., fungal supernatant, bacterial supernatant and the sugarcane husk extract (Thunugunta et al., 2015). In the present study, FTIR analysis was done to determine the biochemical functional groups in the samples that are directly involved in the reduction of $AgNO_3$ to silver nanoparticles (Faghihzadeh et al., 2016). Free amine groups or cysteine residues in the proteins bind to the silver nanoparticles during their production. This revealed their probable role and mechanism for biosynthesis and stabilization of silver nanoparticles. Carbonyl groups associated with protein peptides and amino acid residues have strong ability to bind silver particles. These proteins could possibly form a coating on nanoparticles to prevent their accumulation at one place and help to stay stabilized in the aqueous medium. Thus, the biological molecules have a dynamic role in the formation and stabilization of the AgNPs in the medium (Iravani et al., 2014). It was noted that biosynthesized silver nanoparticles are comparatively more effective for phytopathogens, such as *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Rhizoctonia solani*, *Macrophomina phaseolina*, *Alternaria alternata*, and *Curvularia lunata* as compared to the chemical- based nanoparticles (Krishnaraj et al., 2012). Silver nanoparticles act via dual mechanism by direct antimicrobial effects or by disruption of polymer subunits in microbial membranes (Yin et al., 2020). In the present study, biogenically-produced silver nanoparticles suppressed growth of the two pathogens, *C. falcatum* and *F. monilliforme*. Reason for this might be the penetration of AgNPs into the cells of fungi that directly interfere respiratory sequence leading to disrupted cell division and finally cell death (Qing et al., 2018).

Moreover, the inhibitory action of AgNPS on pathogens involve the loss of DNA replication capability, resulting in deactivated ribosomal protein expressions, as well as the blocked metabolisms of certain proteins and enzymes (Wang et al., 2017). The present experimental work and analyzed results suggest a simple, economical and rapid way to synthesize AgNps through biological means.

4.5. Conclusions

In the present study it was noticed that biological materials can be used as an active reducing agent to produce nanoparticles and exhibit unique physicochemical and biochemical properties. Dispersed dendritic nature nanoparticles with nano size of 45.41 nm, 49.06 nm, and 42.75 nm were observed after treatment of silver nitrate solution with supernatant of bacteria, fungus and sugarcane husk solution, respectively. FTIR spectrum revealed the presence of functional groups (amines, benzenes, alcohols and alkenes) that act as capping and reducing agents to produce stable AgNPs. Highest antifungal activity was shown by nanoparticles produced by aqueous solution of sugarcane husk. This might be due to the relatively small size of the particles as compared to the other samples. Effects of all nanoparticles were dominant in the suppression *F. monilliforme*. This biological synthesis approach of AgNPs has many benefits obviated by its economic viability and simple processing.

Chapter 5. Greenhouse Experiments

Experiment 1: Effects of inoculated bacteria on plant growth regulation and development of sugarcane

5.1. 1. Introduction

Plant growth promoting rhizobacteria (PGPR) are widely used as a substitute to chemical-based fertilizers and fungicides because of their cost-effective and environment-friendly nature. Role of these microbes as bio-antagonistic to suppress plant pathogens is evident from various mechanisms. Induced systemic resistance (ISR) is one of the important mechanisms possessed by various plant growth promoting bacteria to enhance their immunity and secure the plant against a wide range of phytopathogens. Induced systemic resistance is a defensive mechanism established as a result of various physiological changes in the plants. Modification in the structure of cell wall and denovo production of antimicrobial compounds, such as phytoalexins and pathogenesis-related (PR) proteins stop the pathogens dispersion. Antioxidant enzymes like superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) might act as inducers as disease suppression in plants is highly correlated with their increased production (Hassan et al., 2017). Antioxidant enzymes activity mitigate the oxidative stress created by the burst of the reactive oxygen species (ROS) as observed during pathogens infection. The connection of a bacteria or its released metabolites to regulate its host defense aptitude is insufficiently known and needs to be discovered.

During the long developmental stages, many plants, such as sugarcane might face multiple biotic and abiotic stresses. These environmental stresses adversely affect all the major metabolic activities of plants like reduced photosynthetic rate, cell wall damage, accumulation of compatible solutes, e.g., soluble sugars and proline, effects on membrane stability, that all eventually results in declined germination and stunted growth (Tiwari et al., 2019). Microbes associated with sugarcane could enhance plant growth by the production of various growth promoting metabolites as well as by antagonistic traits against *Colletotrichum falcatum* pathogen that cause the severe red rot disease (Backer et al., 2018). Application of bacterial inoculum, which can enhance sugarcane growth along with the suppression of red rot disease, is a desirable and environmentally friendly strategy to cut off the huge input of fertilizers and fungicides by farmers. In this regard, exertions have been channelized toward the growth enhancement of sugarcane plants and the biological approach to inhibit the red rot pathogen by the antagonistic PGPR.

5.1.2. Material and methods

5.1.2.1. Microbes selected for plant growth promoting analysis

Among all screened strains, 7 strains were selected based on antagonistic and various other traits

in order to analyze their effects on sugarcane plants. The selected strains included *Bacillus. gibsonii* (PM11); *Bravibacterium frigoritolerans* (PM12); *Bacillus siamensis* (PM13); *Bacillus xiamenesis* (PM14); *Bacillus sp.* (PM15); *Bacillus sp.* (PM16); *B. tequilensis* (PM17).

5.1.2.2. Greenhouse experiment

Experimental soil was taken from the National Agricultural Research Centre (NARC), Islamabad, Pakistan (33.6701° N, 73.1261° E).

5.1.1. Basic properties of soil and greenhouse conditions

Soil Properties	**Values**
Soil texture	Clayey
Soil pH	7.8
Soil EC dS m^{-1}	12.9
Soil Organic matter (SOM)	0.78%
Total nitrogen (N) ppm	71.24
Available Phosphorus (P) ppm	1.14
Available Potassium (K) ppm	103
Green house conditions	**Range**
Temperature	25 ºC to 35 ± 3 ºC
Photoperiod	10 hrs.
Light intensity	80 µM S^{-1} m^{-2}
Relative humidity	60 ± 10%

EC: Electrical conductivity

The pot experiment was carried out under semi-controlled conditions (Table 5.1.1). In order to examine the efficacy of these bacterial strains, experiment was performed in three replicates and designed with eight treatments i) C; as control plants without bacterial inoculation; ii) **T1:** plants with *B. gibsonii* inoculation (PM11); iii) **T2:** *Bravibacterium frigoritolerans* (PM12); iv) **T3:** *B. siamensis* (PM13); v) **T4:** *B. xiamenesis* (PM14); vi) **T5:** *Bacillus sp.* (PM15); vii) **T6:** *Bacillus sp.* (PM16); viii) **T7:** *B. tequilensis* (PM17). Bacterial strains were applied to plants by root inoculation (10^8-10^9 cfu/ml).

5.1.2.3. Measurement of plant growth parameters

While harvesting, plant height, plant fresh weight, cane length, cane weight, cane diameter and root length of sugarcane plants grown under various treatments were measured (Ferreira et al., 2017).

5.1.2.4. Photosynthetic pigments

Fresh leaf material (0.05 g) was homogenized in a mortar with 10 ml of 80% acetone. Homogenized material was incubated in the dark to extract the pigment. Tubes were then centrifuged for 15 min at 5000 rpm. Supernatant was used to quantify the photosynthetic pigments by using spectrophotometer (Agilent 8453 UV–visible Spectroscopy System) (Amna et al., 2019).

5.1.2.5. Quality control parameters of sugarcane

For qualitative control parameters of sugarcane (Brix, Pol and Commercial Cane Sugar%), the canes were collected from each replicate (Meade and Chen, 1977). These canes were washed and crushed with a cutter grinder. Then five hundred grams of crushed canes processed through hydraulic press. Obtained juice of sugarcane was collected in a 500 ml glass beaker.

Brix (%) To determine the level of brix i.e. concentration of total soluble solids a drop of extracted cane juice was positioned on the prism of Refractometer (PR-101, ATAGO Co. Ltd, Japan) with the help of pipette.

Pol (%) In this case the extracted sugarcane juice was treated by following Horns Lead Acetate method. To obtain good juice clearness, lead acetate (4 g) was carefully mixed in juice (100 ml) with the help of glass rod. Juice was carefully filtered using Whattman filter paper No.1 and used further to take the POL% reading. (Magwaza and Opara, 2015).

Commercial Cane Sugar (CCS) of the extracted sugarcane juice was determined based on the values of brix and pol (Meade and Chen, 1977).

5.1.2.6. Re-isolation of microbial strains

To make sure the presence and survival of inoculated bacterial strains in soil of sugarcane plants re-isolation was done. By using selected antibiotics (Table 3.1), the screening and presence of bacterial strains was reconfirmed. Moreover, analysis of general appearance, morphology, color, and gram staining was also done.

5.1.2.7. Statistical analysis

All experiments and analysis were done in triplicates. Excel was used to form a database of obtained results. Statistical analysis of data was conducted by analysis of variance using software Statistics 8.1. (Duncan, 1955).

5.1.3. Results

5.1.3.1. Effects on plant growth parameters and yield

Differences in plant height, plant fresh weight, cane length, cane weight, cane diameter, and root length of plants receiving different treatments were observed. All these parameters were significantly influenced by the inoculation of PGPR. Sugarcane plants without the PGPR inoculation (control) showed the least plant height, fresh weight, cane length, cane weight, diameter of cane and root length (Table 5.1.1).

Table 5.1.2. Effects of PGP bacterial inoculation (seven treatments and a control) on sugarcane plant growth parameters (plant height, fresh weight, cane length, cane weight, cane diameter, root length, chlorophyll a, chlorophyll b, carotenoids, Brix, POL, CSS)

Parameters	C	T1	T2	T3	T4	T5	T6	T7
Plant height	255e	290c	279d	**326a**	294c	273d	256e	310b
Plant fresh weight	0.67f	0.81cd	0.74def	**1.01a**	0.86bc	0.73cde	0.71ef	0.73ab
Cane length	153.24f	170cd	164e	**200a**	176c	164de	159ef	188.2b
Cane weight	0.61f	73bcd	0.71def	**0.91a**	0.83abc	0.75cde	0.68ef	0.87aab
Cane Diameter	0.51bc	5.4ab	5.3bc	**6.142a**	5.7ab	5.57ab	5.572c	6a
Root length	66e	83c	74d	**106.33a**	97b	79cd	73d	101.3ab
Chlorophyll a	1.53e	3.1bc	2.53cd	**4.13a**	3.64ab	2.87c	2.12de	1.53a
Chlorophyll b	0.63d	1.48ab	1.22bc	**1.67a**	1.53ab	1.36ab	0.93cd	1.58ab
Carotenoids	6.9a	10.9ab	8.7cd	**12.27a**	11.35a	9.3bc	7.36d	11.78a
Brix	24.56g	35.48cd	31.43ef	**40.1a**	36.53bc	33.12de	28.56f	39.23ab
POL	68.69d	87.25ab	83.41bc	**90.13a**	89.72a	85.47abc	80.76c	89a
CSS	12.86d	19.83ab	17.13bc	**21.76a**	21.31a	17.98bc	16cd	18.64abc

C: Control; T1: PM11, T2: PM12; T3: PM13; T4: PM14; T5: PM15; T6: PM16; T7: PM17. Brix: soluble solid contents, POL: sucrose content. Values in bold showed highest response of PM13 (*B. siamenesis*).

Sugarcane plants receiving various bacterial innoculation were: (T1: *B. gibsonii* (PM11)*;* T2: *Bravibacterium frigoritolerans* (PM12); T3: *B. siamensis* (PM13); T4: *B. xiamenesis* (PM14); T5: *Bacillus sp.* (PM15); T6: *Bacillus sp.* (PM16); T7: *B. tequilensis* (PM17). Examination of the growth parameters of these plants revealed that the application of bacterial strain PM13 *B. siamensis* resulted in the highest values for all plant growth parameters (21.77%, 33.66%, 23.38%, 32.96%, 16.9%, 36.9 %, 62.68%, 62.27%, 43.76%, 38.73%, 23.7%, 40.9% for plant height, plant fresh weight, cane length, cane weight, cane diameter, root length, Chl a, Chl b, carotenoids, Brix, POL and CCS, respectively) as compared to control plants without inoculation of bacterial strains (Table 5.1.1). Lowest growth enhancement was noticed in the sugarcane plants inoculated with *Bacillus sp.* PM16 with increased values of0.39%, 5.6%, 5.76%, 10.27%, 8.77%, 8.21%, 27.83%, 32.25%, 6.25%, 13.39%, 14.94%, 19.62% for plant height, plant fresh weight, cane length, cane weight, cane diameter, root length, Chl a, Chl b, carotenoids, Brix, POL and CCS, respectively, as compared to control without inoculation of bacterial strains.

5.1.4. Discussion

Native phytomicrobiome provide a wide range of services to associated plants that lead to enhanced plant growth via various mechanisms and minimize the use of synthetic fertilizers and agrochemicals. In the present study, inoculation of bacterial strains *Bacillus gibsonii* PM11*, Bravebacterium frigoritolerans* PM12*, Bacillus siamensis* PM13*, Bacillus xiamenesis* PM14*, Bacillus sp.* PM15*, Bacillus sp.*PM16 and *Bacillus tequilensis* PM17 showed increased plant height, plant weight, cane length, cane weight, cane diameter, root length, photosynthetic pigments, Brix, POL and commercial cane sugar. Highest growth parameters among all the studied *Bacillus* strains were noted in sugarcane plants inoculated with *B. siamensis* (T3), followed by *B. tequilensis* (T4), and *B. xiamenesis* (T5).

The reason behind the improved growth of bacteria-inoculated sugarcane plants may be the mechanisms of associated microbes that enhance plant growth via phytohormone production (IAA). Generally, IAA affects cell division, extension and differentiation of plant cells, increases the developmental rate of xylem and root; enhances vegetative growth; triggers the formation of lateral and adventitious root; directly or indirectly affects various metabolic processes such as photosynthesis and respiration, increases root surface area and length that directly provide the plant with greater access to soil micro- and macronutrients (Ahemad and Kibert, 2014). Phosphorus

is directly involved in the plant energy reactions, photosynthesis and genetic transfer; thus, its enhanced uptake plays a role in the increased growth of bacteria- inoculated sugarcane plants. Chelation of insoluble iron through release of siderophores results in the formation and uptake of iron-siderophore complex (Sharma and Johri, 2003). Iron plays a vital role in various metabolic processes, such as photosynthesis and respiration. Role of iron in the synthesis of chlorophyll and maintenance of structure and function of chloroplasts is evident from previous findings. Thus, increased iron uptake directly effects the growth of plants. Hydrogen cyanide (HCN) and ammonia production by PGPR have positive relationship to nitrogen and phosphorus accumulation, elongation of roots and shoots and higher biomass production (Marques et al., 2010).

Increased photosynthetic pigments in plants as compared to control conditions can be linked with the application of PGPR as they aid in solubilization and mobilization of minerals like phosphorus and iron. Iron is an important constituent of chlorophyll (Mahmood et al., 2016). Higher chlorophyll content boosts the photosynthetic activity of plants that can be related to increased fresh weight of sugarcane plants as well as the cane. The present research revealed the benefits of PGPR inoculation in sugarcane plants to increase the fresh weight, yield and productivity in the form of increased Brix, POL and CCS %age in comparison to control plants without bacterial inoculation. During maturation and ripening stage of sugarcane plant, photo assimilates produced in sugarcane leaves are transported from phloem to other heterotrophic sink organs (cane) for plant growth and development (Sachdeva et al., 2011). When those assimilates surpass the plant's metabolism demands, they start being stored inverted to other carbohydrates in storage form (Bihmidine et al., 2013). Compartmentation of sucrose between vacuoles and apoplast act as control step for the accumulation of sucrose in sugarcane. Increased photosynthetic activity and enhanced growth result in the accumulation of higher sugar contents and sucrose compartmentation in inoculated plants as compared to un-inoculated plants (Santos et al., 2018).

5.1.5. Conclusion

The present study suggests that growth promoting bacterial strains, such as *B. gibsonii* PM11*, Bravebacterium frigoritolerans* PM12*, B. siamensis* PM13*, B. xiamenesis* PM14*, Bacillus sp.* PM15*, Bacillus sp.* PM16 and *B. tequilensis* PM17 have the potential for use to improve growth, photosynthetic pigments and cane quality. Highest values of growth parameters were noticed in sugarcane plants that were treated with *B. siamensis* PM13 (21.77%, 33.66%, 23.38%, 32.96%, 16.9%, 36.9 %, 62.68%,62.27%, 43.76%, 38.73%, 23.7%, and 40.9%) increased values for plant

height, plant fresh weight, cane length, cane weight, cane diameter, root length, Chi a, Chi b, carotenoids, Brix, POL and CCS, respectively) followed by *B. tequilensis* PM17 and *B. xiamenesis* PM14. These results provide evidence for use of native microflora to produce high yield and productivity in sugarcane plants.

Experiment 2:

Plant growth promoting rhizobacterial strains activating sugarcane (*Saccharum officinarum* L.) *Colletotricum falcatum* disease resistance

5.2.1. Introduction

Plant growth promoting rhizobacteria (PGPR) are widely used as a substitute to chemical-based fertilizers and fungicides because of their cost-effective and environment-friendly nature. Role of these microbes as bio-antagonistic to suppress plant pathogens is evident from various mechanisms. Induced systemic resistance (ISR) is one of the important mechanisms possessed by various plant growth promoting bacteria to enhance their immunity and secure the plant against a wide range of phytopathogens. Induced systemic resistance is a defensive mechanism established as a result of various physiological changes in the plants. Modification in the structure of cell wall and denovo production of antimicrobial compounds, such as phytoalexins and pathogenesis-related (PR) proteins stop the pathogens dispersion. Antioxidant enzymes like superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) might act as inducers as disease suppression in plants is highly correlated with their increased production (Hassan et al., 2017). Antioxidant enzymes activity mitigate the oxidative stress created by the burst of the reactive oxygen species (ROS) as observed during pathogens infection. The connection of a bacteria or its released metabolites to regulate its host defense aptitude is insufficiently known and needs to be discovered. During the long developmental stages, many plants, such as sugarcane might face multiple biotic and abiotic stresses. These environmental stresses adversely affect all the major metabolic activities of plants like reduced photosynthetic rate, cell wall damage, accumulation of compatible solutes, e.g., soluble sugars and proline, effects on membrane stability, that all eventually results in declined germination and stunted growth (Tiwari et al., 2019). Microbes associated with sugarcane could enhance plant growth by the production of various growth promoting metabolites as well as by antagonistic traits against *Colletotrichum falcatum* pathogen that cause the severe red rot disease (Backer et al., 2018). Application of bacterial inoculum, which can enhance sugarcane growth along with the suppression of red rot disease, is a desirable and environmentally friendly strategy to cut off the huge input of fertilizers and fungicides by farmers. In this regard, exertions have been channelized toward the growth enhancement of sugarcane plants and the biological approach to inhibit the red rot pathogen by the antagonistic PGPR.

5.2.2. Material and methods

5.2.2.1. Selection of microbes antagonistic for red rot

Among all screened strains, 4 bacterial strains were selected in order to analyze their role in disease suppression. The selected strains were *Bravibacterium frigoritolerans* (PM12); *B. siamensis* (PM13); *B. xiamenesis* (PM14) and *B. tequilensis* (PM17).

5.2.2.2. Glass house experiment: biocontrol of red rot disease

Experimental soil was taken from National Agricultural Research Centre, Islamabad, Pakistan (33.6701° N, 73.1261° E). The- soil related parameters have been mentioned previously in Table 5.1.1. In order to examine the efficacy of the above mentioned as the bio-control agents, experiment was performed by maintaining three replicates and designed as follows: Control; T1: *C. falcatum*; T2: *B. siamensis*+ *C. falcatum*; T3: *B. tequilensis*+ *C. falcatum*; T4: *B. xiamenensis*+ *C. falcatum*; T5: *Braviectrium frigoritolerans* + *C. falcatum*. Bacterial strains were applied to plants by root inoculation (10^8-10^9 cfu/ml). At the end of the experiment, plants were harvested and preserved for further analysis. After the dilution of spore suspension, the number of spores were totaled. Spore concentration was finally adjusted to 10^6 spores per mL by using a haemocytometer. At the end of experiment, plants were harvested carefully and preserved for further analysis.

5.2.2.3. Measurement of plant growth parameters

While harvesting, plant height, plant fresh weight, cane length, cane weight, cane diameter and root length of sugarcane plants grown under various treatments were measured (Ferreira et al., 2017).

5.2.2.4. Photosynthetic pigments

Fresh leaf material (0.05 g) was homogenized in a mortar with 10 ml of 80% acetone. Homogenized material was incubated in the dark to extract the pigment. Tubes were centrifuged for 15 min at 5000 rpm. Supernatant was used to quantify the photosynthetic pigments by using spectrophotometer (Amna et al., 2019).

5.2.2.5. Estimation of antioxidant enzyme activity proline content and electrolyte leakage

To assess superoxidase dismutase (SOD) activity, the method developed by Afridi et al. (2019) was followed. Peroxidase (POD) activity was determined by the modified method of Afridi et al. 2019). Production of proline under different treatments was also checked by following the method

of Amna et al. (2015). Electrolyte leakage of leaves was measured by using EC meter and following the protocol of Batool et al. (2019).

5.2.2.6. Disease severity index

Disease severity index of red rot in sugarcane plants was measured by following the protocol of Nadeem et al. (2014).

5.2.2.7 Quality control of sugarcane

For qualitative control parameters of sugarcane (Brix, Pol and Commercial Cane Sugar%), the canes were collected from each replicate (Meade and Chen, 1977). These canes were washed and crushed with a cutter grinder. Then five hundred grams of crushed canes processed through hydraulic press. Obtained juice of sugarcane was collected in a 500 ml glass beaker.

Brix (%) To determine the level of brix i.e. concentration of total soluble solids a drop of extracted cane juice was positioned on the prism of Refractometer (PR-101, ATAGO Co. Ltd, Japan) with the help of pipette.

Pol (%) In this case the extracted sugarcane juice was treated by following Horns Lead Acetate method. To obtain good juice clearness, lead acetate (4 g) was carefully mixed in juice (100 ml) with the help of glass rod. Juice was carefully filtered using Whattman filter paper No.1 and used further to take the POL% reading. (Magwaza and Opara, 2015).

Commercial Cane Sugar (CCS) of the extracted sugarcane juice was determined based on the values of brix and pol (Meade and Chen, 1977).

5.2.2.8. Statistical analysis

All experiments and analysis were done in triplicate. Excel was used to form a database of the obtained results. Statistical analysis of data was conducted by analysis of variance using Statistics 8.1. (Duncan, 1955). Principal component analysis correlation (PCA) was analyzed by using XL-STAT 2010.

5.2.3. Results

5.2.3.1. Effects of PGPR on sugarcane growth parameters

Differences in plant height, plant fresh weight, cane length, cane weight, cane length, cane diameter, and root length of plants were observed under different treatments. All these parameters were significantly influenced by the inoculation of PGPR. Upon the *C. falcatum* application, significant ($p \leq 0.05$) reduced plant growth in terms of biomass production was noted both in control and inoculated sugarcane plants. Sugarcane plants infected with *C. falcatum* without the PGPR

inoculation showed the lowest fresh weight, root length, plant height, cane length, cane weight, and diameter of cane. It was evident from the growth parameter examination that the application of bacteria significantly enhanced the plant growth even with fungal pathogen infection. Application of PGPR PM13, PM17, PM14, PM11 inoculation enhanced plant height (18.39%, 15.88%, 5.36%, 4.4%), fresh weight (36.95%, 45.86%, 12.7, 5.8%) cane length (32.83%, 28.5%, 12.53%, 7.3%) cane weight (46.36%, 37,89%,15.81%, 5.6%) (Fig. 5.2.1); cane diameter (44.31%, 32.05%, 26.12%, 20%) and root length (26.04%, 21.96%, 18.46%, 13.46%) respectively as compared to the diseased sugarcane plants (Fig. 5.2.2).

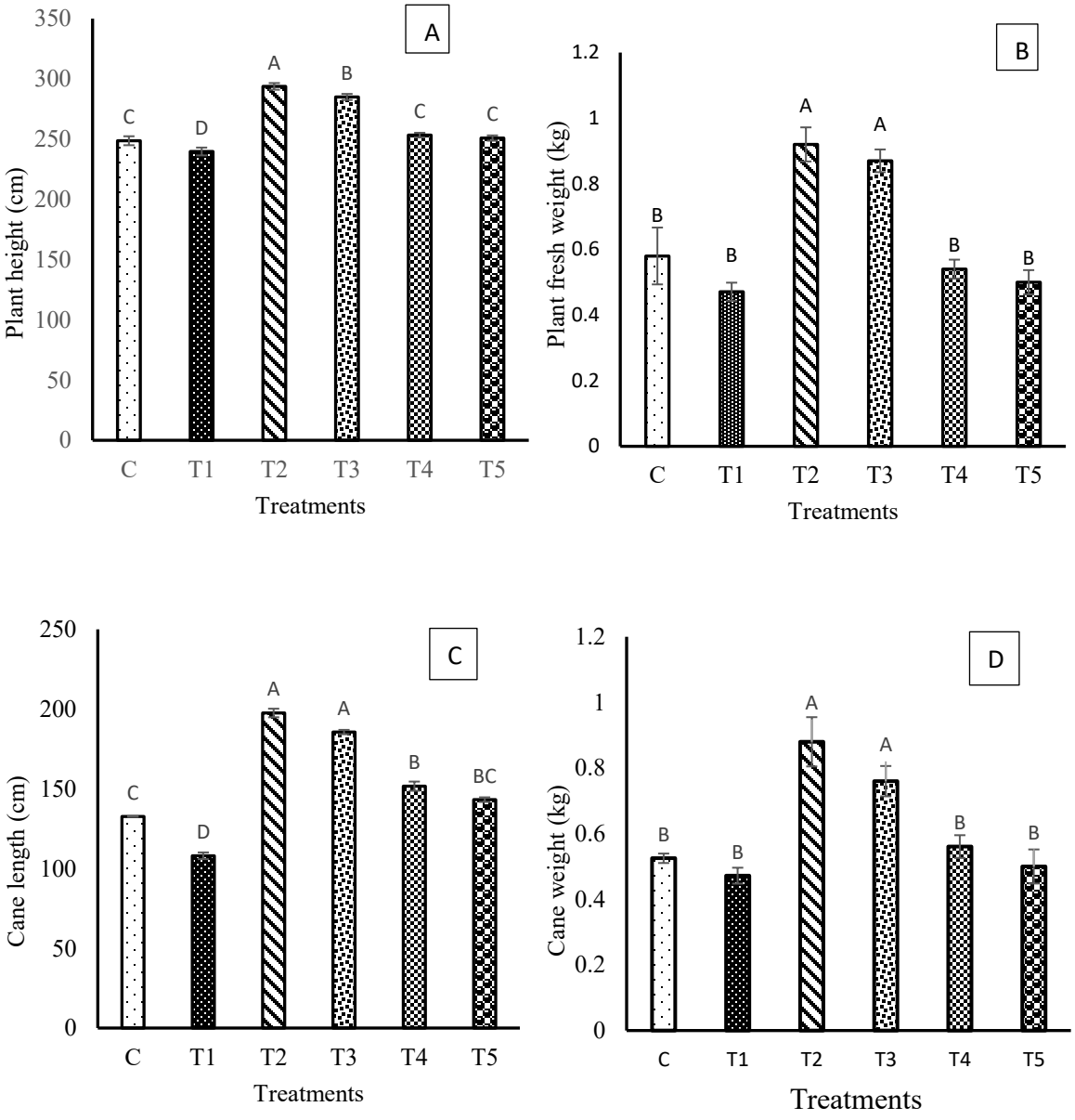


Figure 5.2.1. Effects of PGPR on Plant height (a), Plant fresh weight (b), Cane length (c), Cane weight (d) under biotic stress of *C. falcatum*. C: Control; T1: *C. falcatum*; T2: *B. siamensis*+ *C. falcatum*; T3: *B. tequilensis*+ *C. falcatum*; T4: *B. xiamenensis*+ *C. falcatum*; T5: *Braviectrium frigoritolerans* + *C. falcatum*. All means the sharing common letter(s) are significantly not different ($p < 0.05$) by LSD. Standard error of means was represented by vertical bars (n=3).

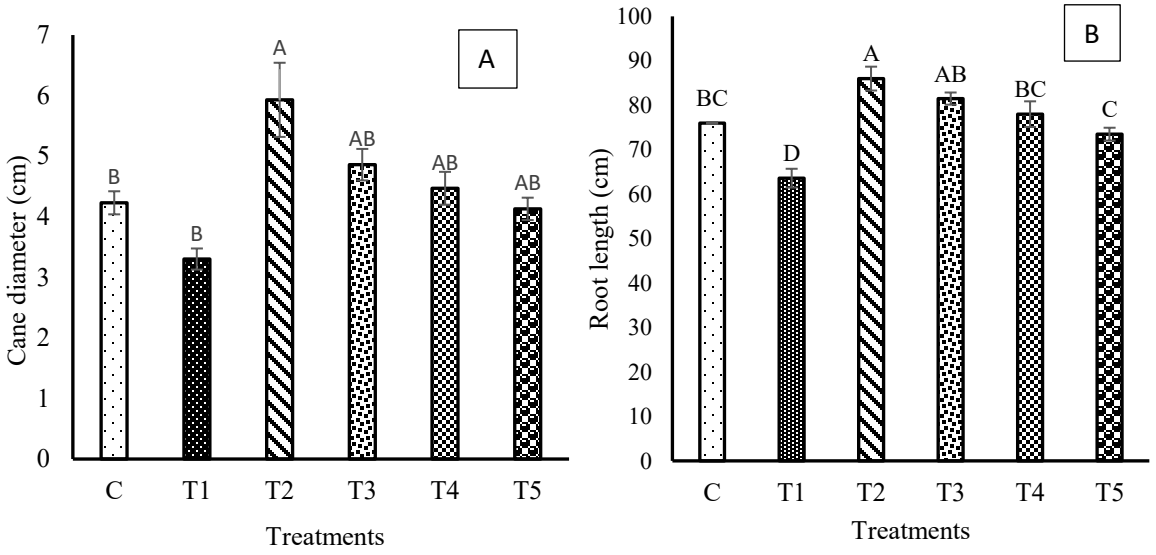


Figure 5.2.2. Effects of PGPR on Diameter of cane (a) Root length (b) of sugarcane under biotic stress of *C. falcatum*. C: Control; T1: *C. falcatum*; T2: *B. siamensis*+ *C. falcatum*; T3: *B. tequilensis*+ *C. falcatum*; T4: *B. xiamenensis*+ *C. falcatum*; T5: *Braviectrium frigoritolerans* + *C. falcatum*. All means the sharing common letter(s) are significantly not different ($p < 0.05$) by LSD. Standard error of means was represented by vertical bars (n=3).

5.2.3.2. Estimation of photosynthetic pigments and proline contents

Bacterial inoculation enhanced Chl a (21.27%, 19.53%, 16.84%, 0%), Chl b (37.30%, 35.45%, 32.04%, 22.54%), carotenoids (39.76%, 37.47%, 33.98%, 23.2%), and proline (23.41%, 21.58%, 20.21%, 17.19%) respectively, as compared to *C. falcatum* diseased plants without any bacterial inoculation (Fig 5.2.3).

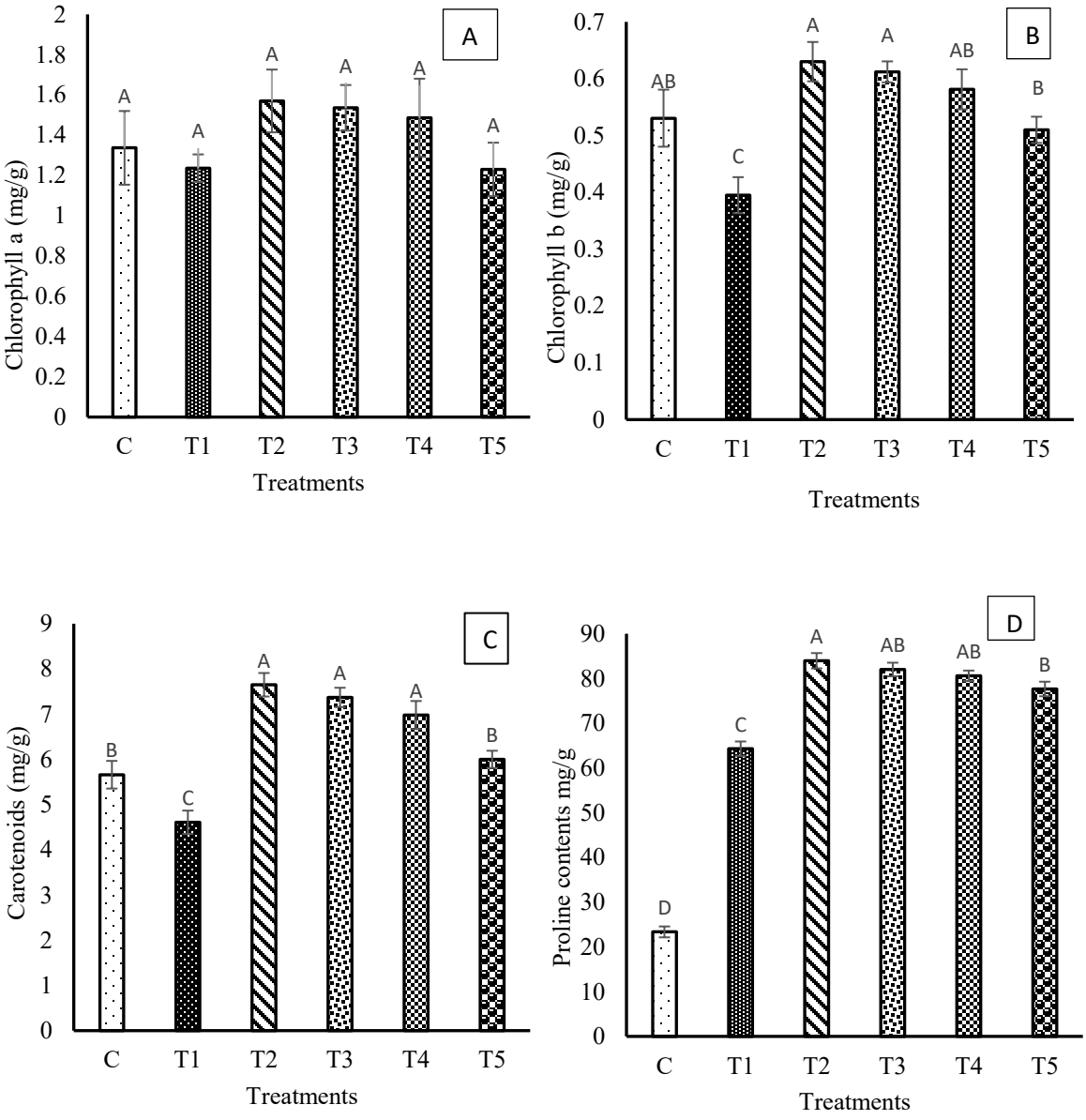
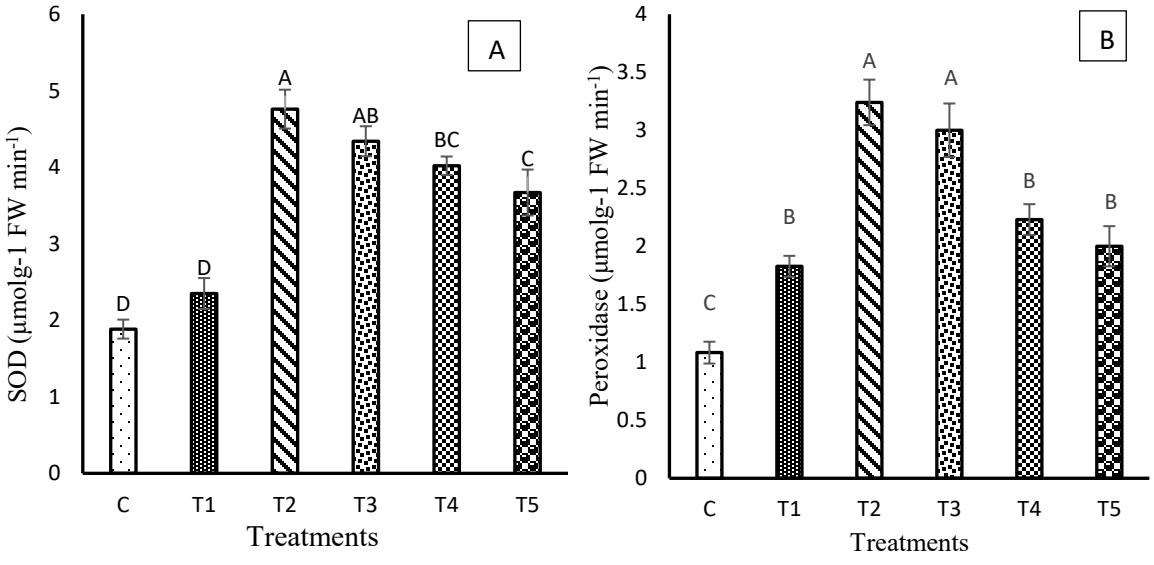


Figure 5.2.3. Effects of PGPR on Chlorophyll a (a), Chlorophyll b (b), Carotenoids (c), Proline contents (d) of sugarcane under biotic stress of *C. falcatum*. C: Control; T1: *C. falcatum*; T2: *B. siamensis*+ *C. falcatum*; T3: *B. tequilensis*+ *C. falcatum*; T4: *B. xiamenensis*+ *C. falcatum*; T5: *Braviectrium frigoritoilerans* + *C. falcatum*. All means the sharing common letter(s) are significantly not different ($p < 0.05$) by LSD. Standard error of means was represented by vertical bars (n=3).

5.2.3.3. Estimation of antioxidant enzymes, electrolyte leakage

Antioxidant enzymse activity (SOD, POD, and CAT) were evaluated in control and un-inoculated plants under diseased and control conditions. Less antioxidant activity was noticed in un-inoculated plants (control) as compared to inoculated and diseased plants. The highest activity was noted in bacterial inoculated plants infected with *C. falcatum*. In *C. falcatum* treated plant (T1), 19.78%, 40.7%, 67% and 14.59 increased SOD, POD, catalase activity and electrolyte leakage were observed as compared to control plants. In PGPR treated diseased plants increased SOD activity (50.63%, 47.04%, 41.54%, 35.96), POD (43.60%, 39.09%, 18.06%, 8.64%) and catalase (57.43%, 46.63%, 30.31%, 3.53%) was noted for T2, T3, T4, and T5%, respectively. Electrolyte leakage was observed to be increase (14.59 %) in pathogen infected sugarcane plants as compared to non-infected and un-inoculated control. Whereas, antagonistic PGPR inoculated sugarcane plants infected with *C. falcatum* exhibited significant decrease in electrolyte leakage up to 57.17%, 46.15%, 22.71 and 18.81% in T2, T3, T4 and T5 respectively as compared to *C. falcatum* treated disaese plants (Fig. 5.2.4).



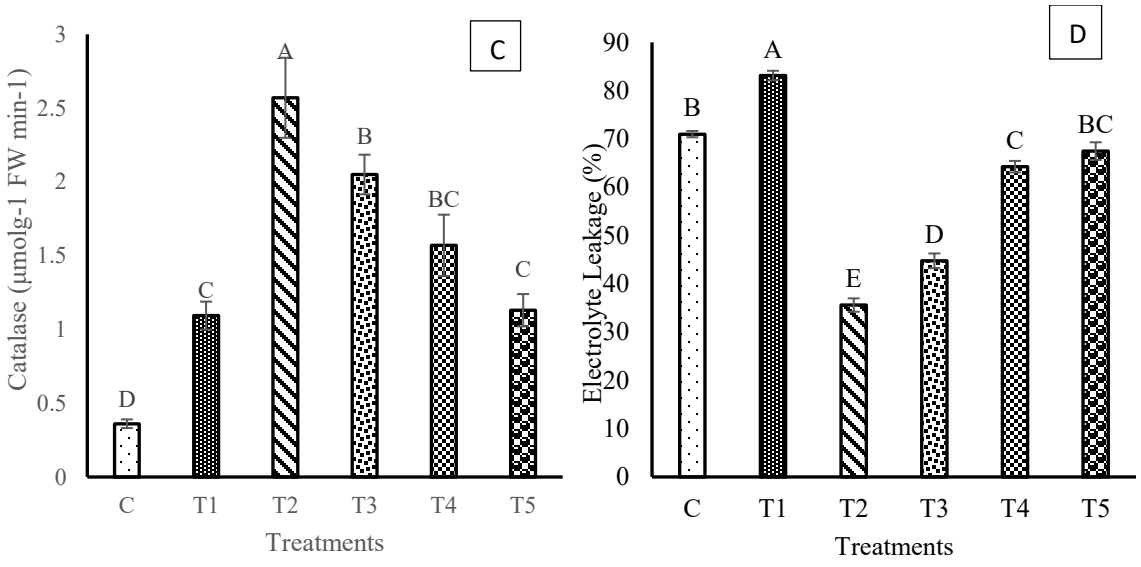


Figure 5.2.4. Effects of PGPR on SOD (a), POD (b), Catalase (c), Electrolyte leakage (d) of sugarcane under biotic stress of *C. falcatum*. C: Control; T1: *C. falcatum*; T2: *B. siamensis*+ *C. falcatum*; T3: *B. tequilensis*+ *C. falcatum*; T4: *B. xiamenensis*+ *C. falcatum*; T5: *Braviectrium frigoritoilerans* + *C. falcatum*. All means the sharing common letter (s) are significantly not different ($p < 0.05$) by LSD. Standard error of means was represented by vertical bars (n=3).

5.2.3.4. Estimation of quality control parameters of sugarcane

It was observed that PGPR could enhance the plant growth and yield as well as quality parameters with up to 27% for Brix and 20.25% for Pol and 30.37%, for CCS (Commercial Cane sugar), respectively (Fig. 4). However, the infection of fungal strains directly affects it's all quality parameters up to 14.46%, 4%, and 4.5% for Brix, Pol and CCS%, respectively. The dual effects of PGPR helped the inoculated sugarcane plants to cope with the biotic stress by suppressing the pathogens and comparatively enhancing plant growth parameters. It was noted than bacterial application in *C. falcatum* infected plants could enhance the brix up to 13.39%, POL 16.49%, CCS 17.5% as compared to uninoculated control (Fig. 5.2.5).

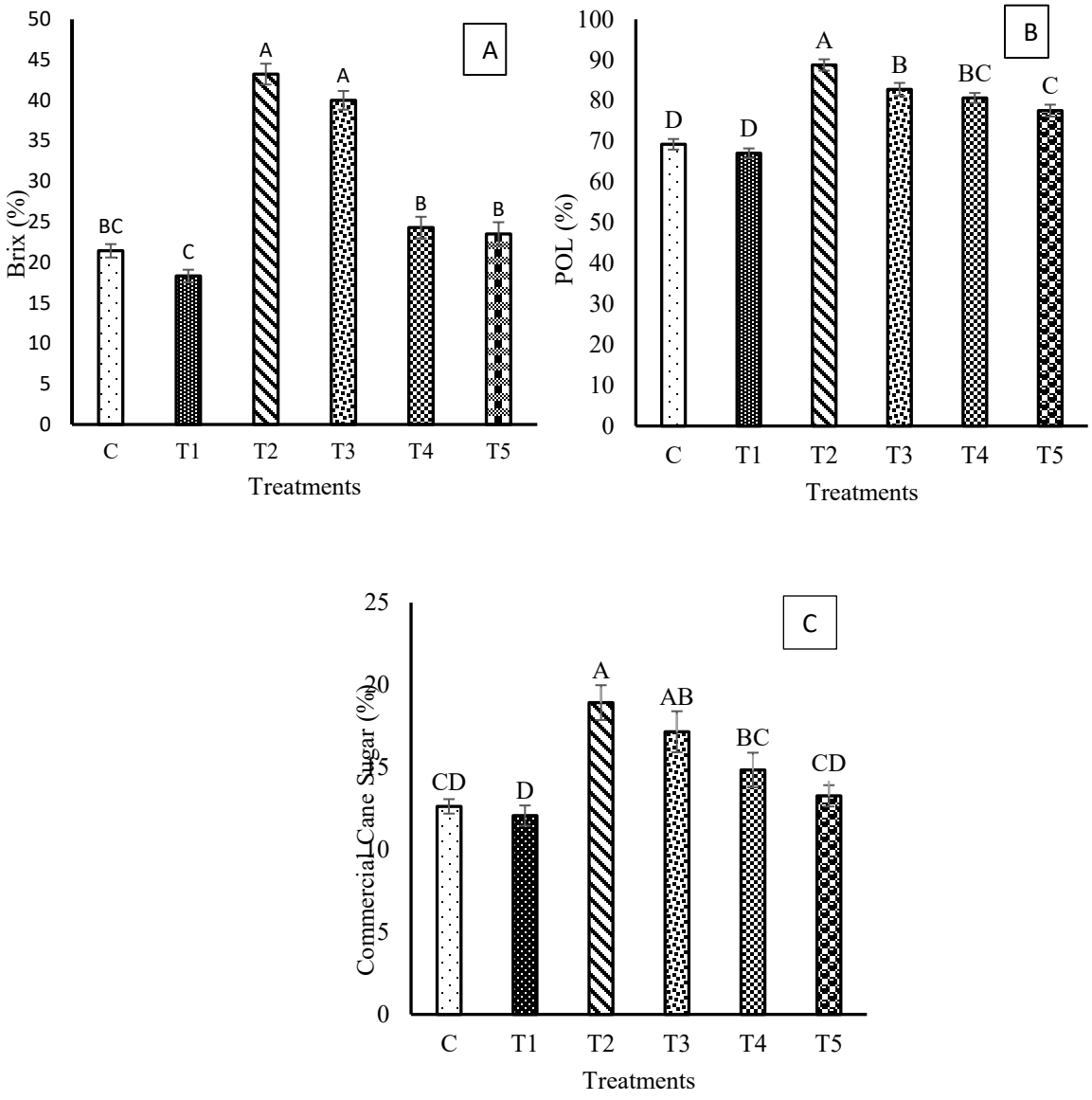


Figure 5.2.5. Effects of PGPR on Brix% (a), POL% (b), CSS% (c) of sugarcane under biotic stress of *C. falcatum*. C: Control; T1: *C. falcatum*; T2: *B. siamensis*+ *C. falcatum*; T3: *B. tequilensis*+ *C. falcatum*; T4: *B. xiamenensis*+ *C. falcatum*; T5: *Braviectrium frigoritoilerans* + *C. falcatum*. All means the sharing common letter(s) are significantly not different ($p < 0.05$) by LSD. Standard error of means was represented by vertical bars (n=3).

5.2.3.5. Disease severity index

Antagonistic activity of PGPR strains against red rot showed their significance in controlling sugarcane red rot disease. Sugarcane plants treated with fungal strain *C. falcatum* (T1) showed clear symptoms of disease in longitudinal section of the cane with nodal transduction up to 6.33 inches, nodal length up to7.53 inches, lesion length up to 17.1 inches, and lesion width up to0.933 inches. *In vivo* suppression of red rot disease was evident from the disease severity index noticed in infected sugarcane plants inoculated with bacterial strains as compared to non-inoculated plants. It was noted that antagonistic bacterial strains decreased nodal transduction (57.34%, 52.60%, 49.44%, 41.5%), nodal length (41.96%, 32.27%, 23.37%, 10.22%), lesion length (79.18%, 75.26%, 68.42%, 64.32%), lesion width (62.22%, 56.66%, 33.3%, 21.11%) in red rot infected plants of T2, T3, T4 and T5, respectively (Fig. 5.2.5).

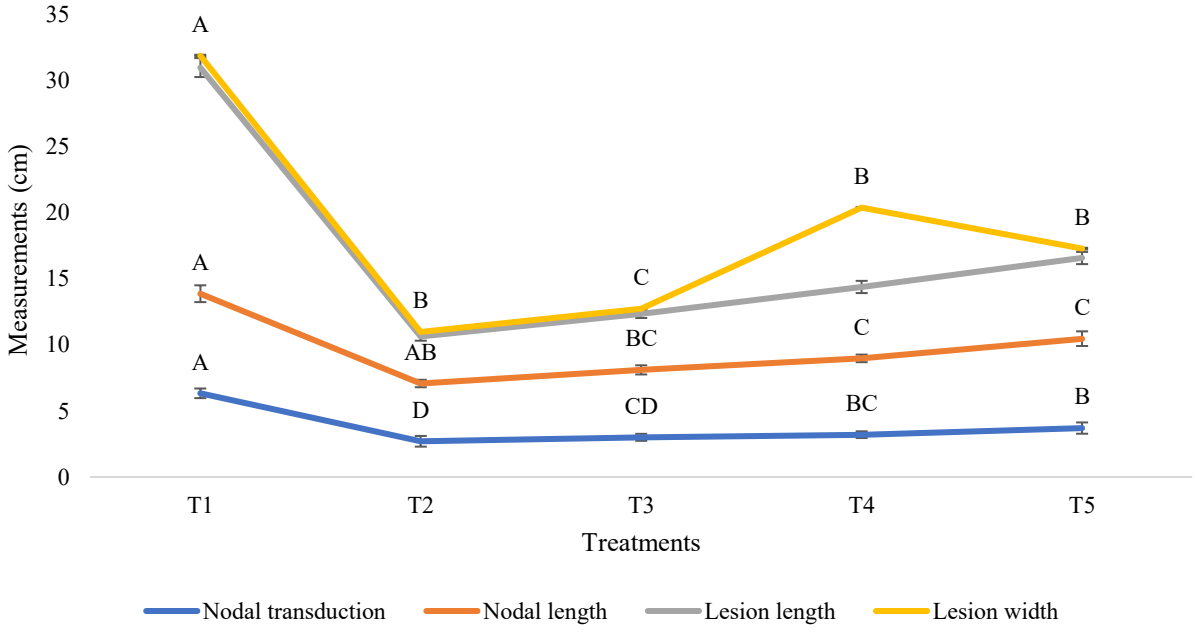


Figure 5.2.5. Effects of antagonistic PGPR on the nodal transduction, node length, lesion length, and lesion width of sugarcane plants infected with *C. falcatum* under bacterial inoculated and non-inoculated conditions. C: Control; T1: *C. falcatum*; T2: *B. siamensis*+ *C. falcatum*; T3: *B. tequilensis*+ *C. falcatum*; T4: *B. xiamenensis*+ *C. falcatum*; T5: *Braviectrium frigoritoilerans* + *C. falcatum*. All the means sharing common letter(s) are insignificantly different at $p < 0.05$ level by LSD. Vertical bars represent standard error of means (n=3).

5.2.3.6. Pearson correlation analysis

It was noticed that PGPR showed a positive impact on sugarcane plant growth, photosynthetic pigments, enzymes activity, disease index, and quality control parameters of sugarcane plants infected with *C. falcatum* or grown in normal conditions. In the Pearson correlation analysis of all parameters done collectively, it was noticed that the variables which were strongly positively correlated were present in the same quadrant and very close to each other. For *B. siamensis* (PM13), *B. tequilensis* (PM17), *B. xiamenensis* (PM14), *Bravibacterium frigoritolerans* (PM12) treated plants, the correlation biplots between the F1 and F2 showed 100% variations in all cases however variation in the contribution of F1 and F2 were observed. In PM13, PM14 and PM17 significant correlation was observed among the parameters SOD, POD, catalase, proline, Plant height, plant weight, cane length, cane weight and cane diameter. While, disease severity index parameters and EL lie on opposite quadrats that showed negative correlation among these parameters. In biplot analysis of *Bravebacterium frigoritolerans* (PM12) Cane diameter, SOD, POD, catalase, nodal length, nodal transduction, nodal length electrolyte leakage and proline were observed in same quadrat which shows a positive correlation with each other. However, POL, Brix CSS, cane length, plant height, root length, cane weight, cane diameter, plant fresh weight, and chlorophyll contents lied in opposite quadrats showing their positive correlation among each other.

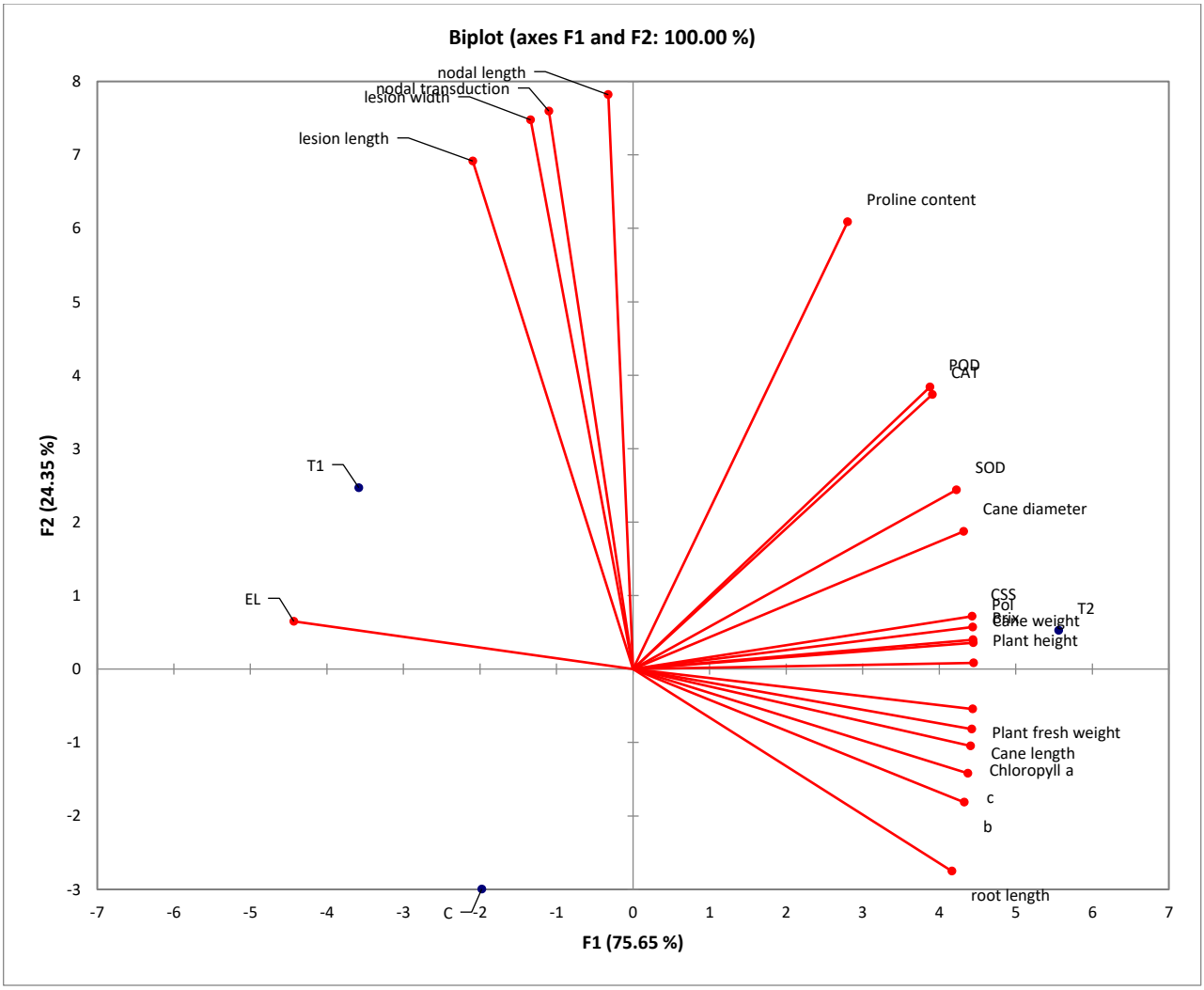


Figure 5.2.6. Pearson correlation plot between the treatments and parameters of sugarcane plants inoculated with *B. siamensis*. The correlation biplot between the F1 and F2 showed 100% variation in which F1 contributed for 24.35% and F2 contributed 75.65%. Strongly and positively correlated variables were very close to each other and were present in the same quadrat. Red dots showed correlation between parameters while blue dots show correlation within treatments. The variables which were strongly positively correlated are present in the same quadrant and are very close to each other.

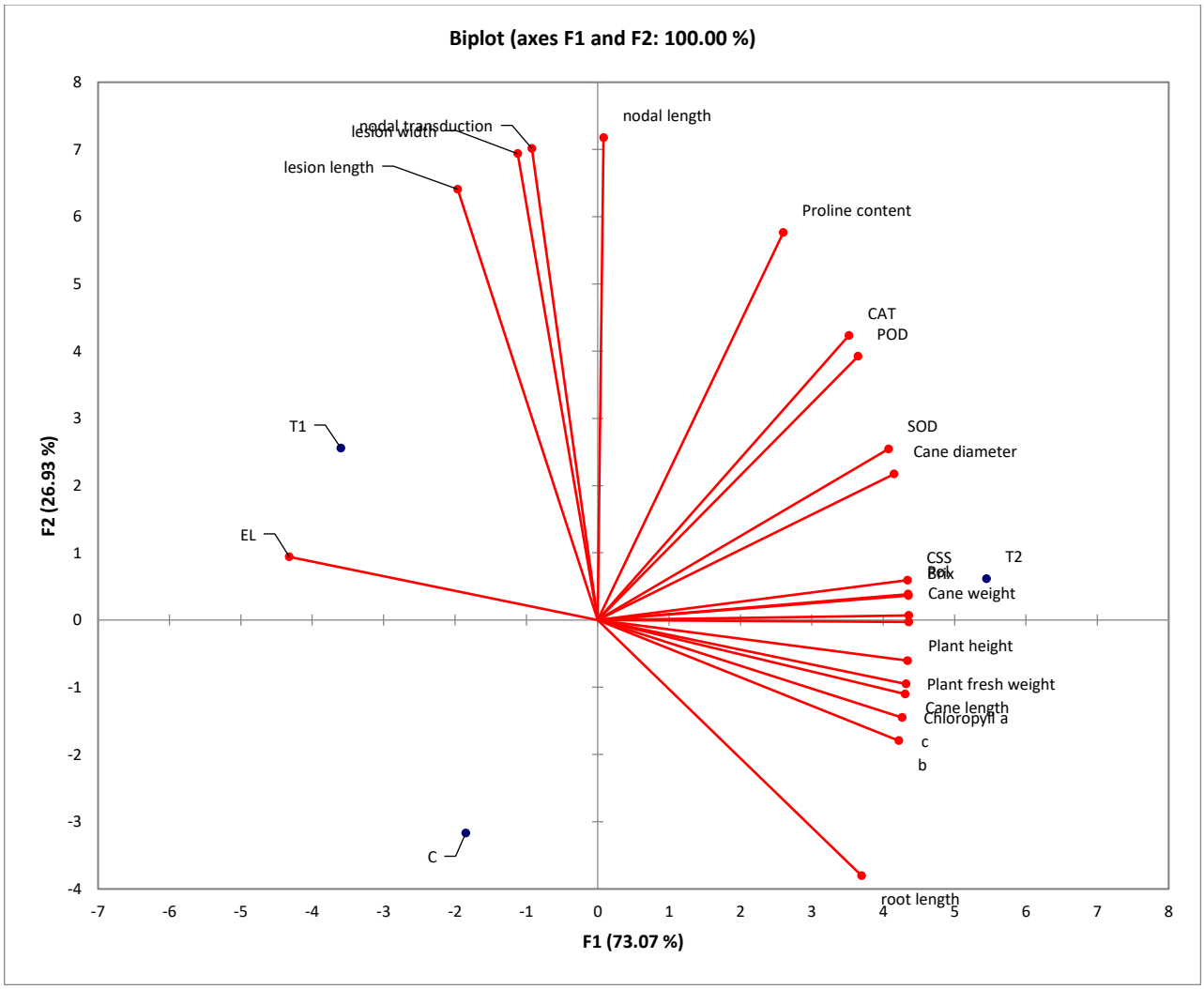


Figure 5.2.7. Pearson correlation plot between the treatments and parameters of sugarcane plants inoculated with *B. tequilensis*. The correlation biplot between the F1 and F2 showed 100% variation in which F1 contributed for 26.93% and F2 contributed 73.07%. Strongly and positively correlated variables were very close to each other and are present in the same quadrat. Red dots showed correlation between parameters, while blue dots showed correlation within treatments. The variables which had strong positive correlation are present in the same quadrant and are very close to each other.

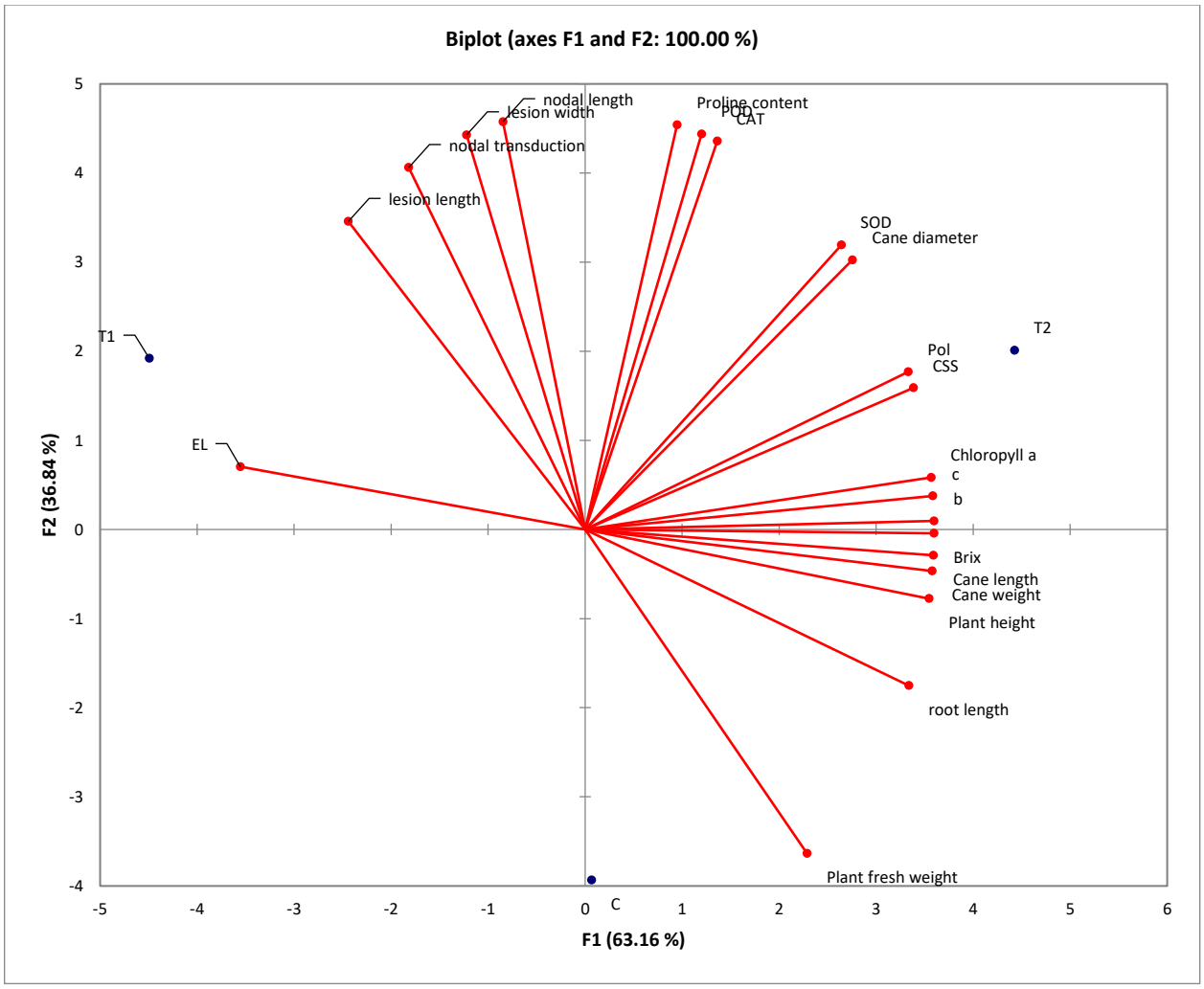


Figure 5.2.8. Pearson correlation plot between the treatments and parameters of sugarcane plants inoculated with *B. xiamenensis*. The correlation biplot between the F1 and F2 showed 100% variation in which F1 contributed 36.84% and F2 contributed 63.16%. Strongly and positively correlated variables were very close to each other and present in the same quadrat. Red dots show correlation between parameters, while blue dots showed correlation within treatments. The variables which had strong positive correlation are present in the same quadrant and are very close to each other.

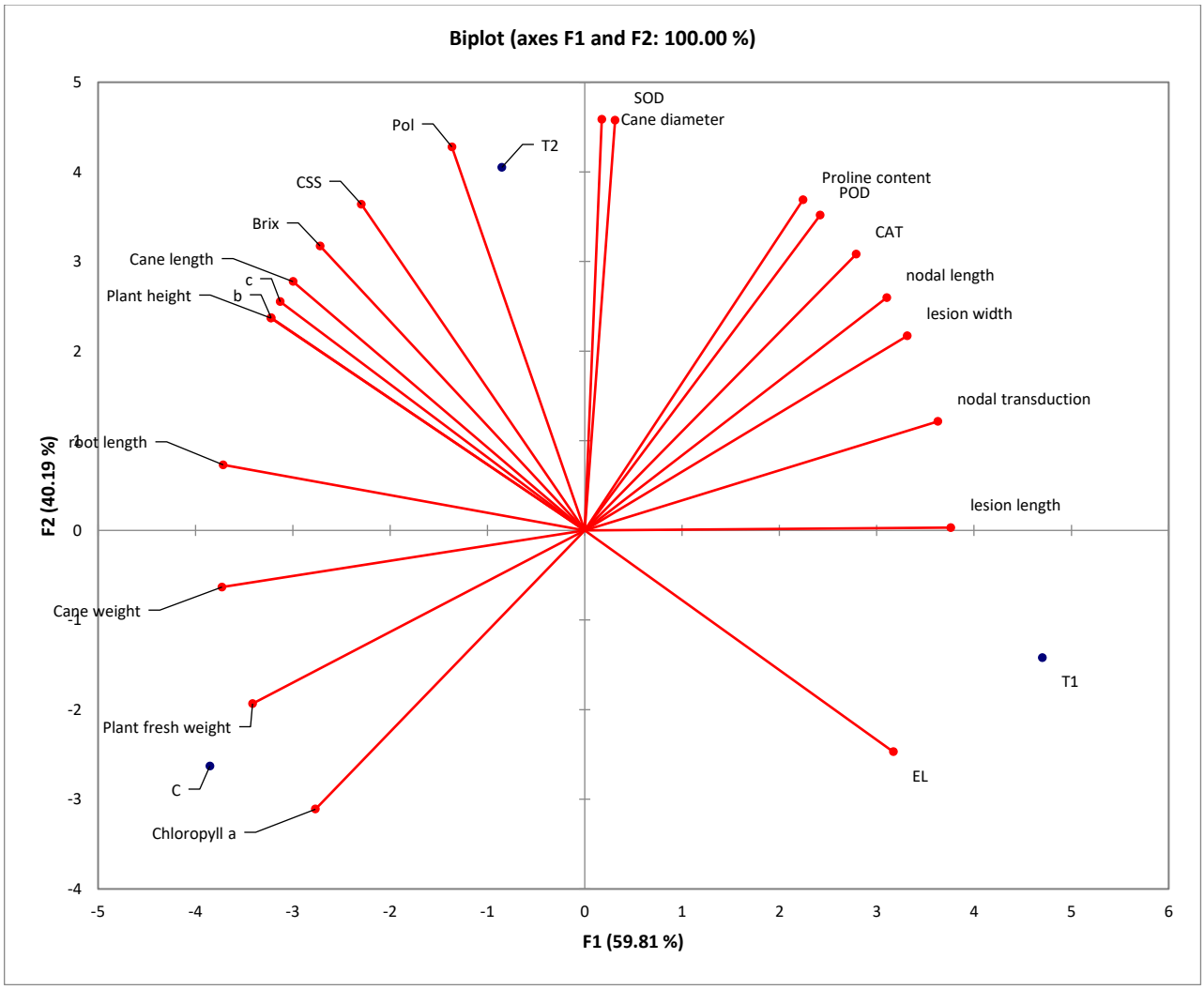


Figure 5.2.9. Pearson correlation plot between the treatments and parameters of sugarcane plants inoculated with *Bravibacterium frigoritolerans*. The correlation biplot between the F1 and F2 showed 100% variation in which F1 contributed 40.19% and F2 contributed 59.81%. Variables that showed. Strong positive correlation was very close to each other and were present in the same quadrat. Red dots show correlation between parameters, while blue dots show correlation within treatments. The variables which had strong positive correlation are present in the same quadrant and are very close to each other.

5.2.4. Discussion

Low annual crop production due to phytopathogens results in rising food demands (FAO 2018). The use of biocontrol agents for improved farming would considerably help to overcome these issues. Moreover, such a strategy also reduces the lethal effects of pesticides in the food chain. Consequently, the current study was attempted to discover the potential of antagonistic PGPR

(*Bacillus siamensis* PM13*, Bacillus tequilensis* PM17*, Bacillus xiamenesis* PM14 and *Bravebacterium frigoritolerans* PM12) along with plant growth promoting effects in red rot diseased sugarcane plants infected with *Colletotricum falcatum*.

Reduced growth of sugarcane plants was noticed in all diseased plants and the lowest growth rate was observed in T1 (sugarcane infected with pathogen without bacterial inoculation). However, a remarkable increase in root and shoot length, plant height, cane length and fresh weight was observed in PGPR-inoculated plants infected with red rot. PGPR can induce disease resistance in plants and facilitate them to maintain high productivity under the attack of different phytopathogens. These bacteria with multiple plant growth promoting (PGP) traits benefit plants via various mechanisms. For instance, enhanced fertility of soils by solubilization of phosphorus (P) and chelation of iron (Fe) by siderophore production (Souza et al., 2015), activity of ACC-deaminase (Zainab et al., 2020), generation of phytohormones like IAA which can enhance plant growth. The pathogen and disease control can be achieved by the generation of extracellular enzymes (chitinase, cellulase) that can hydrolyze the fungal cell wall; possessing a broad spectrum of antibiotic resistance, competition for nutrients (iron, phosphorus) in the rhizosphere, and induction of systemic resistance (ISR) against pathogen infections in plants (Marquez et al., 2020).

The production of various extracellular antimicrobial compounds as diffusible and/or volatile molecules may act on phytopathogenic fungi by exerting fungicidal effects, such as the inhibition of germination or the lysis of fungal mycelia as observed in an initial study of bacterial screening for PGP activities and suppression of phytopathogens (Slama et al., 2019). Various PGPRs genera, including *Bacillus, Pseudomonas, Azospirillum, Flavobacterium, Sinorhizobium, Chryseobacterium, Achromobacter, Bradyrhizobium, Aeromonas, Acetobacter*, and many more, have been reported to sustain the growth and yield of different crop plants when cultivated in stressed conditions (Etesami and Maheshwari 2018).

PGPR stimulate plant growth and chlorophyll content by increased nutrient uptake like phosphorus and iron that are an important part of various metabolic processes (chloroplast, photosynthesis) and phytohormone production, lowering of stress ethylene levels by ACC deaminase activity, production of microbial-based siderophores that enhance plant iron uptake. Increased levels of proline via up-regulation of proline biosynthesis pathway defend plants from stress by membrane protection and maintaining cell water content. Whereas, increase in superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) activity in sugarcane plants could be due to activation of plant defense mechanisms and confers resistance to plants against pathogenic attack (Swarnakumari et al., 2011). The rapid and fastest production of reactive oxygen species (ROS) after pathogenic attack is considered as an initial defense response of plants. Stunted growth was observed in fungal infected sugarcane plants due to interaction of ROS with biomolecules, poor uptake of water and nutrients, loss of electrolytes with change in membrane permeability, shifting of the host metabolism towards disease control and also due to the negative effects of fungal toxins. Low chlorophyll contents observed in the red rot infected sugarcane plants might be due to the increased accumulation of ROS or it could be linked with the inactivation of enzymes responsible for its synthesis (Batool et al., 2019). Mentioned antioxidant enzymes catalyze the conversion of free radicals and their enhanced activity contributes to bio protection of plants against pathogens and insects.

Reduction in POL, Brix and CSS content was observed in infected plants as compared to uninfected ones. The fungal pathogen, *C. falcatum,* attacks sucrose accumulating parenchyma cells of cane stalk that leads to severe losses in extraction, sugarcane recovery, CSS, polarity and purity of sugar content (Nayyar et al., 2017).

Red rot infected sugarcane plants give low sugar recovery due to diminished sucrose assimilation metabolism. The red rot infection also reduces total carbohydrates in the diseased canes that results in reduced plant growth and the quality of plant as synthesis and translocation of phyto assimilates get disturbed (Santos et al., 2018). Moreover, the pathogen produces profuse quantities of acids like invertases that break the sucrose content into simple sugars glucose and fructose for the consumption of *C. falcatum*. Fungal infection results in increased levels of soluble salts, reducing sugars, acids, gums and simultaneous decrease in pH, sucrose and purity of cane juice in infected canes. Similarly, the present study has revealed that pathogen infection showed drastically reduced

Brix, purity and commercial cane sugar (CCS) percent in the diseased canes. The affected canes recorded 25 to 75% reduced sucrose content as compared to the healthy canes (Viswanathan and Samiyappan, 1999). Moreover, during the milling process, mixing of juice from healthy and diseased canes results in decomposition of entire juice due to inversion of sucrose. The quality control parameters of all PGPR inoculated sugarcane plants were observed elevated that revealed the antagonistic role of bacteria in suppression of drastic effects of red rot. The present study with the evaluation of antagonistic activity and PGP activity confirmed that these native bacterial strains can effectively be deployed in extreme environments to reveal rhizosphere proficiency and saprophytic competitive ability to benefit the sugarcane plants with enhanced health and yield.

5.2.5. Conclusion

Subsiding of ecological problems and stresses using eco-friendly approach is of vital importance. Based on the present study, it is concluded that use of antagonistic PGPR with extracellular enzyme production can be a good option to control red rot of sugarcane. Maximum disease suppression and plant growth was observed in sugarcane plants treated with strain *B. siamensis* PM13 enhance plant height (18.39%), plant fresh weight (36.95), cane length (32.83), cane weight (46.36), cane diameter (26.09), chl a (21.27), chl b (37.30) carotenoids (39.76). And, the disease index was also observed to be reduced (nodal transduction 49.5%; nodal width 19.5%; lesion length 68.4%; lesion width 36%). Following PM13 strain, *B. tequilensis*>*B. xiamenesis*>*Bravebacterium frigoritolerans* also showed same trend of enhanced growth and disease suppression.

Experiment 3:

Suppression of Sugarcane *Fusarium moniliforme* disease through selected antagonistic PGPR

5.3.1. Introduction

Sugarcane is an economically important cash crop of many tropical and subtropical countries that is used to produce sugar and biofuels. It is the world's largest sugar-producing crop and leads sixth in production quantity in Pakistan. The crop is affected by many biotic and abiotic stresses challenging the sugar production in Pakistan. Among the biotic stresses and diseases such as red rot, wilt, grassy shoot, pineapple disease, smut, leaf scald and pokkah boeng have been considered as serious constraints. Among the foliar diseases, pokkah boeng was recorded to range from 1% to 90% during 2007–2017 in the most affected cultivars leading to serious threats for commercial sugar production. Although the disease has been a minor problem, the epidemiological threats during last few years indicated the growing concern for this disease. Nowadays, the incidence and severity of the disease has been reported from in major sugarcane growing areas in Pakistan and many other sugarcane-growing countries (Whittle and Irawan, 2000). Pokkah boeng, derived from a Javanese term, describes a disease affecting the sugarcane tops. Disease was first reported in Java by Walker and Went in 1886. The disease is associated with sett rot or stem rot disease as both are caused by the same pathogen that is*, Fusarium moniliforme*. The chlorotic phase, acute phase or top-rot phase and knife-cut phase (associated with top rot phase) have been reported as common symptoms of the disease. The pathogen affects the stalk tissues, but the symptoms were variable in appearance and intensity among cultivars of hybrid varieties. Various biocontrol agents have been frequently exploited for the biological control of soil borne as well as foliar pathogens. Various species of *Bacillus, Enterobacter*, *Micromonospora*, *Pseudomonas* and *Streptomyces* have been studied for their broad-spectrum antagonistic activity against various phytopathogens. However, the selected bio-agent should be able to enhance or maintain already existing defense mechanisms operating in the environment. There is increasing need for novel and environmentally safe strategies to manage this disease because it is cost-effective for developing country like Pakistan. Keeping in view the pensiveness of the problem, the present research emphasized upon the disease management practices by the beneficial effect of biocontrol bacterial strains along with plant growth promoting effects.

5.3.2. Material and methods

5.3.2.1. Selection of microbes antagonistic for

Among all screened strains, 4 bacterial strains were selected in order to analyze their role in disease suppression caused by *F. moniliforme*. Selected strains were *B. gibsonii* (PM11), *B. siamensis* (PM13); *Bacillus sp.* (PM15), *B. tequilensis* (PM17).

5.3.2.2. GlasshouseExperiment: Biocontrol of pokha boeng disease

Experimental soil was taken from National Agricultural Research Centre, Islamabad, Pakistan (33.6701° N, 73.1261° E). The soil related parameters were mentioned previously in Table 5.1.1. In order to examine the efficacy of above mentioned as the bio-control agents, experiment was performed in three replicates and designed as follows: Control; T1: *C. falcatum*; T2: *B. siamensis*+ *C. falcatum*; T3: *B. tequilensis*+ *C. falcatum*; T4: *B. gibsonii*+ *C. falcatum*; T5: *Bacillus sp.* + *C. falcatum*. The spore suspension was diluted, and spores were counted. The final spore concentration was adjusted up to 10^6 spores per mL using a haemocytometer. At the end of experiment, plants were harvested carefully and preserved for further analysis.

5.3.2.3. Measurement of plant growth parameters

While harvesting, plant height, plant fresh weight, cane length, cane weight, cane diameter and root length of sugarcane plants grown under various treatments were measured (Ferreira et al., 2017).

5.3.2.4. Photosynthetic pigments

Fresh leaf material (0.05 g) was homogenized in a mortar with 10 ml of 80% acetone. Homogenized material was incubated in the dark to extract the pigment. Tubes were centrifuged for 15 min at 5000 rpm. Supernatant was used to quantify the photosynthetic pigments by using spectrophotometer (Amna et al., 2019).

5.3.2.5. Estimation of antioxidant enzyme activity proline content and electrolyte leakage

To access superoxidase dismutase (SOD) activity, the method developed by Afridi et al., (2019) was followed. Peroxidase (POD) activity was determined by the modified method of (Afridi et al., 2019). Production of proline under different treatments was also checked by following the method of Amna et al., (2015). Electrolyte leakage of leaves was measured by using EC meter by following the protocol of Batool et al., (2019).

5.3.2.6. Disease severity index

Disease severity index of pokha boeng in sugarcane plants was measured by following the protocol of Zhang et al., (2017).

5.3.2.7. Quality control of sugarcane

For qualitative control parameters of sugarcane (Brix, Pol and Commercial Cane Sugar%), the canes were collected from each replicate (Meade and Chen, 1977). These canes were washed and crushed with a cutter grinder. Then five hundred grams of crushed canes processed through hydraulic press. Obtained juice of sugarcane was collected in a 500 ml glass beaker.

Brix (%) To determine the level of brix i.e. concentration of total soluble solids a drop of extracted cane juice was positioned on the prism of Refractometer (PR-101, ATAGO Co. Ltd, Japan) with the help of pipette.

Pol (%) In this case the extracted sugarcane juice was treated by following Horns Lead Acetate method. To obtain good juice clearness, lead acetate (4 g) was carefully mixed in juice (100 ml) with the help of glass rod. Juice was carefully filtered using Whattman filter paper No.1 and used further to take the POL% reading. (Magwaza and Opara, 2015).

Commercial Cane Sugar (CCS) of the extracted sugarcane juice was determined based on the values of brix and pol (Meade and Chen, 1977).

5.3.2.8. Statistical analysis

All experiments and analysis were done in triplicates. Excel was used to form a database of obtained results. Statistical analysis of data was conducted by analysis of variance using Statistics 8.1. (Duncan, 1955). Principal component analysis correlation (PCA) was analyzed by using XL-STAT 2010.

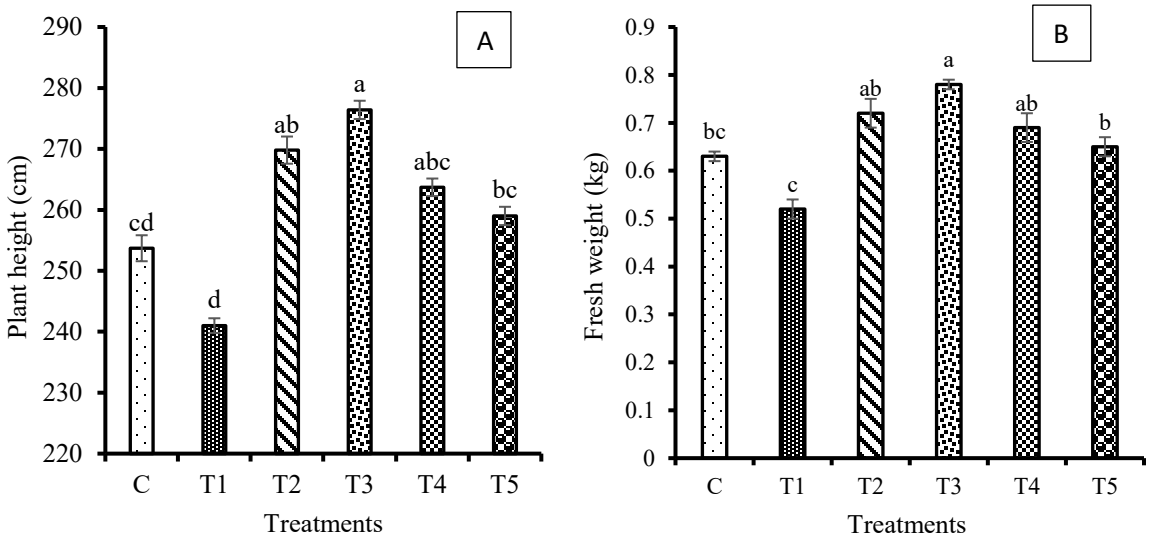
5.3.3. Results

5.3.3.1. Effects of plant growth parameters

An experiment was conducted to analyze the role of selected antagonistic PGPR in the enhancement of plant growth parameters both under normal and infected conditions. After harvesting it was noted that inoculation of *F. moniliforme* pathogen decreases the growth showed disease symptoms and have negative effects on plant growth. Disease inoculation showed decreased plant height (5%), plant fresh weight (27.7), cane length (17.31), cane weight (26.31), cane diameter (14.58), root

length (14.58), Chl a (15.48), Chl b (27.33), carotenoids (28.98), Brix (11.36), Pol (3.36) and CCS contents (7.9). While it was observed that antioxidant enzyme activities SOD (36.6%), POD (51.26%), CAT (71.81%), proline content (62%) and electrolyte leakage (13.82%) increased in disease infected plants

While inoculation of bacterial strains showed dual effects of both plant growth promotion and disease suppression. Different treatments T2, T3, T4, T5 enhanced sugarcane plant height (10.67%, 12.8%, 8.6%, 6.9%), plant fresh weight (27.7%, 33.33%, 24.63%, 6.9%), cane length (17.31%, 20.95%, 15.81%, 12.59%), cane weight (19.67%, 23.43%, 16.94%14.03%) (Fig. 5.3.1). cane diameter (26.31%, 32.25%, 17.64%, 14.28%), root length (14.58%, 19.9%, 9.2%, 9.26%) (Fig. 5.3.2). as compared to diseased plants without any bacterial inoculation. Highest growth promoting response was showed in treatment T3 (diseased plants inoculated with *B. tequilensis*).



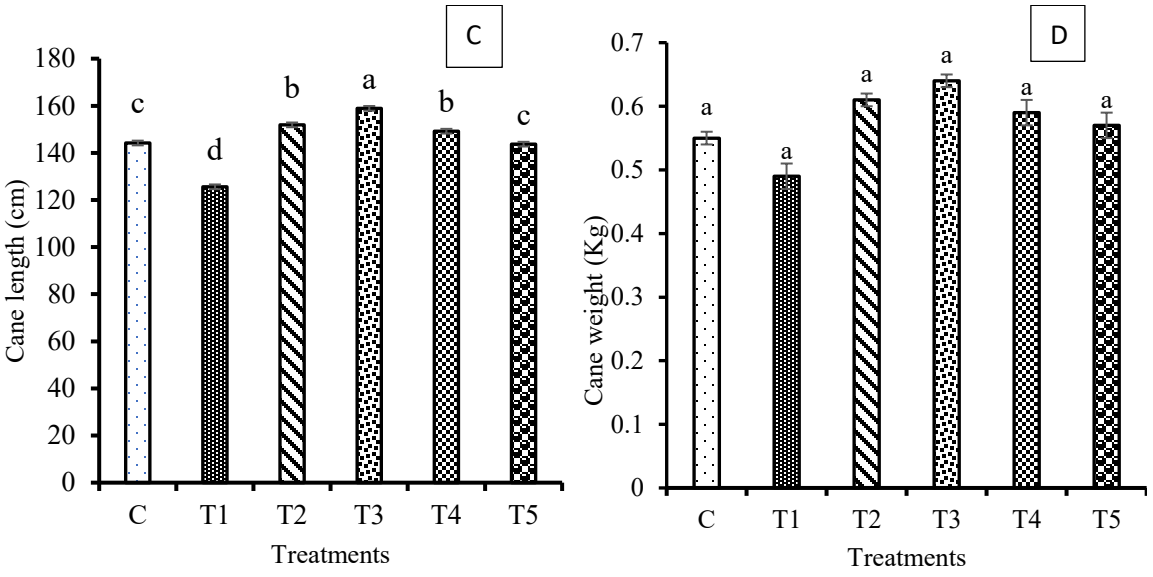


Figure 5.3.1. Effects of PGPR on Plant height (a), Plant fresh weight (b), Cane length (c), Cane weight (d) of sugarcane under biotic stress of *F. moniliforme*. C: Control; T1: *F. moniliforme*; T2: *B. siamensis*+ *F. moniliforme*; T3: *B. tequilensis*+ *F. moniliforme*; T4: *B. gibsonii*+ *F. moniliforme*; T5: *Bacillus sp.* PM 15+ *F. moniliforme*. All means the sharing common letter(s) are significantly not different ($p < 0.05$) by LSD. Standard error of means was represented by vertical bars (n=3).

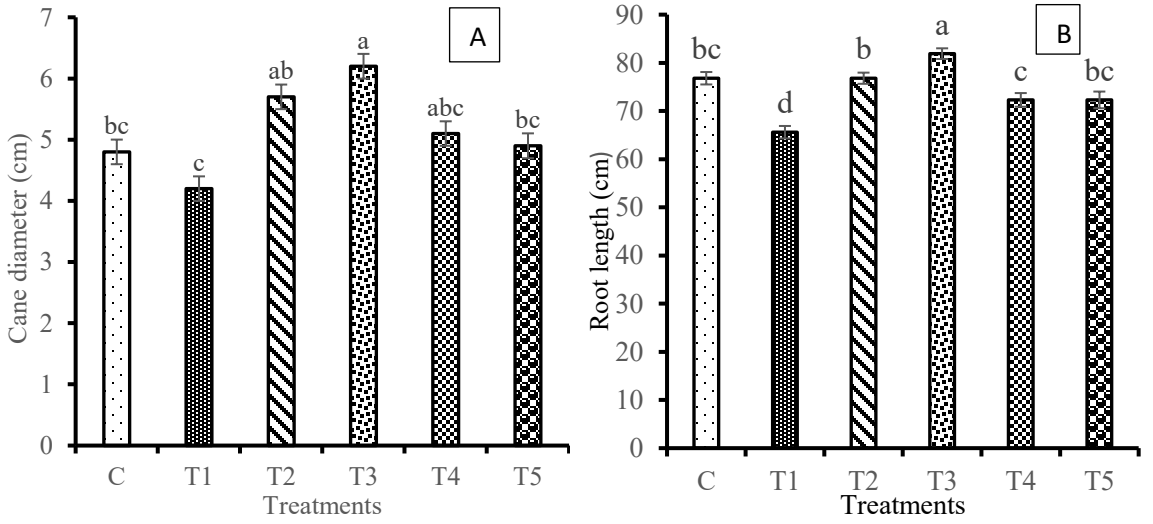
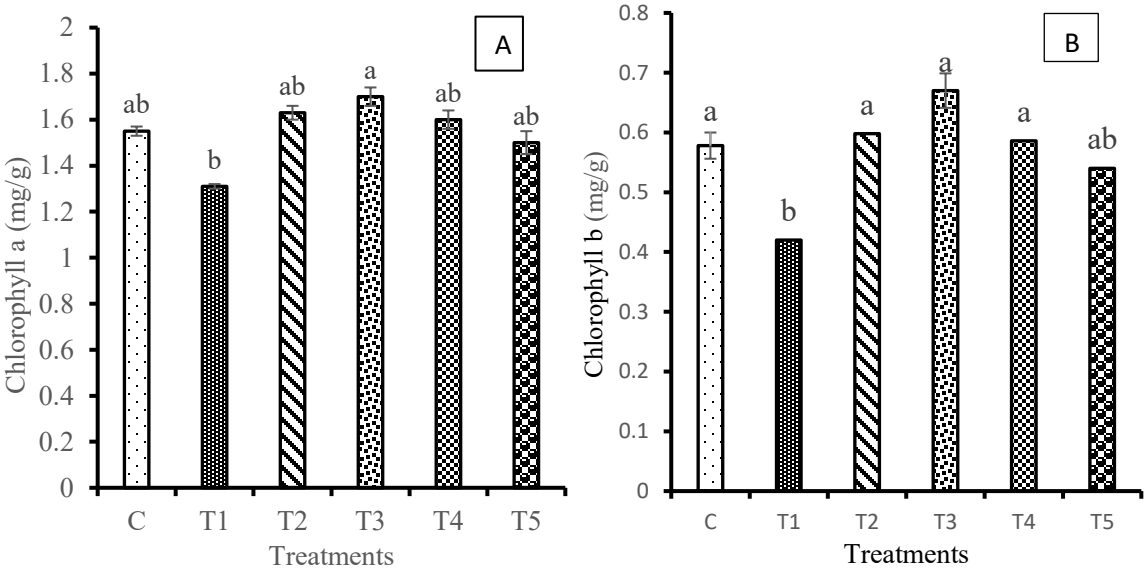


Figure 5.3.2. Effects of PGPR on Diameter of cane (A) Root length (B) of sugarcane under biotic stress of *F. moniliforme*. C: Control; T1: *F. moniliforme*; T2: *B. siamensis*+ *F. moniliforme*; T3: *B. tequilensis*+ *F. moniliforme*; T4: *B. B. gibsonii*+ *F. moniliforme*; T5: *Bacillus sp.* PM 15+ *F.*

moniliforme. All means the sharing common letter(s) are significantly not different ($p < 0.05$) by LSD. Standard error of means was represented by vertical bars (n=3).

5.3.3.1. Effects on chlorophyll and proline contents

Application of disease have negative effects on photosynthetic pigments. Decrease in Chl a (15.48%), Chl b (27.33%), carotenoids (28.98%) was observed. While increase in proline contents (62%) in diseased infected plants showed the defensive mechanism of sugarcane plants. However, it was noticed that applied antagonistic PGPR under different treatments T2, T3, T4, T5 enhance Chl a (19.63%, 22.94%, 18.1%, 12.6%), Chl b (10.74%, 29.76%, 28.32%, 22.22%), carotenoids (32.87%, 39.5%, 28.98%, 24.61%) and proline contents (27.34%, 21.36%, 18.11%, 16.44%) respectively (Fig. 5.3.3).



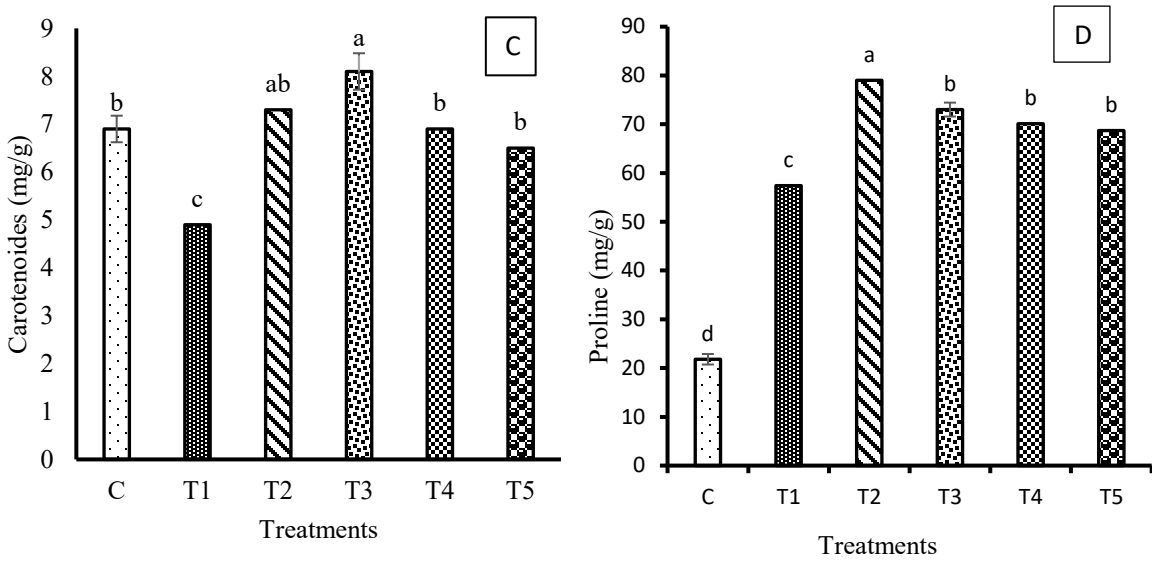


Figure 5.3.3. Effects of PGPR on Chlorophyll a (a), Chlorophyll b (b), Carotenoids (c), Proline (d) of sugarcane under biotic stress of *F. moniliforme*. C: Control; T1: *F. moniliforme*; T2: *B. siamensis*+ *F. moniliforme*; T3: *B. tequilensis*+ *F. moniliforme*; T4: *B. B. gibsonii*+ *F. moniliforme*; T5: *Bacillus sp.* PM 15+ *F. moniliforme*. All means the sharing common letter(s) are significantly not different ($p < 0.05$) by LSD. Standard error of means was represented by vertical bars (n=3).

5.3.3.2. Effects on antioxidant enzyme activities and electrolyte leakage

Increase in antioxidant enzymes under stress conditions revealed their role in scavenging reactive oxygen species (ROS). Same in the case of the current study where pokha boeng infected sugarcane plants showed increased SOD (36.6), POD (51.26%) and catalase activity (71.81%). Under various treatments T2, T3, T4, T5 showed elevated levels of SOD (20.58%, 12.9%, 7.4%, 3.5%), POD (17.91%, 12.5%, 10.65%, 10.18%), and catalase (30.88%, 21.42%, 0%, 9%) enzyme activity in bacterial inoculated diseased plants as compared to disease infected plants without any bacterial inoculation. Electrolyte leakage was prominent in disease infected plants i.e. 13.82% higher than control plants. Under various treatments decreased electrolyte leakage was noticed up to 26.52%, 24.03%, 21.54% 14.81% for T2, T3, T4 and T5, respectively (Fig. 5.3.4).

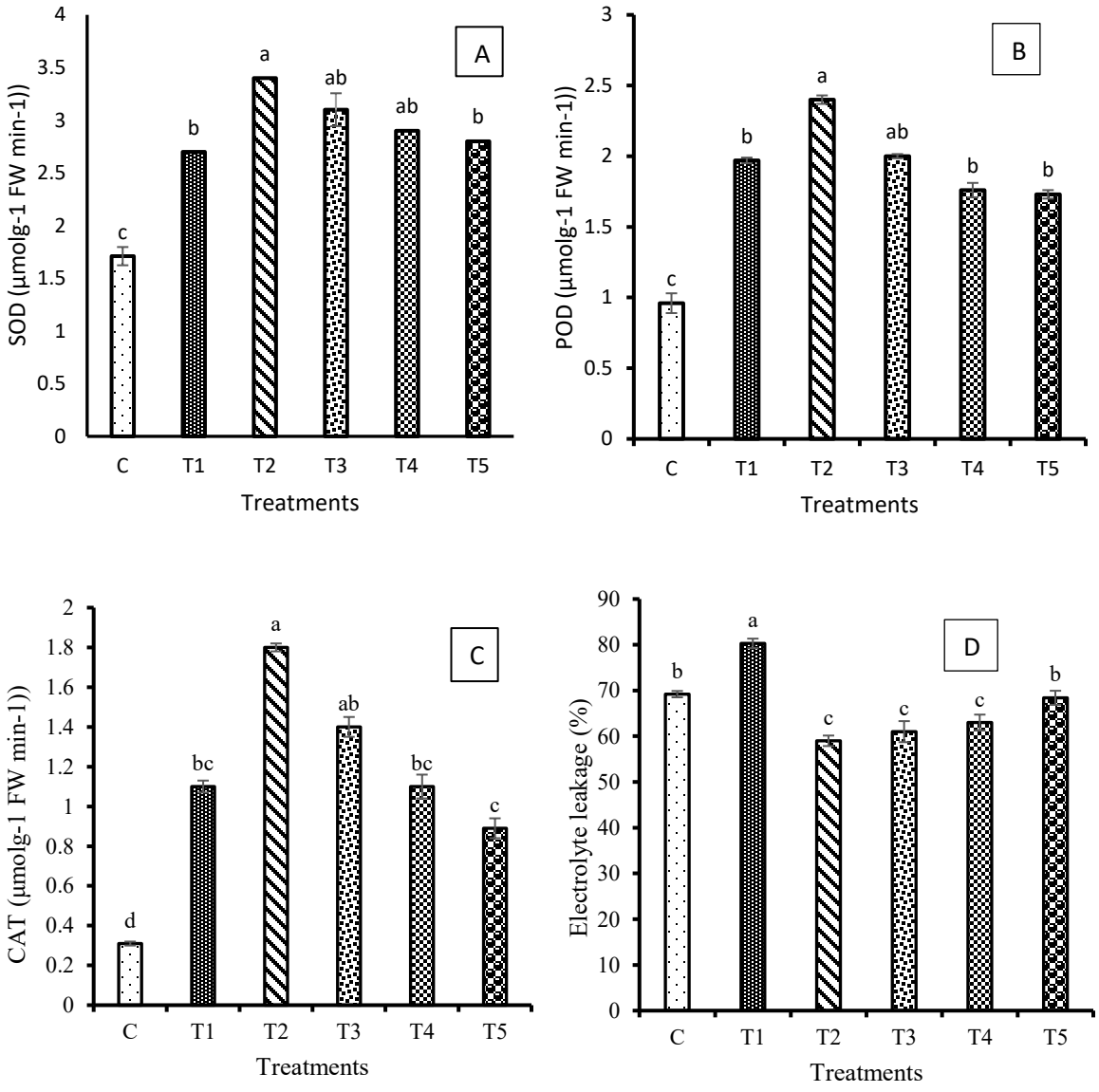


Figure 5.3.4. Effects of PGPR on SOD (a), POD (b), Catalase (c), Electrolyte Leakage (d) of sugarcane under biotic stress of *F. moniliforme*. C: Control; T1: *F. moniliforme*; T2: *B. siamensis*+ *F. moniliforme*; T3: *B. tequilensis*+ *F. moniliforme*; T4: *B. B. gibsonii*+ *F. moniliforme*; T5: *Bacillus sp.* PM 15+ *F. moniliform.* All means the sharing common letter(s) are significantly not different ($p < 0.05$) by LSD. Standard error of means was represented by vertical bars (n=3).

5.3.3.3. Effects of disease and bacterial application on quality control parameters of sugarcane plants

Due to fungal stress decrease in sugarcane quality control parameters Brix (11.36%), Pol (3.36) and CCS (7.9%) was observed. Under treatments T2, T3, T4, T5 comparative increase was observed in Brix (22.92%, 27.23%, 21.68%, 15.58%), POL (16.78%, 18.65%, 15.25%, 13.98%) and CCS (29.69%, 35.19%, 26.58%, 21.62%) as related to diseases plants without any bacterial application (Fig. 5.3.5).

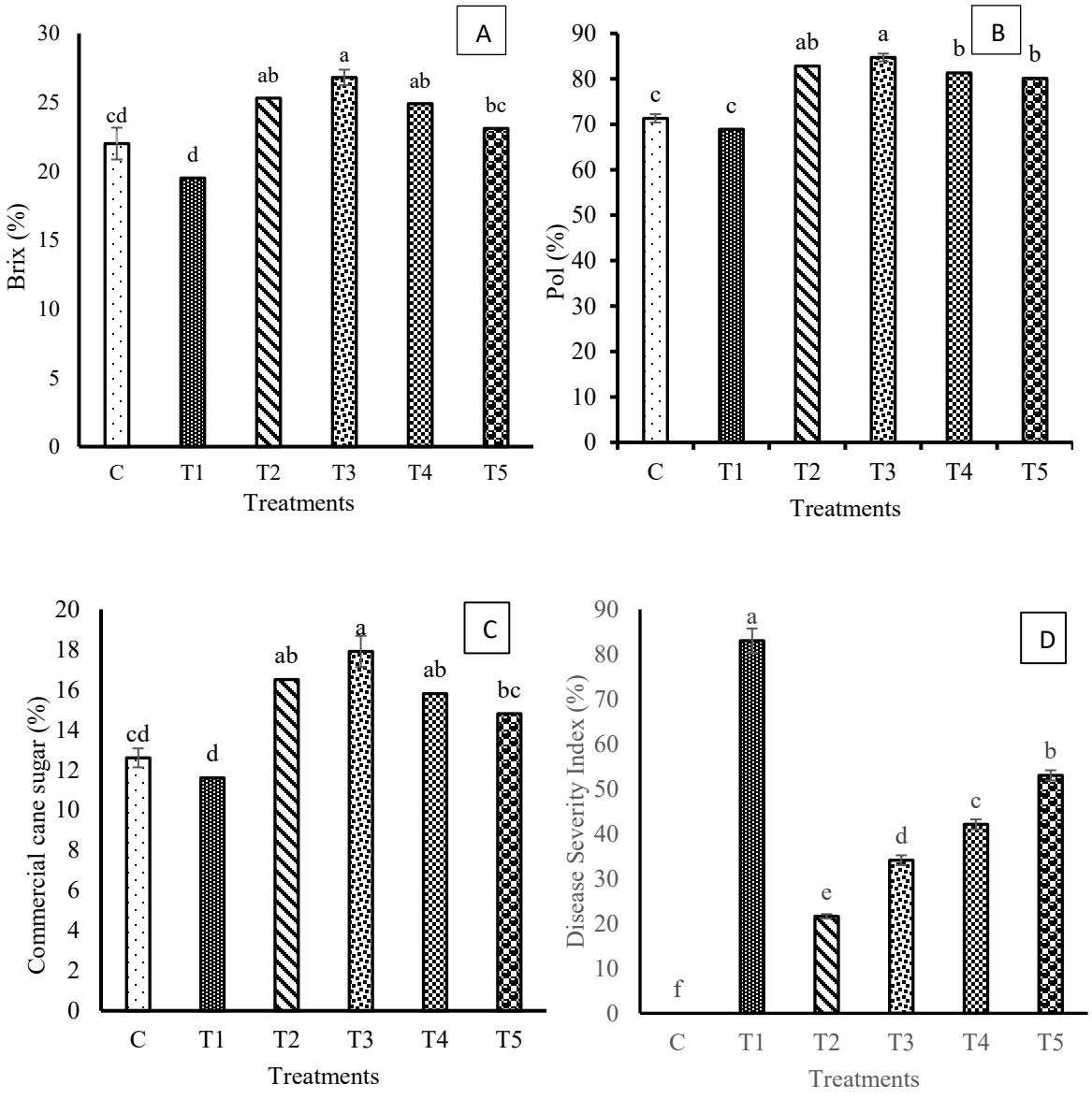


Figure 5.3.5. Effects of PGPR on Brix (a), POL (b), CCS (c) and Disease index (d) of sugarcane under biotic stress of *F. moniliforme*. C: Control; T1: *F. moniliforme*; T2: *B. siamensis*+ *F. moniliforme*; T3: *B. tequilensis*+ *F. moniliforme*; T4: *B. B. gibsonii*+ *F. moniliforme*; T5: *Bacillus sp.* PM 15+ *F. moniliforme*. All means the sharing common letter(s) are significantly not different ($p < 0.05$) by LSD. Standard error of means was represented by vertical bars (n=3).

5.3.3.4. Effects on Pokha boeng disease control

Under control conditions there were no symptoms of pokha beong disease. However, incidence of disease was observed up to 83% as compared to control that confirms the virulence of inoculated *F. monilliforme.* Application of antagonistic PGPR in T2 (*B. siamensis*), T3 (*B. tequilensis*), T4 (*B. gibsonii*), T5 (*Bacillus sp*.) control pokha boeng disease up to 73.97%, 58.9%, 49.27%, 36.14% respectively as compared to diseases plant (Fig. 5.3.5).

5.3.3.5. Correlation analysis

Pearson's correlation biplot between the parameters and treatments under inoculation of antagonistic bacteria were presented in Fig 5.3.6-Fig 5.3.9. Correlation between the parameters showed by red dots while correlation among all treatments are shown by blue dots. Under fungal infection, it was observed that bacterial stains showed positive response towards different plant enhancement parameters through principal component analysis. Variables present in the same quadrat strongly correlated with each other. In Fig 5.3.6 all growth parameters plant height, plant fresh weight, cane length, cane weight, cane diameter, root length, chlorophyll contents and enzyme activities show significant correlation as lies in the same quadrat. Parameters like nodal transduction, nodal length, lesion length and lesion width show correlation among each other. Same trend was observed in Fig 5.3.9. Figure 5.3.7 showed the presence of disease severity index and EL leakage in same quadrat, significantly relate with each other. Presence of SOD, proline, chlorophyll contents, POL, plant height, cane length and root length showed significant relation among each other. However, with all other parameters (POD, catalase, plant fresh weight, cane weight, cane diameter, Brix, commercial cane sugar and disease severity index) reveals its negative correlation present in adjacent but opposite quadrat in bi-plot analysis.

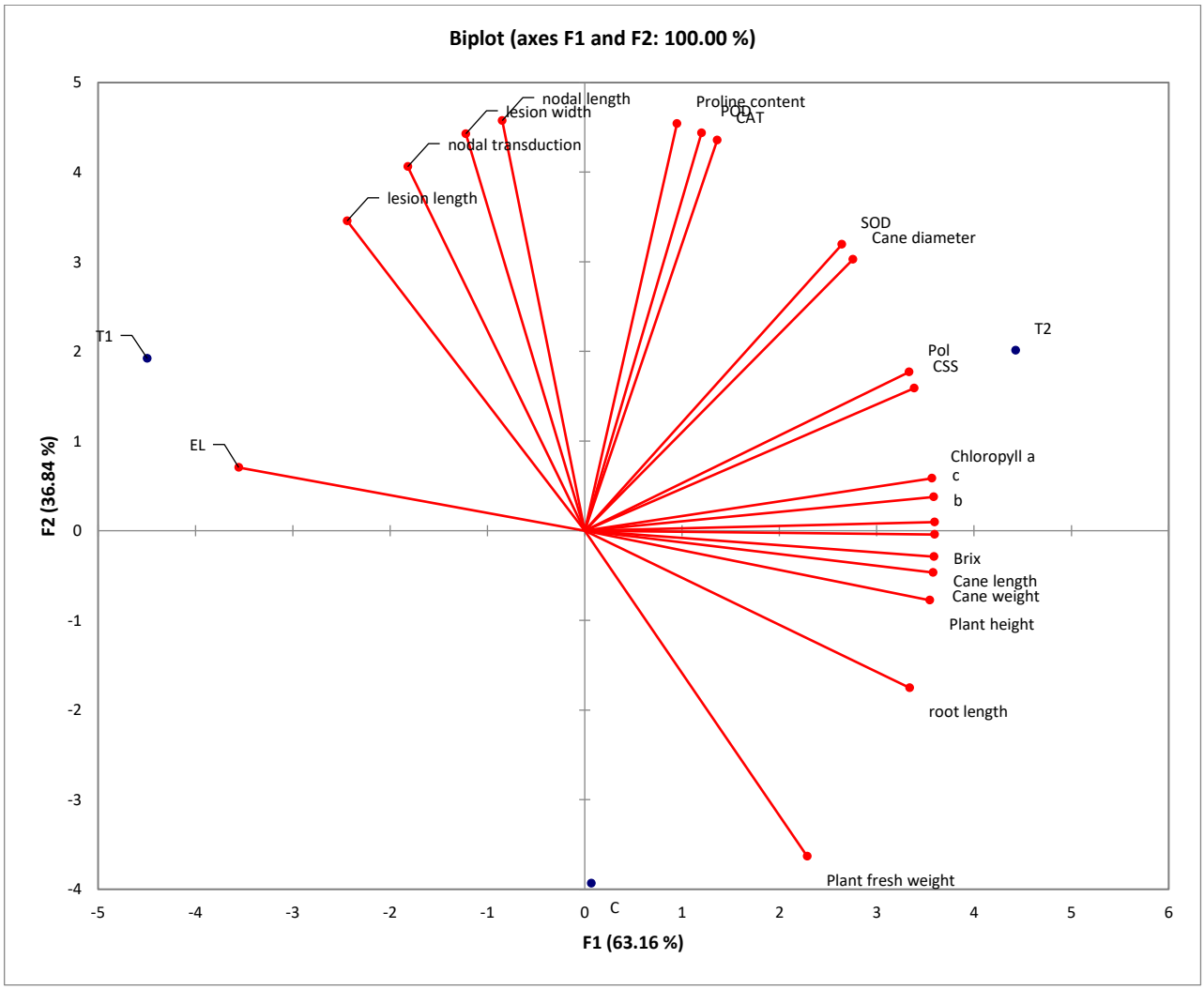


Figure 5.3.6. Pearson correlation plot between the treatments and parameters of sugarcane plants inoculated with *B. siamensis*. The correlation biplot between the F1 and F2 showed the 100% variation in which F1 contributed for 36.84% and F2 contributed 63.16%. Strongly and positively correlated variables were very close to each other and present in the same quadrat. Red dots show a correlation between parameters, while blue dots showed correlation within treatments. The variables which had strong positive correlation are present in the same quadrant and are very close to each other.

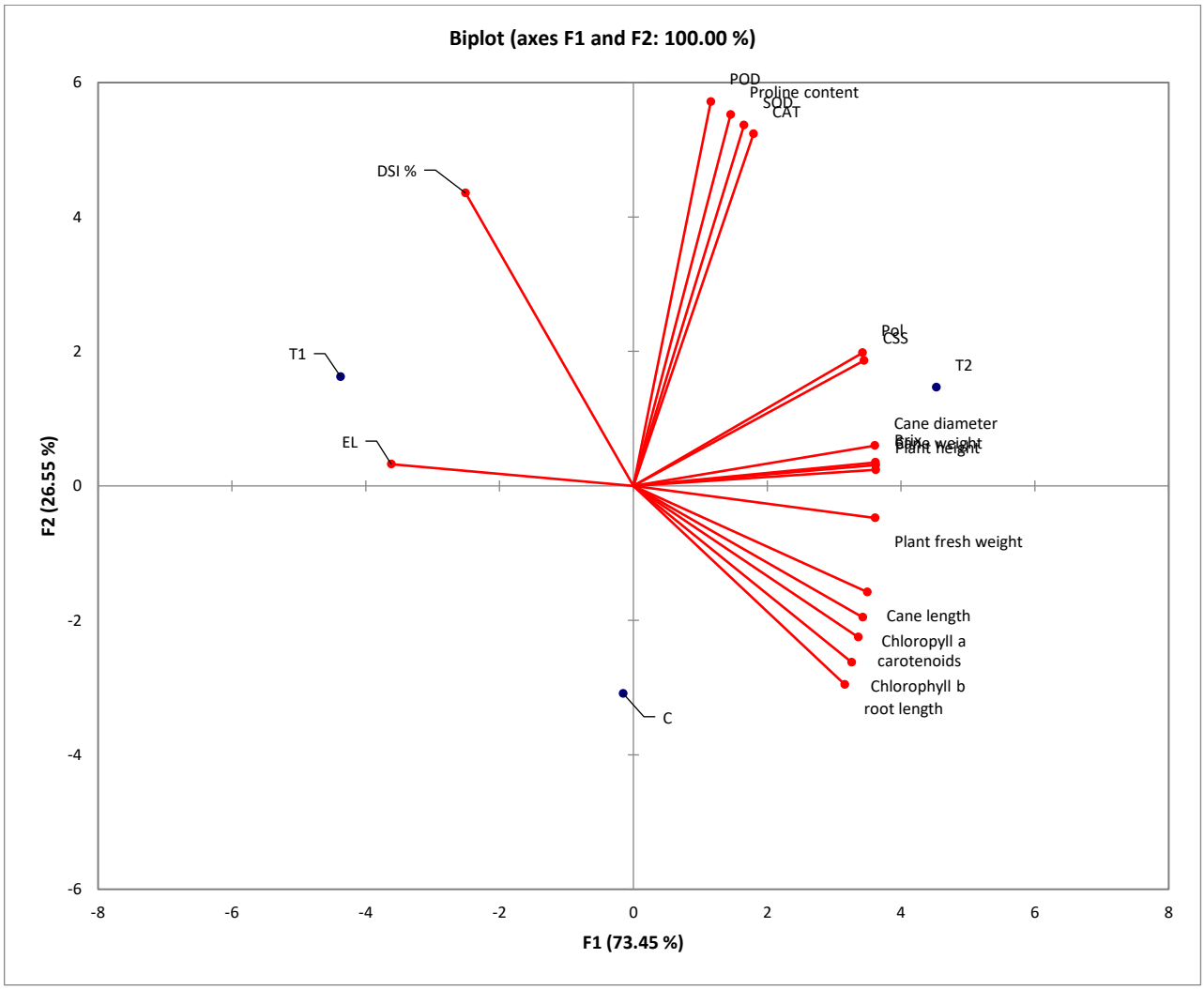


Figure 5.3.7. Pearson correlation plot between the treatments and parameters of sugarcane plants inoculated with *B. tequilensis*. The correlation biplot between the F1 and F2 showed the 100% variation in which F1 contributed for 36.84% and F2 contributed 63.16%. Strongly and positively correlated variables were very close to each other and present in the same quadrat. Red dots show a correlation between parameters, while blue dots showed correlation within treatments. The variables which had strong positive correlation are present in the same quadrant and are very close to each other.

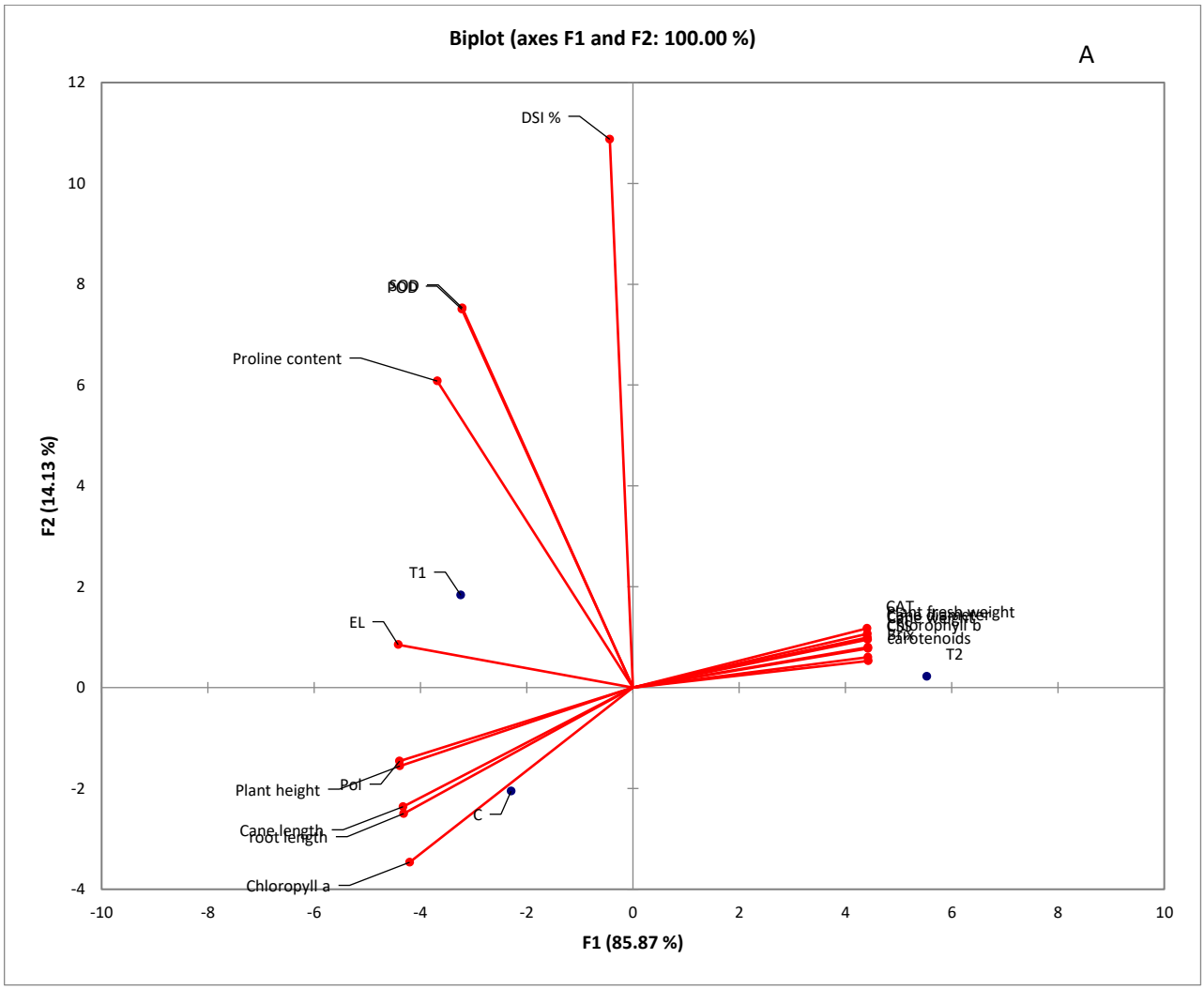


Figure 5.3.8. Pearson correlation plot between the treatments and parameters of sugarcane plants inoculated with *B. gibsonii*. The correlation biplot between the F1 and F2 showed the 100% variation in which F1 contributed for 36.84% and F2 contributed 63.16%. Strongly and positively correlated variables were very close to each other and present in the same quadrat. Red dots show a correlation between parameters, while blue dots showed correlation within treatments. The variables which had strong positive correlation are present in the same quadrant and are very close to each other.

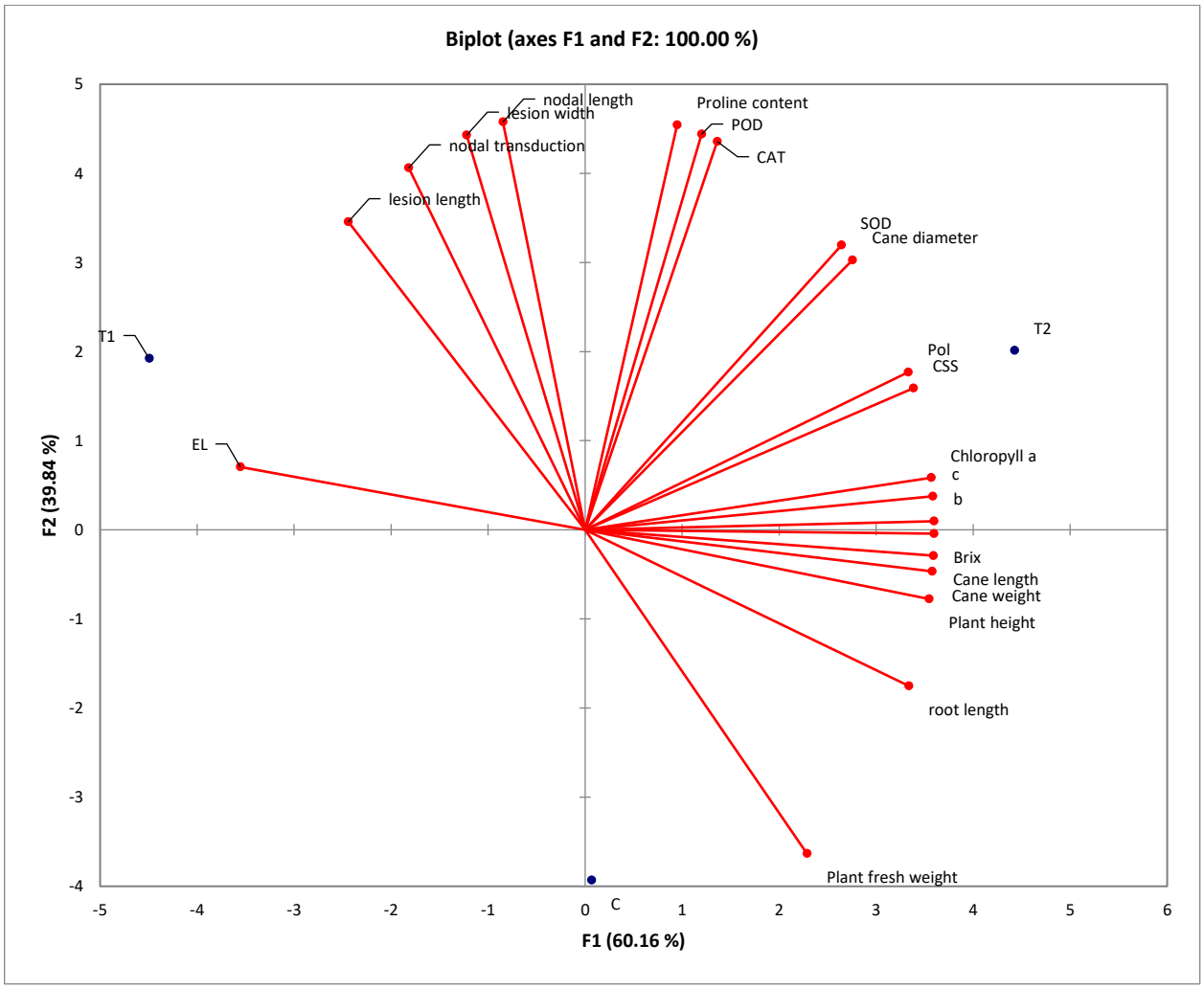


Figure 5.3.9. Pearson correlation plot between the treatments and parameters of sugarcane plants inoculated with *Bacillus sp.* The correlation biplot between the F1 and F2 showed the 100% variation in which F1 contributed for 36.84% and F2 contributed 63.16%. Strongly and positively correlated variables were very close to each other and present in the same quadrat. Red dots show a correlation between parameters, while blue dots showed correlation within treatments. The variables which had strong positive correlation are present in the same quadrant and are very close to each other.

5.3.4. Discussion

Plant pathogens are the most important factors that cause huge losses and damages to agricultural products every year. Sugarcane, a major commercial crop, is susceptible to many microbial diseases like pokha boeng. Biological control of plant diseases using microorganisms, including bacteria, is an effective ecofriendly alternative (Singh 2014). Four bacterial strains (*Bacillus siamensis* PM13*, Bacillus tequilensis* PM14*, Bacillus gibonii* PM11 and *Bacillus sp.* PM15) isolated from different rhizosphric soil samples of sugarcane plant were screened for the antifungal activity against phytopathogenic fungi *Fusarium moniliforme*; the causal agent of sugarcane pokha boeng disease.

In our study, the experimental plants infected with pokha boeng showed reduced growth as it retards the internodal elongation in stalks of the sugarcane plant. Shortened internodes directly affect the plant growth and cane yield. In severe cases *F. monilliforme* affects the growing points of cane that can't be recovered (Avelino et al., 2020). Moreover, metabolism of plants shifts towards the management of infection that retard the growth of plant. Previously, reduction in weight, length, total juice, POL%, cane diameter, plant internode was observed in various varieties of the sugarcane (Singh et al., 2018). However, the application of antagonistic bacterial strains alleviates the negative effects of fungal infection. Bacterial strains *Bacillus siamensis, B. tequilensis, B. gibsonii; Bacillus sp.* showed various activities like phosphate solubilization, IAA and siderophore production demonstrating that these strains have plant growth promotion abilities by enhancing photosynthetic and respirational activities of plants (Singh et al., 2008). Enhancement of growth is correlated with the production of phytohormones (IAA) by PGPR in the rhizosphere. Ammonia production ability indicates that these strains have more nitrate producing ability and play role in plant growth promotion (Backer et al., 2018). Mineralization is carried out by various phosphatase enzymes secreted by rhizospheric bacteria. As evident by results given in the initial parts of our research work indicates the production of hydrolytic enzymes (cellulase and chitinase) that are also involved in fungal cell wall damage. This mechanism might be used as a defense strategy against phytopathogens by inoculated bacterial isolates PM11, PM13, PM14, and PM15. Pectinase production by PGPR is reported to have dual advantage over other sources because it can be subjected to genetic and environmental manipulations to increase yield under various stress conditions including biotic stress (Pandey et al., 2017). Most *Bacillus spp.* were reported to enhance the production of pectinase (Hirose *et al*., 1999). From our results, it is clearly

demonstrated that PGPR treated plants showed an increment in plant height, shoot length, root length, cane length, cane diameter and total biomass as compared to control.

Along with the disease suppression, PGPR under current study showed the ability of phytohormone production like IAA which directly enhanced plant growth parameters like plant growth, fresh weight, cane length, cane weight, cane diameter and root length (Guerrieri et al., 2020). It also plays a beneficial role in enhanced growth of superficial or interior of roots deep in the soil that increase the surface area for nutrients (phosphorus and iron) and water uptake that plays a vital role in various metabolic processes.

It was also observed that under biotic stress photosynthetic pigments (Chl a, Chl b and carotenoids) of plants decreased as compared to control while PGPR application enhanced their levels under biotic stress alleviating negative effects of fungal infection and boost up the photosynthetic activities (Gouda et al., 2018). Elevated levels of proline defend infected plants by maintaining cell water content and membrane protection that also prevent electrolyte leakage in bacterial inoculated fungal infected plants as compared to only bacterial inoculated plants.

Results from spectrophotometer analysis indicated that *Fusarium* disease resulted in an increment of antioxidant enzymes in diseased plant. These antioxidant enzymes catalyze the conversion of free radicals and their enhanced activities contributed to bio-protection of plants against pathogens and insects. In the present study, an increment in SOD activity was observed in plants under disease stress as compared to non-treated control (Sarker and Oba, 2018). Similarly, *Mycosphaerella fragariae* infected leaves of strawberry plants showed maximum SOD activity as compared to control plants and resilient in resistant strawberry plants. Our results are also supported by a study of tomato plants infected with fungus *Botrytis cinerea* where the symptoms of necrosis were observed that results in the increment of SOD and POD in plant leaves (Kuźniak and Skłodowska, 2004). Due to the pathogen infection in the stalk tissues, the affected canes show poor juice quality as observed by Brix, POL% and CCS values in harvested diseased plants as compared to control plants without any fungal infection (Esh et al., 2010). Low juice quality is might be due to the reduction in sucrose content and an increase in reducing sugars, titrable acidity, gums, flavonoids and other soluble salts. Besides, our bacteria play dual role they deteriorate the fungal attack and its negative effects and side by side increase plant growth and cane quality that leads to quality juice production (Frey-Klett et al., 2011). Highest fungal disease control was observed in plants

treated with antagonistic PGPR *Bacillus siamensis* PM13 followed by *Bacillus tequilensis* PM17 > *Bacillus gibsonii* PM11 > *Bacillus sp*. PM15. As screened earlier these strains possess the potential to produce antifungal metabolites (siderophores, chitinase, cellulase and HCN) that play a vital role in pathogen suppression. Antagonistic bacteria also alter host defense mechanisms that accounts for the suppression of fungal diseases. Efficacious bacterial antagonists often show a synergistic combination of various mechanisms responsible for an effective antifungal interaction as also observed in our study.

5.3.5. Conclusion

It is concluded that inoculated PGPR *Bacillus gibsonii* PM11, *Bacillus siamensis* PM13, *Bacillus sp.* PM15 and *Bacillus tequilensis* PM17 having biocontrol ability to overcome the drastic effects of *F. moniliforme* attack and revealed the potential role in enhanced growth. Maximum enhanced growth was observed in sugarcane plants treated with strain *B. tequilensis* PM17 that showed increase in plant height (12.8%), plant fresh weight (33.33%), cane length (20.95%), cane weight (23.43%), cane diameter (32.25%), root length (19.9%), chl a (22.99%), chl b (29.76%) carotenoids (39.5%) and proline contents (21.36%) as compared to other treatments. However, more values of SOD (20.58%), POD (17.91%), catalase (30.88%) and electrolyte leakage (26.52%) were observed in pokha boeng infected plants inoculated with *B. siamensis* PM13 that revealed their role in maximum disease control (73.97%) as compared to other treatments. Following PM13 strain, *Bacillus. tequilensis*> *Bacillus xiamenesis*>*Bravebacterium frigoritolerans* also showed same trend of enhanced growth and disease suppression. This paves the way to explore out more proficient bacterial isolates that could perform in different biotic conditions and provide more tolerance in such conditions. This will also provide a gateway to test these strains at field level for sustainable agricultural practices.

Experiment 4:

Application of biologically produced nanoparticles on plant growth and sugarcane fungal disease control

5.4.1. Introduction

Plants showed a remarkable ability to develop specific mechanisms to maintain its proper growth and development either under favorable or unfavorable conditions. Nowadays, research is focused to develop new methods and techniques that could be suitable for plants to boost their native functions under fluctuating environments. In the current scenario, scientists observe the use of nanoparticles (NPs) as a very attractive approach to handle in biological systems. From previous reports, NPs are found to be very suitable in sensing and detection of biological structures and systems (Singh et al., 2008). Beyond other uses, NPs showed a promise in diverse fields of agricultural biotechnology (Majumder et al., 2007). They showed exclusive biological and physicochemical properties and the potential to boost up plant metabolisms (Giraldo et al., 2014). Using different fertilizers is very important for plant growth and development, but most of the used fertilizers are rendered unavailable to plants due to many factors, such as leaching, degradation by photolysis, hydrolysis, and decomposition. Therefore, it is necessary to minimize nutrient losses in fertilization and to increase the crop yield through the exploitation of new applications with the help of nanotechnology and nanomaterials. Nano-fertilzers or nano encapsulated nutrients might have properties that are effective to crops, release the nutrients on demand, control the release of chemical fertilizers that regulate plant growth, and enhance target activity (Cossins et al., 2014). Agricultural application of NPs is currently an interesting areato minimize the use of chemical fertilizers and improves growth and yield of crops (Majumder et al., 2007; Usman et al., 2020). The introduction of nanoparticles into plants might have a significant impact, and thus, they can be used for agricultural applications for better growth and yield (Sadak et al., 2019). However, a thorough understanding of the role of nano-sized engineered materials in plant physiology to combat fungal diseases is scarce (Siddiqui and Al- Whaibi 2014). Moreover, the mode of action of nanoparticles on plant growth and development is also limited (Wang et al., 2001). Silver nanoparticles (AgNPs) are currently the most produced engineered nanomaterials (Davies 2009). AgNPs have been implicated in agriculture for improving crop growth and yield. Several reports indicate that appropriate concentrations of AgNPs play an important role in enhanced seed germination (Shelar and Chavan 2015) and plant growth (Sharma et al., 2012; Kaveh et al., 2013; Vannini et al., 2013), improving photosynthetic quantum efficiency and chlorophyll content (Sharma et al., 2012; Hatami and Ghorbanpour 2013), increasing water and fertilizer utilization efficiency (Lu et al., 2002) and to control phytopathogens (Mousa et al., 2015). Therefore, the aim

of this work is to study the role of using biologically produced AgNPs in growth enhancement, some biochemical attributes like antioxidant enzyme activities, proline content, and yield quantity of sugarcane plant.

5.4.2. Material and methods

5.4.2.1. Selection of silver nanoparticles and their application

Silver nanoparticles produced by using an aqueous solution of sugarcane husk was used for their application on sugarcane plants to control two pathogens of sugarcane (*Colletotricum falcatum* and *Fusarium moniliforme*). The reason behind the selection of sugarcane husk-based nanoparticles is their highest *invitro* antifungal activity (Table 4.1) as compared to other biologically produced nanoparticles. Reason might be the smallest size of nanoparticles produced in earlier experiments. Applications of 50 ppm of nanoparticles to sugarcane plants was done by foliar spray (El-Waseif et al., 2019)

5.4.2.2. Glasshouse Experiment: application of nanoparticles

Experimental soil was taken from National Agricultural Research Centre, Islamabad, Pakistan (33.6701° N, 73.1261° E). The soil related parameters were mentioned previously in Table 5.1.1. To scrutinize the efficacy of bio fabricated AgNPs as antimicrobial agents, experiment was designed with four treatments and 3 replicates for each treatment. i) C was taken as control without AgNPs application/fungal disease, ii) T1; Plants treated with AgNPs iii) T2; Plants inoculated with fungal pathogen *C. falcatum* iv) T4; Plants inoculated both with AgNPs and *C. falcatum* v) T5; Plants inoculated with fungal pathogen *F. monilliforme* vi) T6; Plants inoculated both with AgNPs and *F. monilliforme*. After completion of experiment, plants were harvested and preserved for further analysis.

5.4.2.3. Measurement of plant growth parameters

While harvesting, plant height, plant fresh weight, cane length, cane weight, cane diameter and root length of sugarcane plants grown under various treatments were measured (Ferreira et al., 2017).

5.4.2.4. Photosynthetic pigments

Fresh leaf material (0.05 g) was homogenized in a mortar with 10 ml of 80% acetone. Homogenized material was incubated in the dark to extract the pigment. Tubes were centrifuged for 15 min at

5000 rpm. Supernatant was used to quantify the photosynthetic pigments by using spectrophotometer (Amna et al., 2019).

5.4.2.5. Estimation of antioxidant enzyme activity proline content and electrolyte leakage

To access superoxidase dismutase (SOD) activity, the method reported by Afridi et al., (2019) was followed. Peroxidase (POD) activity was determined by the modified method of (Afridi et al., 2019). Production of proline under different treatments was also checked by following the method of Amna et al. (2015). Electrolyte leakage of leaves was measured by using EC meter by following the protocol of Batool et al., (2019).

5.4.2.6. Disease Severity index

Disease severity index of red rot and pokha boeng in sugarcane plants was measured by following the protocol of Nadeem et al. (2014) and Zhang et al. (2017) respectively.

5.4.2.7. Quality control of sugarcane

For qualitative control parameters of sugarcane (Brix, Pol and Commercial Cane Sugar%), the canes were collected from each replicate (Meade and Chen, 1977). These canes were washed and crushed with a cutter grinder. Then five hundred grams of crushed canes processed through hydraulic press. Obtained juice of sugarcane was collected in a 500 ml glass beaker.

Brix (%) To determine the level of brix i.e. concentration of total soluble solids a drop of extracted cane juice was positioned on the prism of Refractometer (PR-101, ATAGO Co. Ltd, Japan) with the help of pipette.

Pol (%) In this case the extracted sugarcane juice was treated by following Horns Lead Acetate method. To obtain good juice clearness, lead acetate (4 g) was carefully mixed in juice (100 ml) with the help of glass rod. Juice was carefully filtered using Whattman filter paper No.1 and used further to take the POL% reading. (Magwaza and Opara, 2015).

Commercial Cane Sugar (CCS) of the extracted sugarcane juice was determined based on the values of brix and pol (Meade and Chen, 1977).

5.4.2.8. Statistical analysis

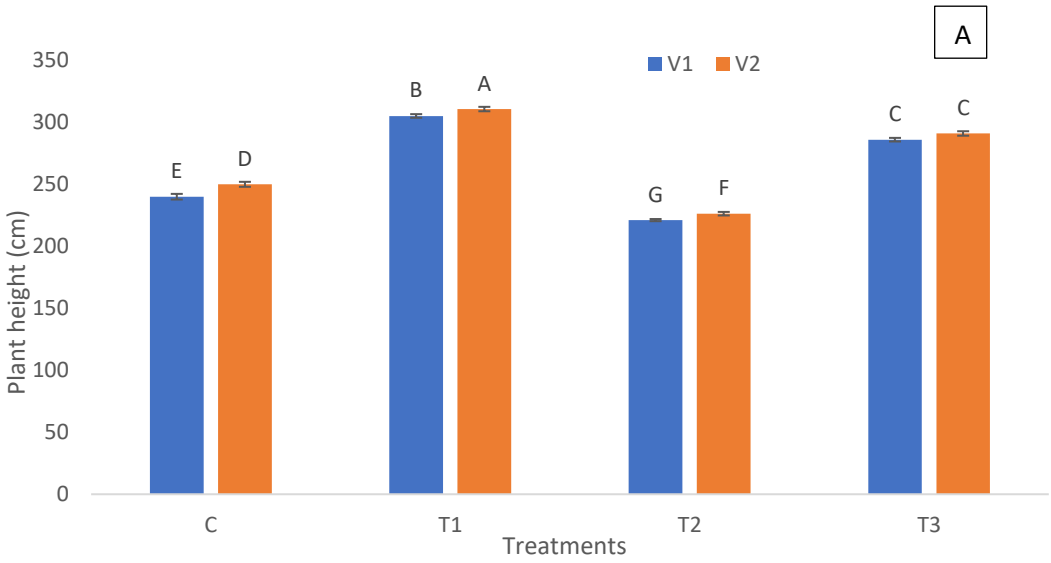
All experiments and analysis were done in triplicates. Excel was used to form a database of obtained results. Statistical analysis of data was conducted by analysis of variance using Statistics 8.1. (Duncan, 1955). Principal component analysis correlation (PCA) was analyzed by using XL-STAT 2010.

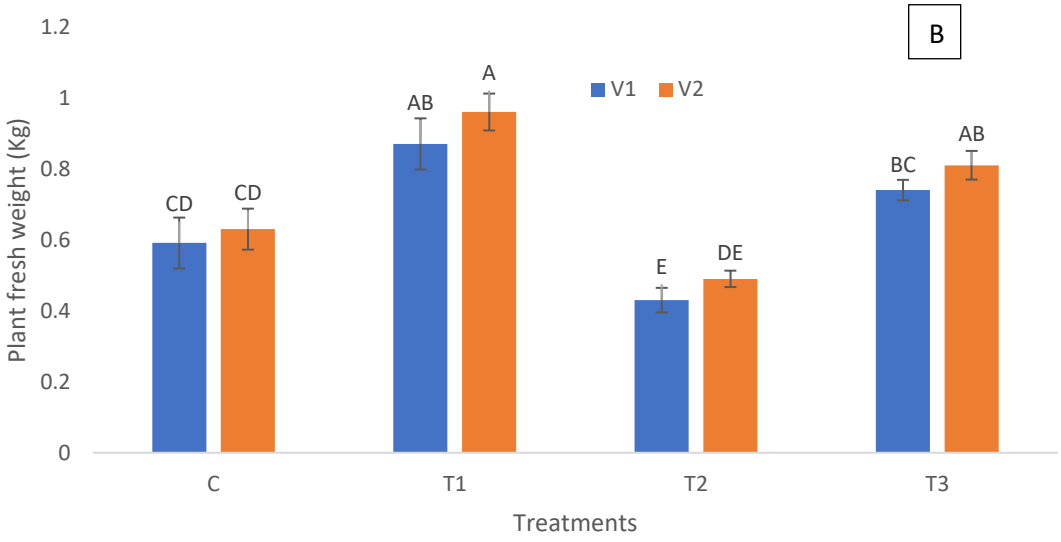
5.4.3. Results

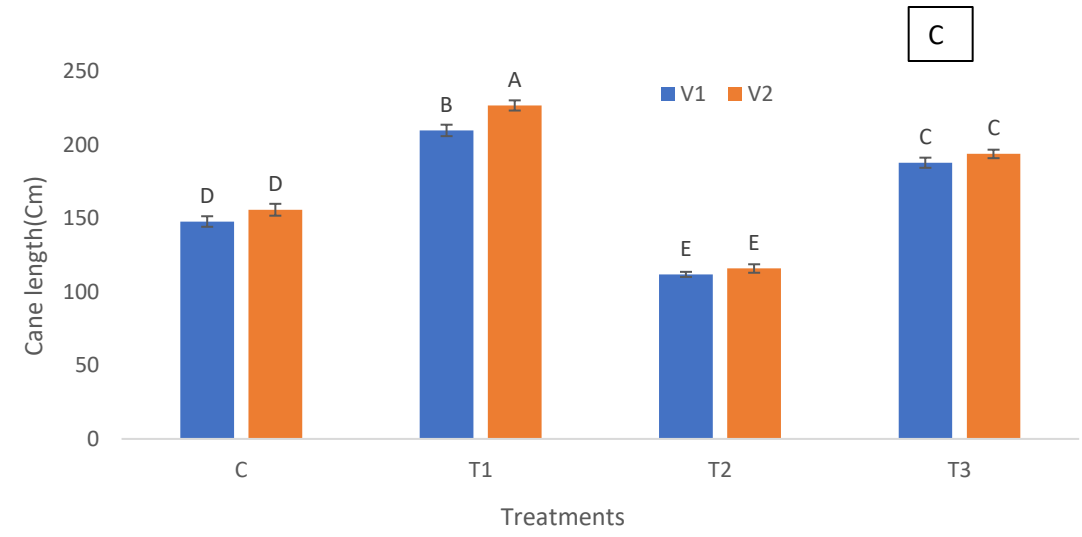
There were positive effects of biogenically produced AgNP's on all growth parameters of two varieties of sugarcane both under normal and diseased conditions. Foliar application of sugarcane plants with AgNP's increased plant height (21.34%), plant fresh weight (32.06), cane length (25.86), cane diameter (25.86), cane weight (27.39), and root length (21.34) in variety 1. In case of variety 2 same trend was observed for increased plant height (19.58%), plant fresh weight (34.375%), cane length (31.27%), cane diameter (15.15%), cane weight (32.14%), and root length (19.35%). Variety 2 showed comparatively better response towards the application of silver nanoparticles as compared to variety 1 (Fig. 5.4.1).

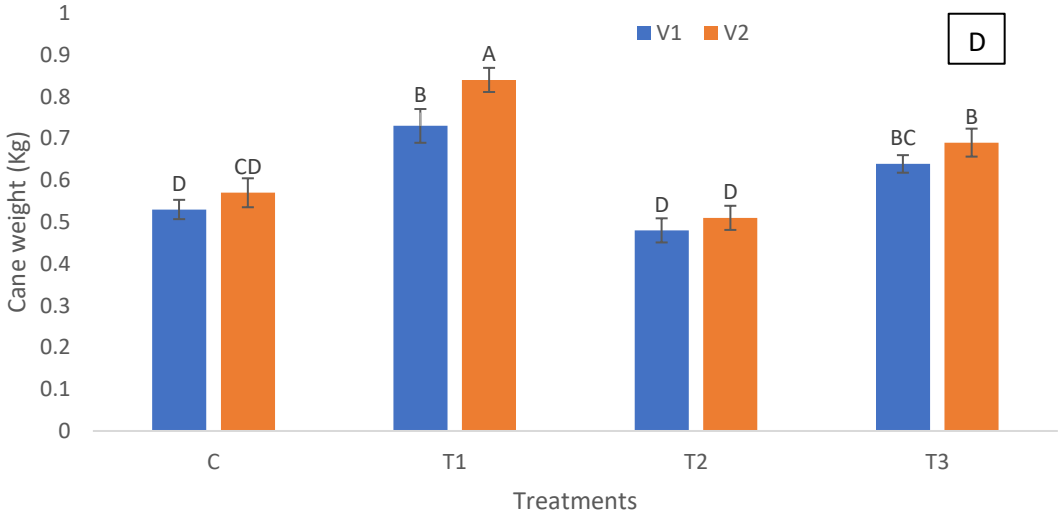
5.4.3.1. Effects of nanoparticle application on sugarcane plant growth

Data clearly revealed the gradual increase of growth parameters even under disease stress with application of nanoparticles as compared to diseased plants without any application of nanoparticles. Under disease stress, application of nanoparticles enhanced plant height (22.68%, 22.23%), plant fresh weight (41.89%, 39.5%), cane length (40.42%, 40.20%), cane fresh weight (24.88%, 26%), cane diameter (25.49%, 30%), and root length (23.80%, 21.83%) in both varieties V1 and V2 respectively (Fig. 5.4.1).


A
V1
V2
Plant height (cm)
350
300
250
200
150
100
50
0
E
D
B
A
G
F
C
C
C
T1
T2
T3
Treatments


B
V1
V2
Plant fresh weight (Kg)
1.2
1
0.8
0.6
0.4
0.2
0
CD
CD
AB
A
E
DE
BC
AB
C
T1
T2
T3
Treatments


C
250
200
150
100
50
0
Cane length(Cm)
V1
V2
D
D
B
A
E
E
C
C
C
T1
T2
T3
Treatments


D
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Cane weight (Kg)
V1
V2
D
CD
B
A
D
D
BC
B
C
T1
T2
T3
Treatments

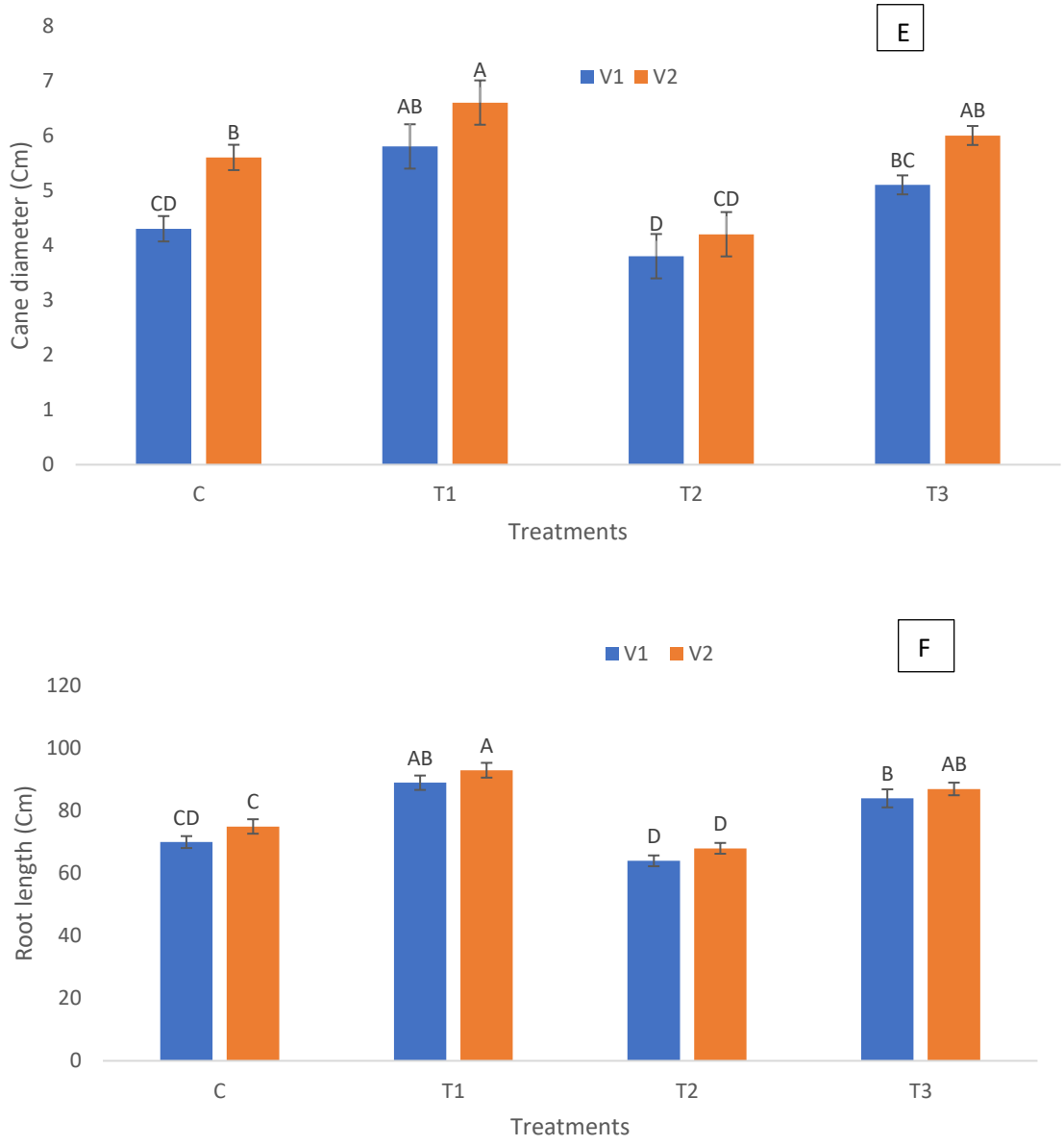
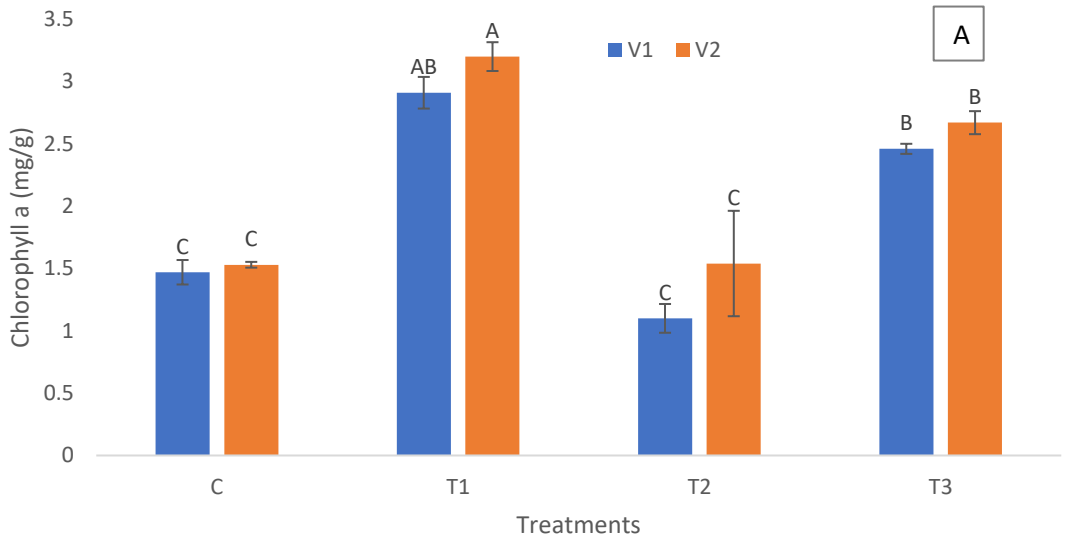
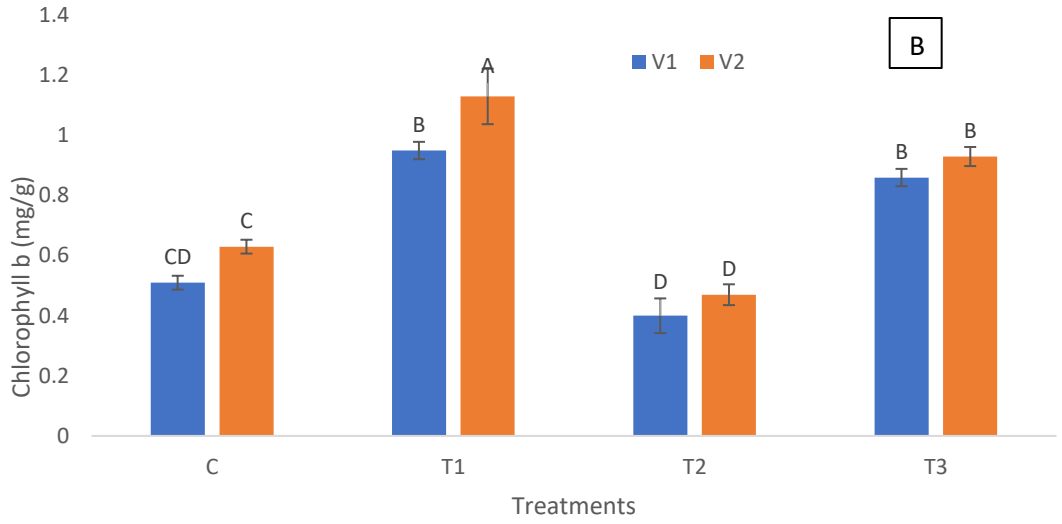


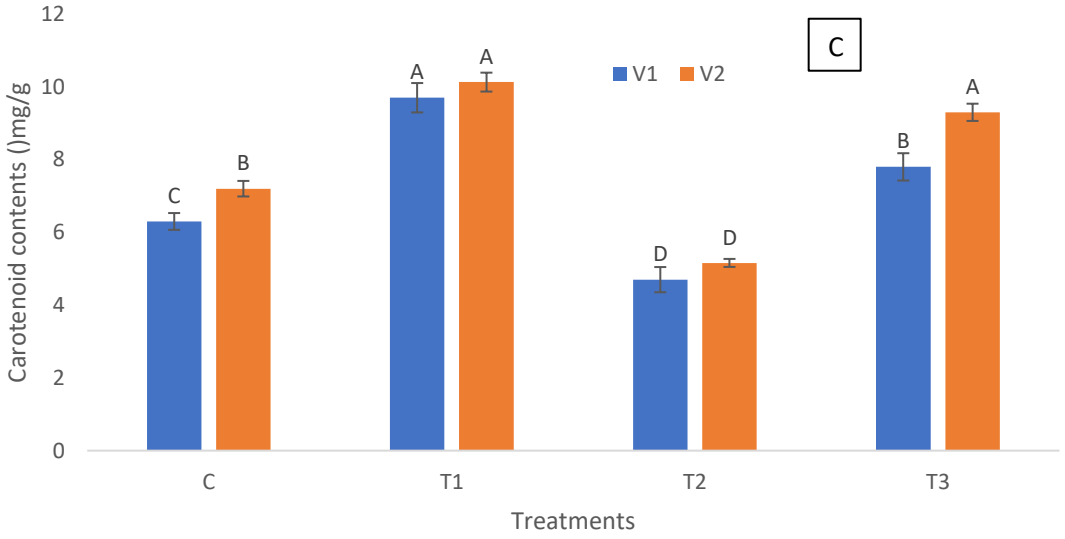
Figure 5.4.1. Effects of silver nanoparticles on Plant height (a), Plant fresh weight (b), Cane length (c), Cane weight (d) Diameter of cane (e) Root length (f) of two sugarcane varieties under biotic stress of *C. falcatum* and *F. moniliforme*. C: Control; T1: treated with nanoparticles; T2: Fungal infection T3: fungal infection+ Nanoparticles. V1: variety susceptible to *C. falcatum*; V2: variety susceptible to *F. moniliforme.* All means the sharing common letter(s) are significantly not different ($p < 0.05$) by LSD. Standard error of means was represented by vertical bars (n=3).

5.4.3.2. Effects of nanoparticles on photosynthetic pigments and proline contents

The response of photosynthetic pigments of sugarcane leaves sprayed with different concentrations of AgNPs are shown in Fig 3. The results revealed the significant increases in all photosynthetic pigment contents (chlorophyll a, chlorophyll b, carotenoids) in response to treatment with AgNPs. Under disease stress, application of nanoparticles increased Chl a (55.28%, 42.32%), Chl b (53.48%, 49.46%), carotenoids (39.74%, 44.51%), and proline contents (41.56%, 44%) in V1 and V2 respectively (Fig. 5.4.2)




B
V1
V2
Chlorophyll b (mg/g)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CD
C
B
A
D
D
B
B
C
T1
T2
T3
Treatments


C
V1
V2
Carotenoid contents ()mg/g
12
10
8
6
4
2
0
C
B
A
A
D
D
B
A
C
T1
T2
T3
Treatments

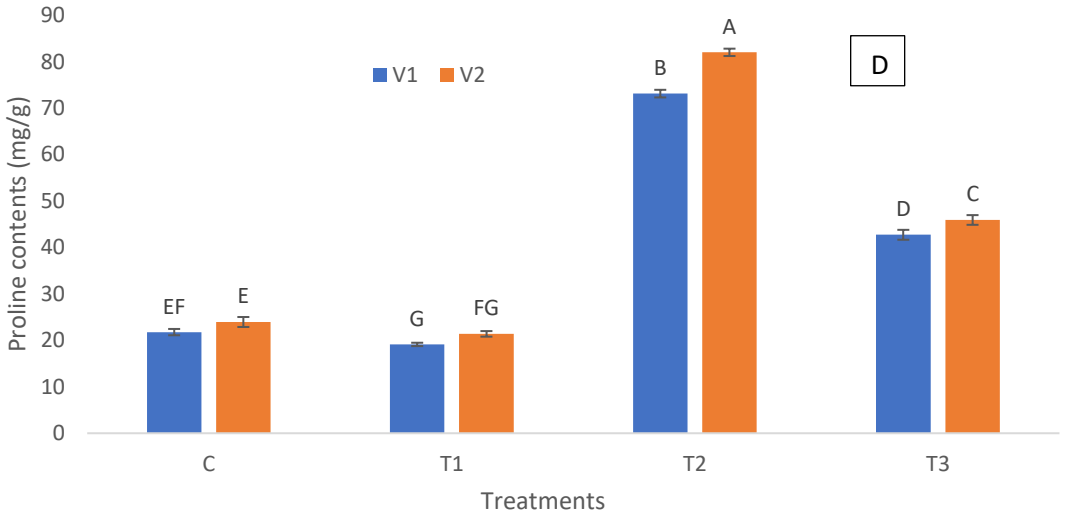
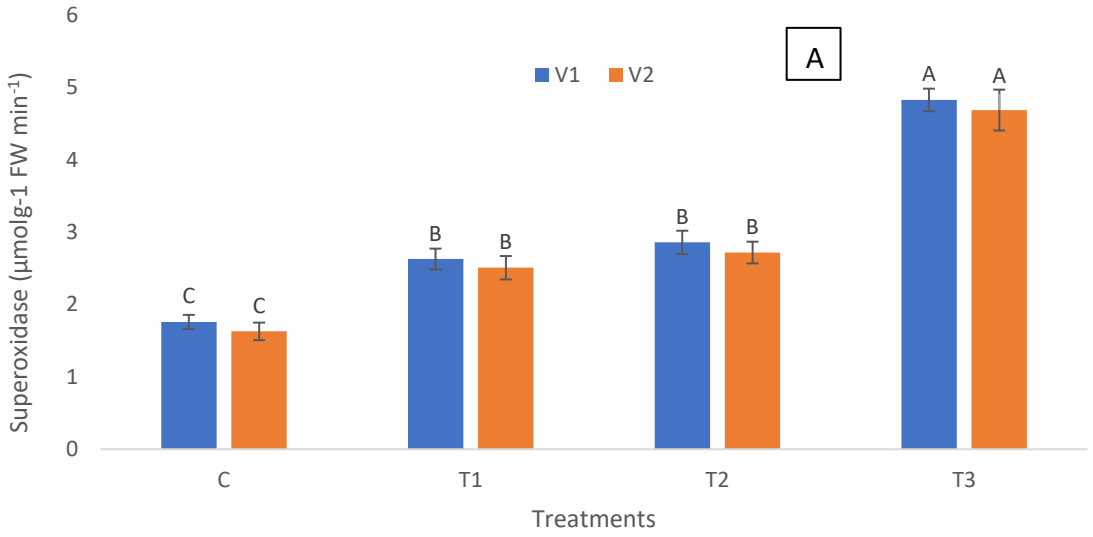
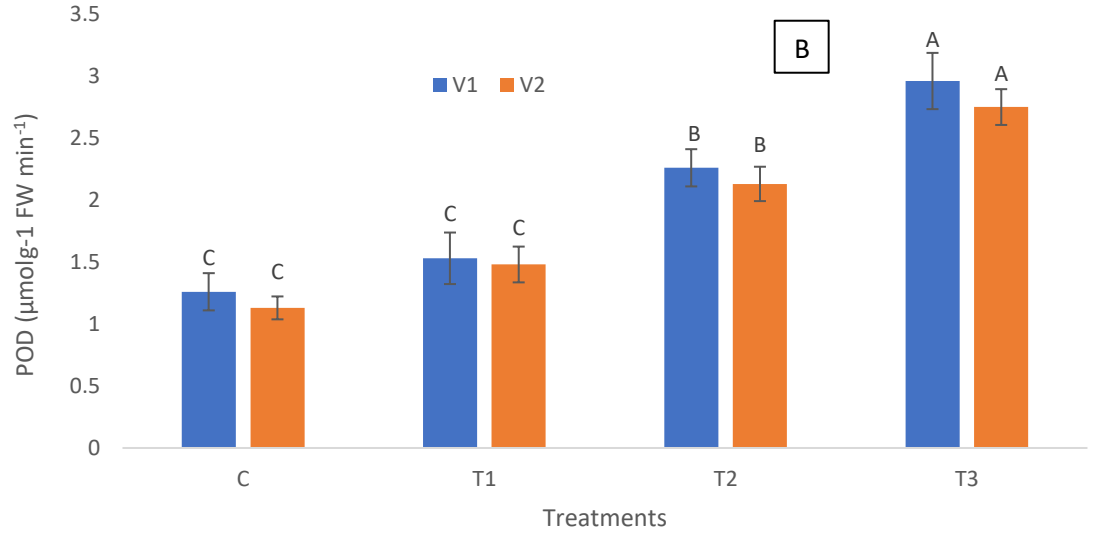


Figure 5.4.2. Effects of silver nanoparticles on Chlorophyll a (a), Chlorophyll b (b), Carotenoids (c), Proline contents (d) of two sugarcane varieties under biotic stress of *C. falcatum* and *F. moniliforme*. C: Control; T1: Treated with nanoparticles; T2: Fungal infection T3: Fungal infection +Nanoparticles. V1: variety susceptible to *C. falcatum*; V2: variety susceptible to *F. moniliforme.* All means the sharing common letter(s) are significantly not different ($p < 0.05$) by LSD. Standard error of means was represented by vertical bars (n=3).

5.4.3.3. Effects on biochemical profiling of antioxidant enzymes

Foliar AgNP's treatment of sugarcane plant led to marked increases in SOD (40.78%, 42%), POD (23.64%, 22.54%), CAT (36.59%, 27.12%) of sugarcane variety V1 and V2 when compared with diseased plants without nanoparticle application. It was noticed that electrolyte leakage was 24.04% decreased in V1 and 24.27% decreased in V2 when infected plants were sprayed with nanoparticles (Fig. 5.4.2).


A
V1
V2
Superoxidase (µmolg-1 FW min-1)
6
5
4
3
2
1
0
C
C
B
B
B
B
A
A
C
T1
T2
T3
Treatments


B
V1
V2
POD (µmolg-1 FW min-1)
3.5
3
2.5
2
1.5
1
0.5
0
C
C
C
C
B
B
A
A
C
T1
T2
T3
Treatments

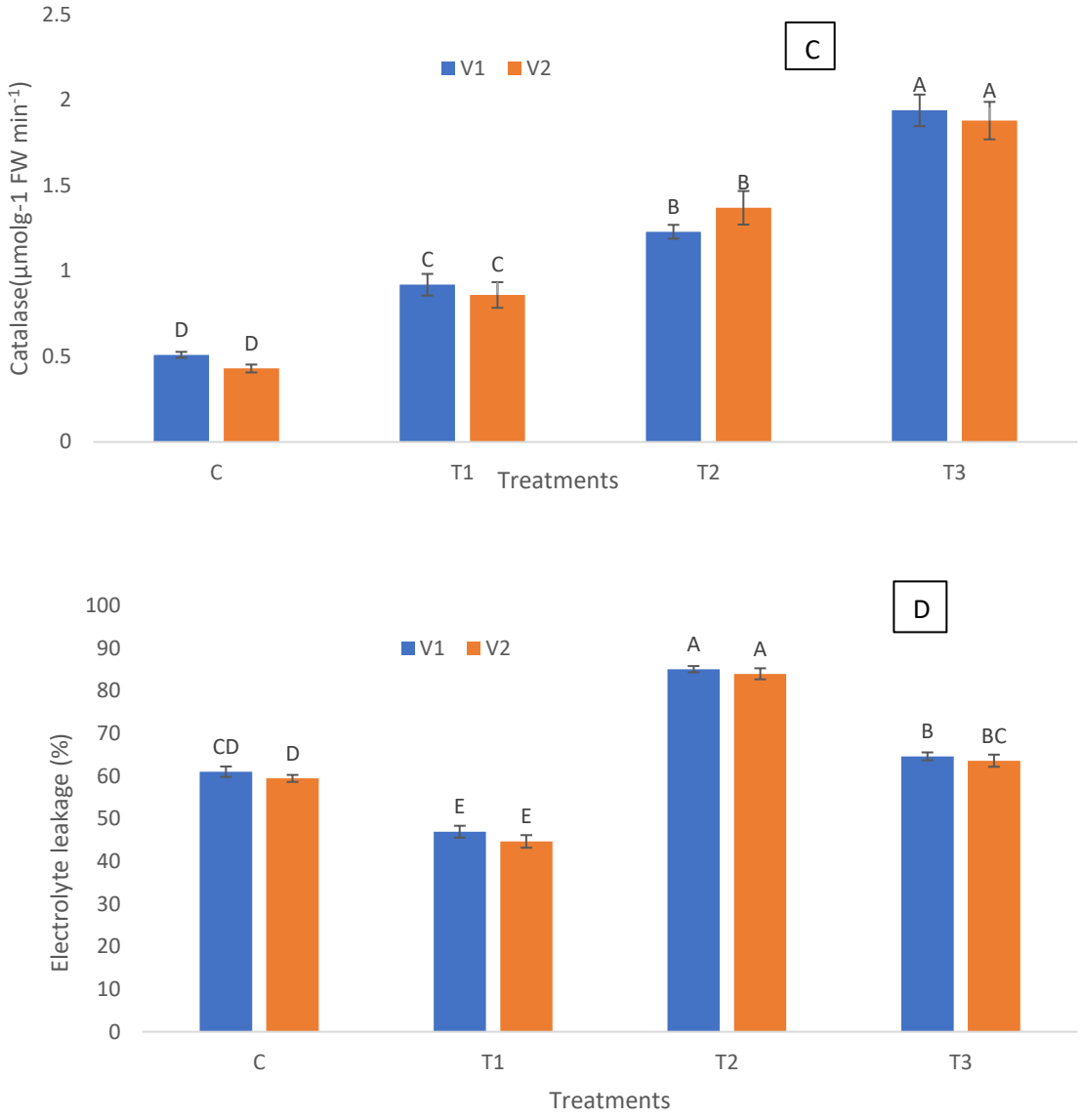
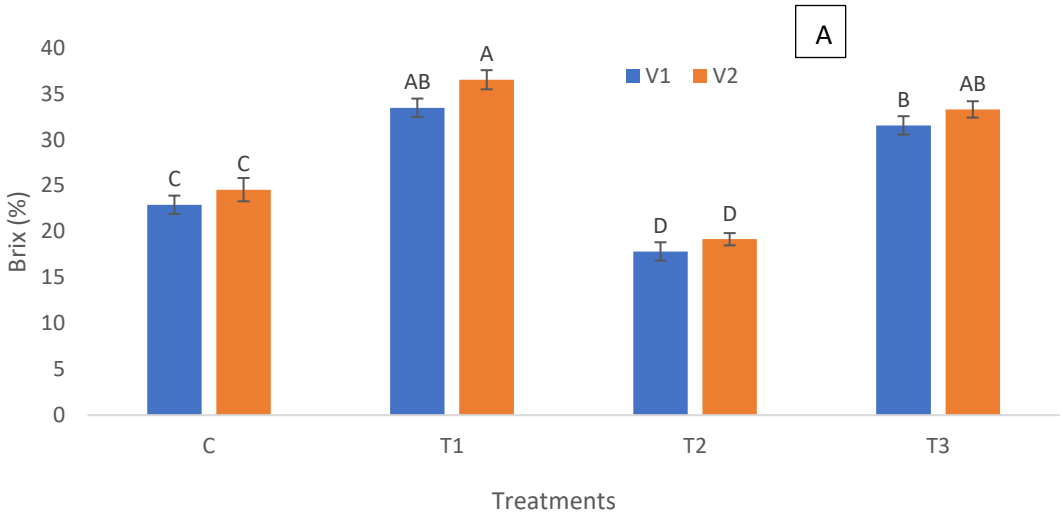
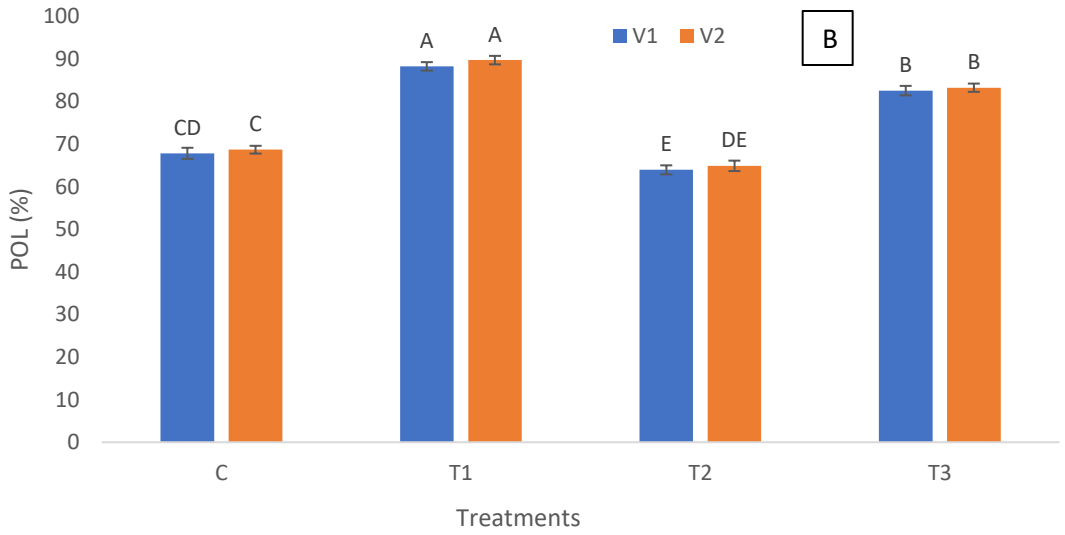


Figure 5.4.3. Effects of silver nanoparticles on SOD (a), POD (b), Catalase (c), Proline contents (d) Electrolyte leakage of two sugarcane varieties under biotic stress of *C. falcatum* and *F. moniliforme*. C: Control; T1: treated with nanoparticles; T2: Fungal infection T3: Fungal infection+Nanoparticles V1: variety susceptible to *C. falcatum*; V2: variety susceptible to *F. moniliforme.* All means the sharing common letter(s) are significantly not different ($p < 0.05$) by LSD. Standard error of means was represented by vertical bars (n=3).

5.4.3.4. Effects of nanoparticle application on sugarcane quality control parameters

It was observed in the current experiment that sugarcane quality control parameters were improved even in diseased plants with the application of silver nanoparticles. This might be due to the increased chlorophyll contents and suppression of disease that further helped to improve sugarcane juice quality. In treatment T4, 43.50 % increased Brix, 22.52% increased POL, 33.87% increased CCS was noticed in V1. However, in V2, 46.45%, 22.03% and 32.76% increased Brix, POL and CCS content was observed (Fig. 5.4.4).





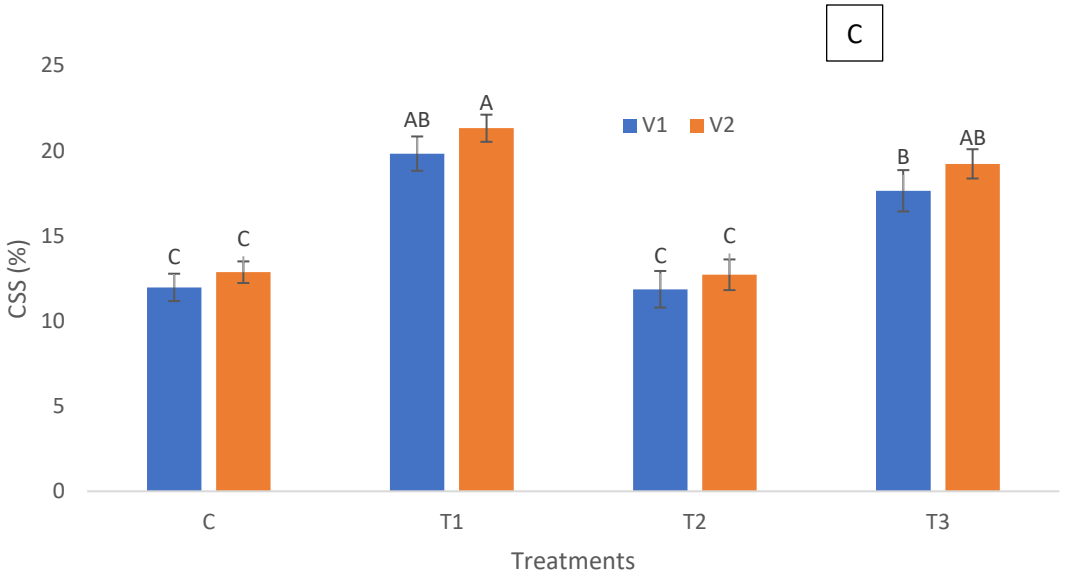


Figure 5.4.4. Effects of silver nanoparticles on Brix (a), POL (b), CCS (c) of two sugarcane varieties under biotic stress of *C. falcatum* and *F. moniliforme*. C: Control; T1: treated with nanoparticles; T2: Fungal infection T3: Fungal infection+Nanoparticles. V1: variety susceptible to *C. falcatum*; V2: variety susceptible to *F. moniliforme.* All means the sharing common letter(s) are significantly not different ($p < 0.05$) by LSD. Standard error of means was represented by vertical bars (n=3).

5.4.3.5. Role of nanoparticles in disease control of redrot and pokha boeng

The activity of AgNP's as antimicrobial agents were further verified by their application on fungal infected varieties of sugarcane. In plants susceptible to *C. falcatum* the diseased index was noticed to be reduced as compared to infected plants without any application of nanoparticles. In V2, AgNP's suppress disease severity index up to 68.34% as compared to diseased plants without nanoparticle application (Fig. 5.4.5 a & b).

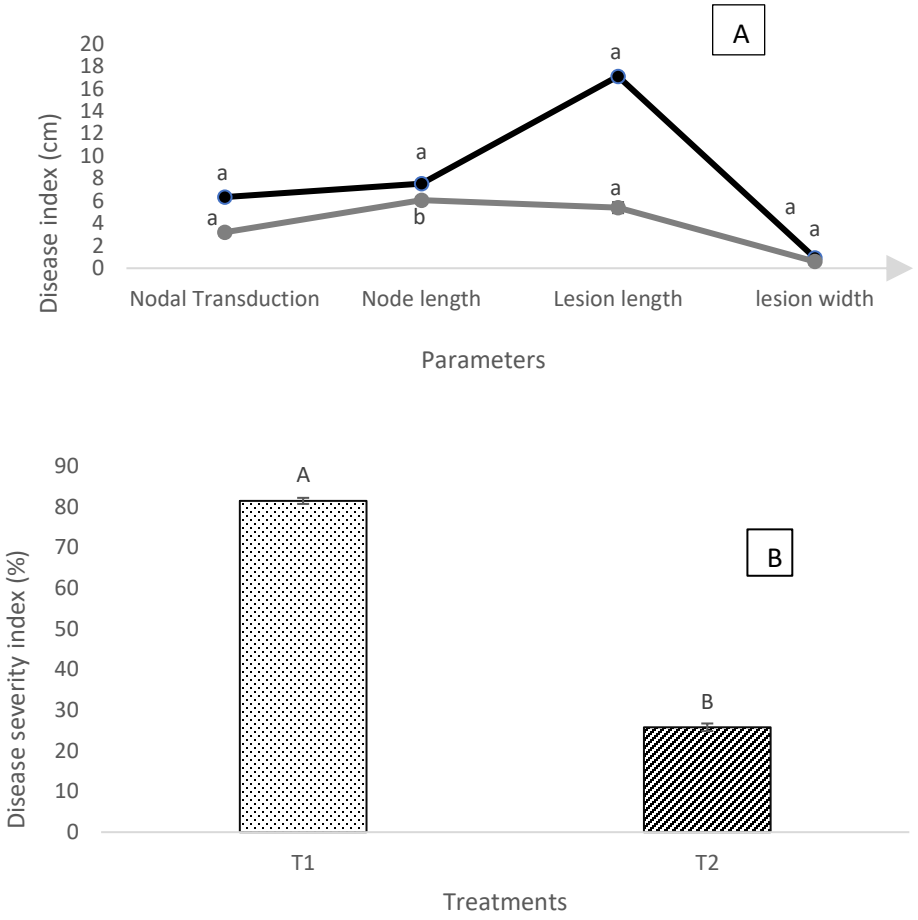


Figure 5.4.5. Effects of silver nanoparticles on Disease severity index of *C. falcatum* (a), Disease severity index of *F. monilliforme* (b) of two sugarcane varieties under biotic stress of *C. falcatum* and *F. moniliforme*. C: Control; T1: treated with nanoparticles; T2: Fungal infection T3: fungal infection+; T4: *B. B. gibsonii*+ *F. moniliforme*; T5: *Bacillus sp.* PM 15+ *F. moniliforme*; V1: variety susceptible to *C. falcatum*; V2: variety susceptible to *F. moniliforme.* All the means sharing common letter (s) are insignificantly different at $p < 0.05$ level by LSD and vertical bars represents standard error of means (n=3).

Discussion

Application of silver nanoparticles on two sugarcane varieties treated with fungal pathogen was done to analyze the role of biogenically produced nanoparticles for disease control and plant growth regulation. Silver nanoparticles (AgNPs) increased all growth criteria when compared with the untreated plants. Moreover, they also help to alleviate the drastic effects of fungal infection as observed in plants of both varieties V1 (CO1148) and V2 (CSSG32) treated with *Colletotricum falcatum and Fusarium monilliforme*, respectively.

It was noted that fungal strains showed virulence as noted by disease symptoms and reduced growth of the sugarcane plants in both varieties. Reduced growth and chlorophyll contents might be due to the alterations in physiological processes of energy generation and consumption like respiration, photosynthesis and photorespiration of infected plants, interference of ROS with the biomolecules like DNA, proteins or membranes that directly affect the integrity of organelle or cell (Fischer et al., 2019). However, it was noticed that application of nanoparticles (50ppm) enhanced the plant height, plant fresh weight, cane length, cane weight, cane diameter, root length and chlorophyll contents. It was previously noted that low concentrations of AgNPs had a stimulating effect on the growth of the common bean and corn plants and increased growth parameters in wheat plants (Salama, 2012; Latif et al., 2017). The impact of AgNPs on the morphology and physiology of plants depends on the size and shape of applied nanoparticles. Though in our knowledge, the mode of action of nanoparticles on growth and physiology of plants is too scarce, however it was revealed from some previous findings that AgNPs play an important role in enhancing seed germination and plant growth, improving chlorophyll contents and photosynthetic efficiency, also increased water and fertilizer use efficiency (Lu et al. 2002). Our results also coincide with the previous findings as AgNPs increased the growth parameters however, more prominent results were noted in the case of chlorophyll contents where significant increase was noted in both varieties that might relate to the alterations in nitrogen metabolism (Sadak, 2019).

In the current experiment, significant increases in all photosynthetic contents i.e. chlorophyll a, chlorophyll b and carotenoids were noticed in response to treatment with of AgNPs in plants of both varieties either infected with *C. falcatum* and *F. monilliforme* or not (Nghia et al. 2017; Latif et al., 2017). According to Govorov and Carmeli (2007), metal nanoparticles can boost the efficiency of photosynthetic systems in the form of chemical energy production. However, higher content of photosynthetic pigments, i.e., chlorophyll a, chlorophyll b, and carotenoids would increase the rate of photosynthesis. In our study it was observed that a high rate of photosynthesis play role in enhanced growth, fresh weight and quality control parameters of plants as compared to plants without AgNPs treatment. It was also previously reported that biosynthesized AgNPs induce synthesis of protein and carbohydrates of *Bacopa monnieri* (Krishnaraj et al., 2012). AgNPs also increased plants growth profile and biochemical attributes (chlorophyll, carbohydrate and protein contents and antioxidant enzymes) of common bean, *Brassica juncea* and corn (Salama 2012a; Sharma et al. 2012). Govorov and Carmeli (2007) analyzed that metal nanoparticles increase the efficiency of chemical energy production in photosynthetic systems. Higher chl a, chl b and carotenoids would increase the rate of photosynthesis, which in turn increased the growth, fresh weight, and sucrose content of sugarcane plants as it was also observed in our study.

Increase in SOD, POD and catalase activity indicates induced antioxidative potential in plants under biotic stress and nanoparticle application. SOD activity scavenges and decreases the risk of ROS formation like OH which is highly reactive and may cause severe damage to DNA, membranes and protein; also produce adverse modifications to cell components (Birben et al., 2012). Induction of cell death and loss of membrane integrity have been noted as common significances of oxidative stress in infected plants (Demidchik et al., 2014). Reduced cellular viability and more accumulation of intracellular ROS has been reported in plant *Lemna gibba* treated with silver nanoparticles (Dewez et al., 2018). Reduced and elevated antioxidative enzymes can be related to oxidative stress. Like our observations, modifications in antioxidative enzyme was observed. Biologically produced nanoparticles upsurge fresh weight, yield and productivity in the form of increased Brix, POL and CCS % age in comparison to control plants without nanoparticle application. Fungal pathogens *C. falcatum* and *F. monilliforme*, impaired plant growth by shifting its metabolism towards the disease control or utilize the photo-assimilates and stored sucrose content, degrade it to simple sugars to be used for their own survival and growth.

Increased photosynthetic activity and enhanced growth (plant height, plant weight, cane length, cane weight, cane diameter and root length) by nanoparticle application result in the accumulation of higher sugar contents and sucrose compartmentation in silver nanoparticle treated plants as compared to plants without nanoparticle treatment (Santos et al., 2018).

Invitro application of silver nanoparticles exposed the suppression of *C. falcatum* and *F. moniliforme* in infected sugarcane plants. It was previously noted that silver nanoparticles affect membrane integrity, DNA replication, expression of ribosomal proteins and proper functioning of membrane bounded enzymes involved in the respiration of targeted fungal pathogens (Kim et al., 2019).

5.5. Conclusion

The present work demonstrated the effect of silver nanoparticles on the sugarcane plant. Application of silver nanoparticles increased plant growth, photosynthetic pigments, antioxidant enzymes, and yield quantity and quality in both varieties. Moreover, its application not only enhances plant growth parameters but also suppress the drastic effects of both sugarcane pathogens i.e. red rot and pokha boeng disease. Sugarcane plants of variety 2 respond better in both aspects of plant growth and disease control towards the application of silver nanoparticles as compared to variety 2 plants infected with *C. falcaum.*

Chapter 6:

General conclusion and Future Perspectives

6.1. General Conclusion

In conclusion, the results suggest that out of 93 finalized strains most of them exhibit PGP activities by producing IAA, siderophores, HCN, phosphate solubilization and catalase activity. Out of 93 strains 10 strains showing best plant growth promoting and antagonistic activities against *Colletotricum faclcatum* and *Fusarium moniliforme*. In our study, occurrence of PGP traits in isolated strains offered an attractive strategy to use these microbial inoculants for further pot experiments**.** Dual performance of bacterial like PGP traits and antagonistic activity could lead to a long term and operative strategy to reduce phytopathogens than chemical pesticides and fertilizers, that employ comparatively sturdier selective pressure for fungal isolates, to improve resistance and improved plant growth. While further screening it was noted that selected strains showed significant tolerance against 15 different antibiotics. This antibiotic profiling will help in the selection of markers that can be used in the reisolation of original microbial inoculants applied for *invitro* experiments/ greenhouse studies. Based on multistress tolerance against high salinity stress, drought, heavy metal toxicity and high temperature along with strong antagonistic potential against fungal strains provide a base for further evaluation of these bacteria at pilot or field scale.

In another study, it was observed that biological materials can be used as active reducing agent to produce nanoparticles and exhibit unique physiochemical and biochemical properties. Dispersed dendritic nature nanoparticles with nano size of 45.41 nm, 49.06 nm, 42.75 nm were observed after treatment of $AgNO^3$ with supernatant of bacteria, fungus and sugarcane husk solution, respectively. FTIR spectrum revealed the presence of functional groups (amines, benzenes, alcohols and alkenes) that act as reducing and capping agents to produce stable silver nanoparticles. Highest antifungal activity was also shown by nanoparticles produced by aqueous solution of sugarcane husk might be due to the smaller size as compared to other samples. Effects of all nanoparticles were dominant in the suppression *F. monilliforme*. This biological synthesis approach of silver nanoparticles has many advantages as revealed by its economic viability and simple processing.

In second phase of our study, growth promoting bacterial strains *Bacillus gibsonii* PM11*, Bravebacterium frigoritolerans* PM12*, Bacillus siamensis* PM13*, Bacillus xiamenesis* PM14*, Bacillus sp.* PM15*, Bacillus sp.* PM16 and *Bacillus tequilensis* PM17 can be potentially used to improve the growth, photosynthetic pigments and cane quality. Highest growth rate was noticed

in sugarcane plants treated with *B. siamensis* PM13 (21.77%, 33.66%, 23.38%, 32.96%, 16.9%, 36.9 %, 62.68%, 62.27%, 43.76%, 38.73%, 23.7%, 40.9% increased values for plant height, plant fresh weight, cane length, cane weight, cane diameter, root length, Chl a, Chl b, carotenoids, Brix, POL and CCS, respectively) followed by *B. tequilensis* PM17 and *B. xiamenesis* PM14. Current results provide evidence for the use of native microflora to produce high yield and productivity in sugarcane plants. Subsiding of ecological problems and stresses using eco-friendly approach is of vital importance. The use of antagonistic PGPR with extracellular enzyme production can be a good option to control red rot of sugarcane. Maximum disease suppression and plant growth was observed in sugarcane plants treated with strain *B. siamensis* PM13 enhance plant height (18.39%), plant fresh weight (36.95), cane length (32.83), cane weight (46.36), cane diameter (26.09), chl a (21.27), chl b (37.30) carotenoids (39.76). And, the disease index is also observed to be reduced (nodal transduction 49.5%; nodal width 19.5%; lesion length 68.4%; lesion width 36%). Following PM13 strain, *B. tequilensis*> *B. xiamenesis*>*Bravebacterium frigoritolerans* also showed same trend of enhanced growth and disease suppression.

It is also finalized that inoculated PGPR *Bacillus gibsonii* (PM11), *Bacillus siamensis* (PM13); *Bacillus sp.* (PM15), *Bacillus tequilensis* (PM17) having biocontrol ability to overcome the drastic effects of *F. moniliforme* attack and plays the potential role in enhanced growth. Maximum enhanced growth was observed in sugarcane plants treated with strain *B. tequilensis* PM17 that showed increase in plant height (12.8%), plant fresh weight (33.33%), cane length (20.95%), cane weight (23.43%), cane diameter (32.25%), root length (19.9%), chl a (22.99%), chl b (29.76%) carotenoids (39.5%) and proline contents (21.36%). As compared to other. However, more values of SOD (20.58), POD (17.91), catalase (30.88) and electrolyte leakage (26.52) were observed in pokha boeng infected plants inoculated with *B. siamensis* PM13 that revealed their role in maximum disease control (73.97%) as compared to others. Following *B. siamensis* PM13 strain, *B. tequilensis* (PM17)> *B. xiamenesis* (PM14)>*Bravebacterium frigoritolerans* (PM12) also showed same trend of enhanced growth and disease suppression. This paves the way to explore out more proficient bacterial isolates that could perform in different biotic conditions and provide more tolerance in such conditions. This will also provide a gateway to test these strains at field level for sustainable agricultural practices. In continuation of current experiment, effects of silver nanoparticles on sugarcane plant were explored. Application of silver nanoparticles increased plant growth, photosynthetic pigments, antioxidant enzymes, and yield quantity and quality in both

varieties either inoculated with silver nano particles or not. Moreover, its application not only enhance plant growth parameters but also suppress the drastic effects of both sugarcane pathogens i.e. red rot and pokha boeng disease. Sugarcane plants of variety 2 respond better in both aspects of plant growth (22.23%, 39.5%, 40.20%, 26%, 30%, 21.83%, 26%, 42.32%, 49.46%, 44.51%, 44%) and disease control towards the application of silver nanoparticles as compared to variety 2 plants infected with *C. falcaum.*

6.2. Future Perspectives Keeping in mind the findings and observations of conducted study relevant to native plant growth promoting and antagonistic bacteria isolated from sugarcane we can further study following aspects.

- Antagonistic bacteria have the potential to intercalate with DNA and proteins of pathogen via various mechanisms. In this prospect, extent of DNA damage of phytopathogens encountered with biocontrol agents can be studied.
- It was previously reported that augmented bacteria can activate cell wall genes in associated plants that lead to their resistance against attacking pathogens. In future, we can investigate the cell wall response of sugarcane crop towards *Colletotricum falcatum and Fusarium moniliforme.*

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Plate 1: Sugarcane cuttings used for sowing

Plate 2: Two leaf germination stage

Plate 3: Growth of sugarcane five leaf stage

Plate 4: Growth of sugarcane at three month stage

Plate 5: Effects of Plant growth promoting bacteria on growth of sugarcane plant C: control, T1: PM11, T2: PM12, T3: PM13, T4: PM14, T5: PM15, T6: PM16, T7: PM17.

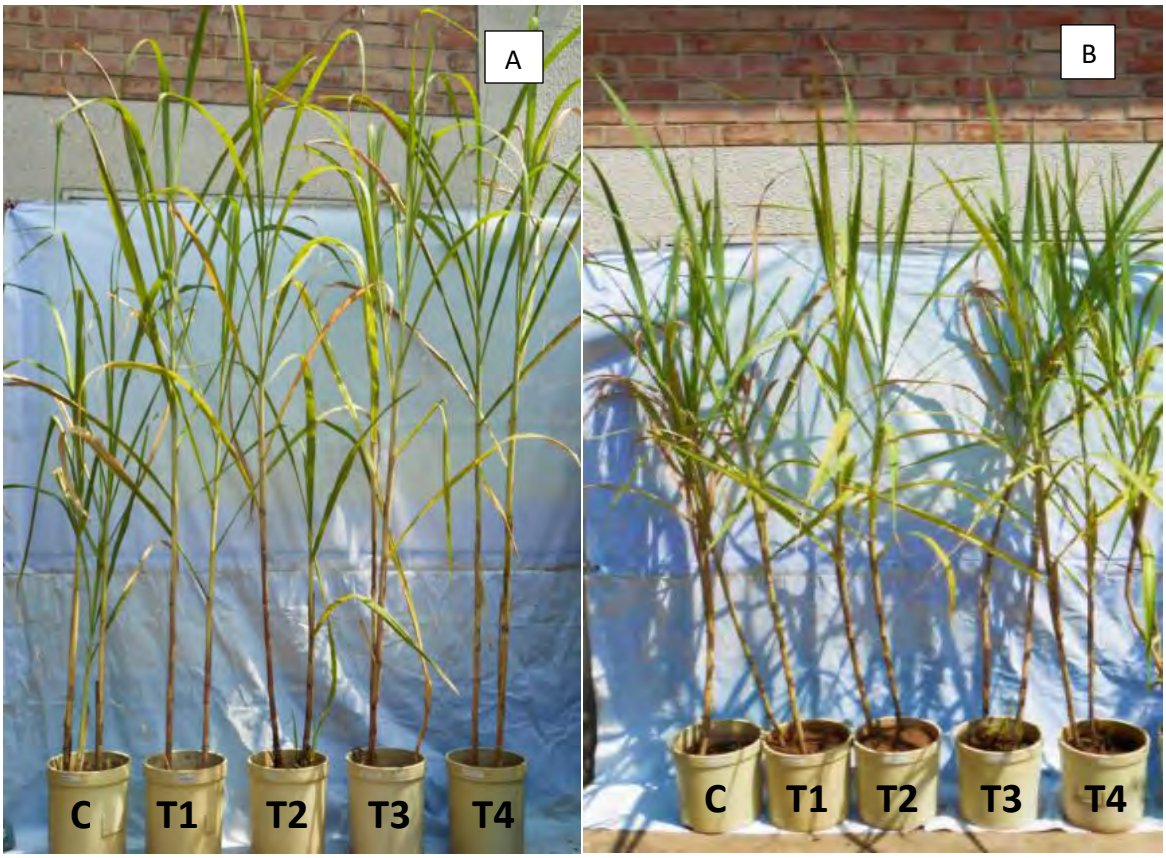


Plate 6: Effects of antagonistic bacteria on growth of sugarcane plant Fig A susceptible variety of red rot: C: control, T1: PM11, T2: PM12, T3: PM13, T4: PM16, Fig B susceptible variety of Pokha boeng: T1: PM13, T2: PM14, T3: PM14, T4: PM16

Plate 7: Effects of nanoparticles on growth of sugarcane plant under diseased conditions

Fig A. susceptible variety of Pokha boeng: Fig B. susceptible variety of red rot

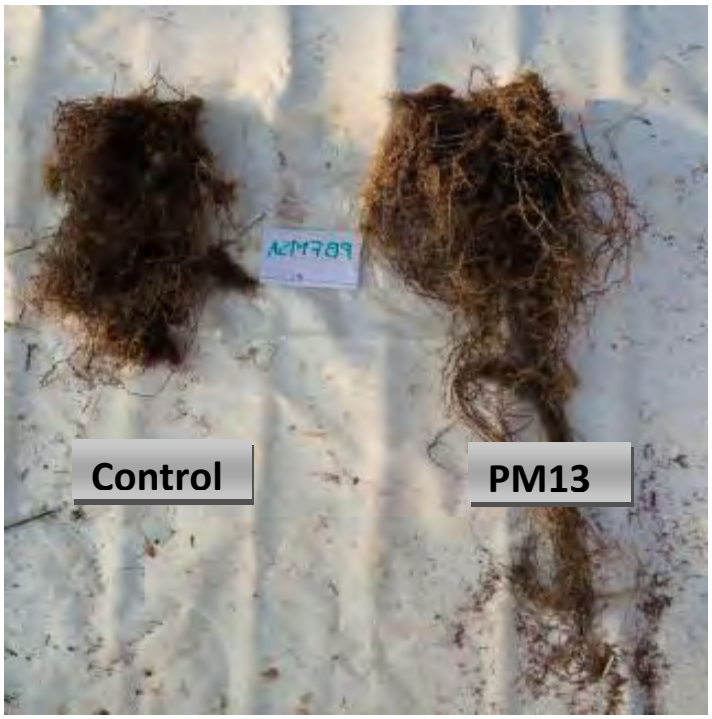


Plate 8. Effects of *B. siamensis* on root growth

Plate 9. Effects of *B. gibsoni* on root growth

Plate 10. Effects of *B. xiamenesis* on root growth

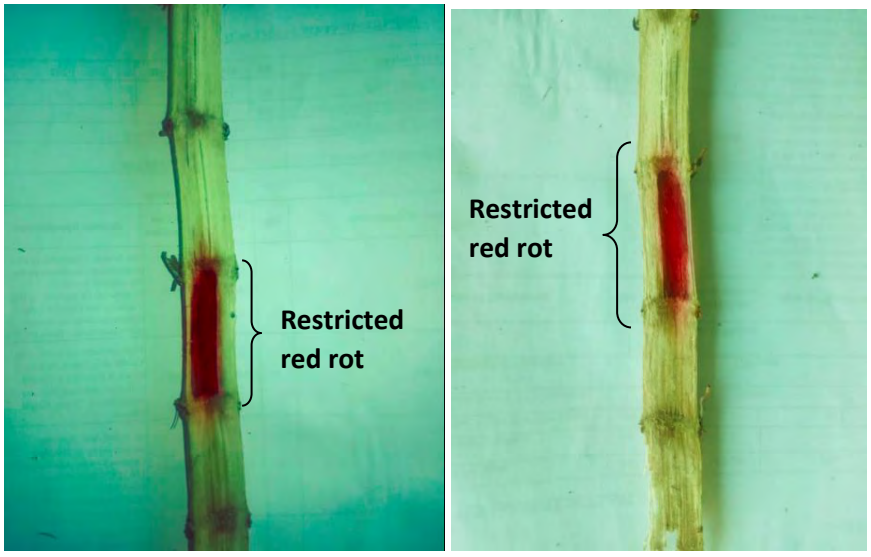


Plate 11. Resistriced infection of red rot in B. siamensis and B. tequilensis inoculated plants

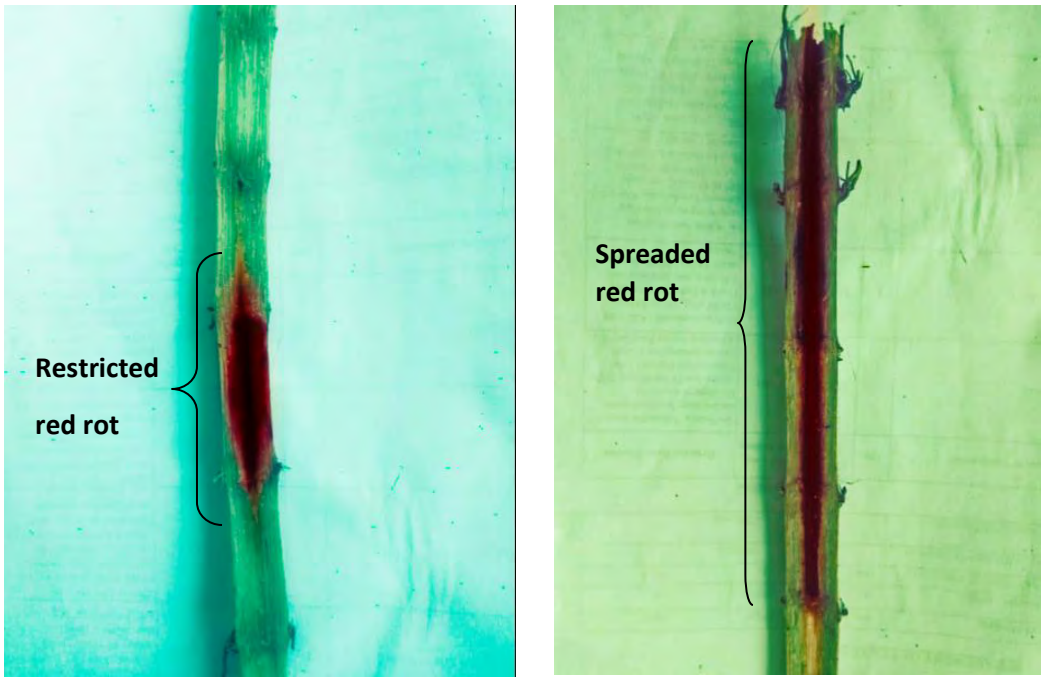
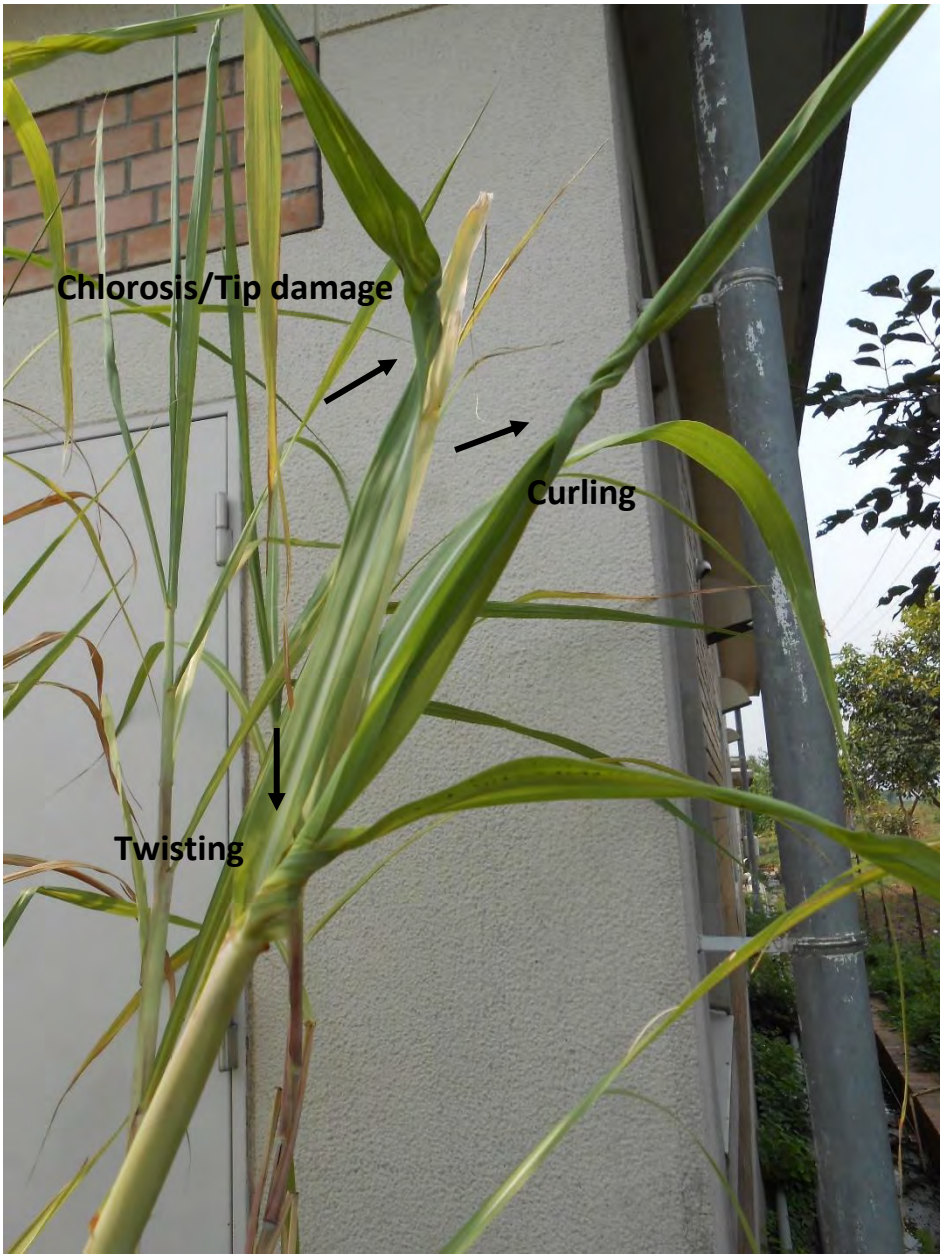
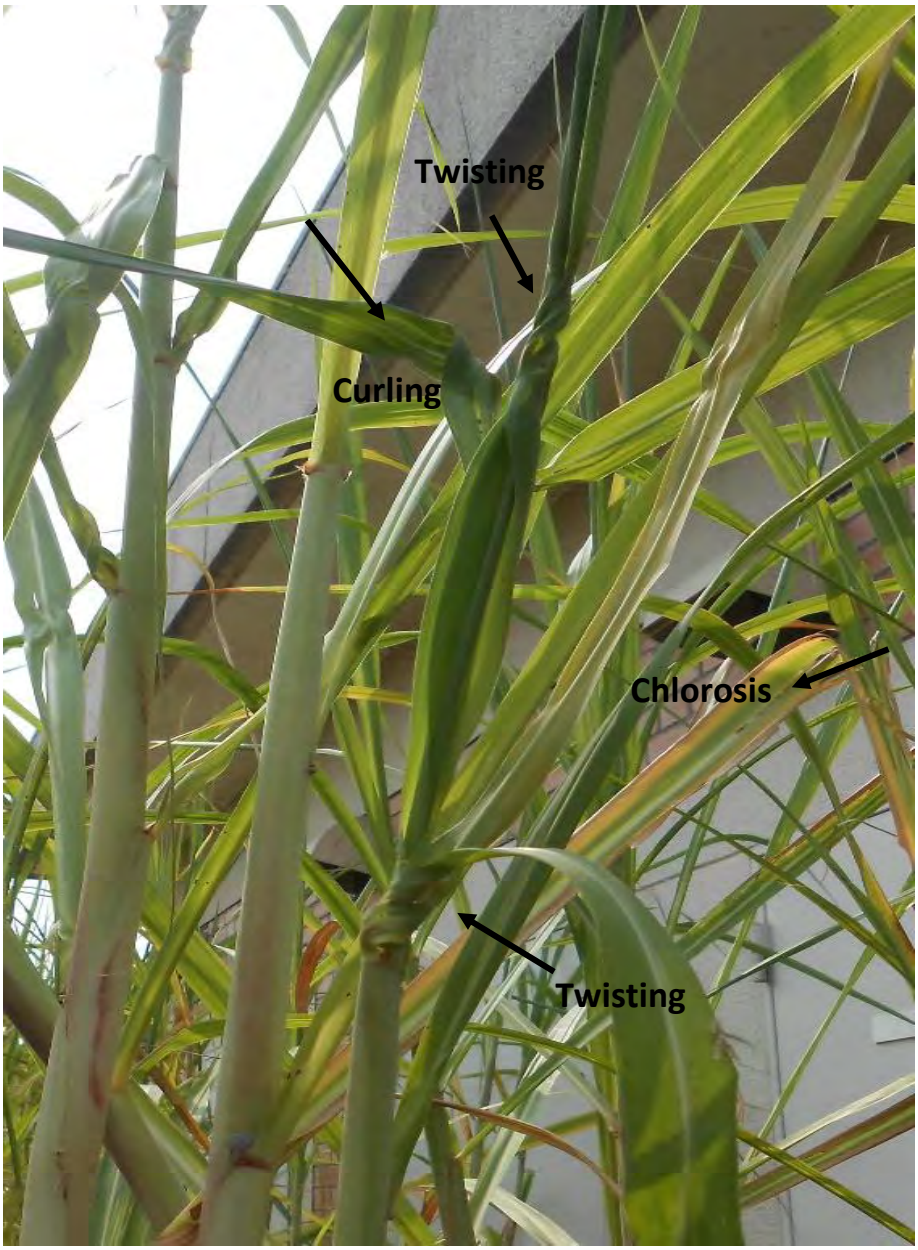


Plate 12. Restricted red rot infection in *B. xiamensis* inoculated plants while second figure showed control plants with spreaded infection (without bacterial treatment


Chlorosis/Tip damage
Curling
Twisting


Twisting
Irregular specks/stripes


Twisting
Curling
Chlorosis
Twisting

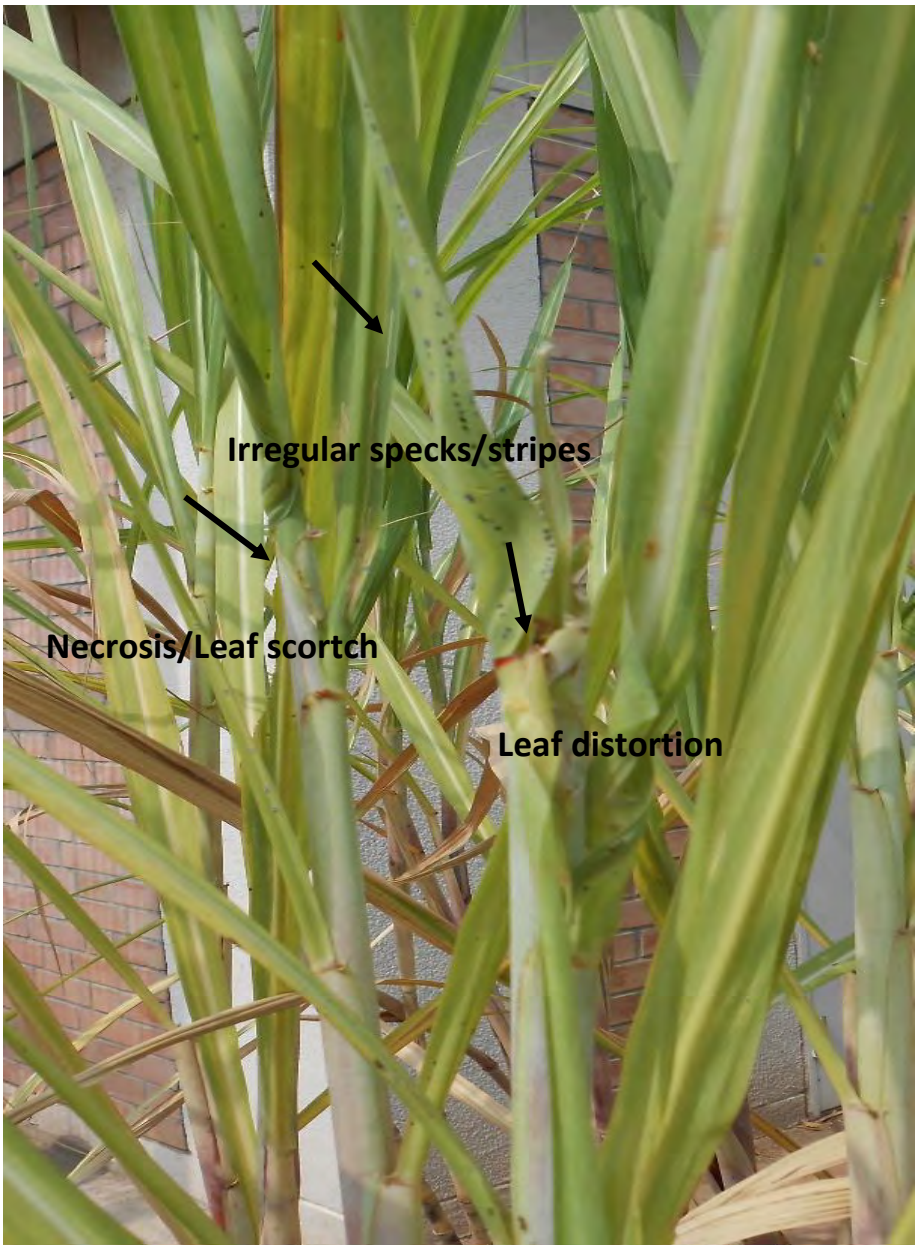


Plate 13: Disease symptoms of Pokha boeng

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Mechanistic elucidation of germination potential and growth of wheat inoculated with exopolysaccharide and ACC- deaminase producing *Bacillus* strains under induced salinity stress

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ARTICLE INFO

Keywords:
ACC-Deaminase
Bacillus
Exopolysaccharide
Germination
Plant growth promoting rhizobacteria
Proline
Salt stress

ABSTRACT

The potential of plant growth regulating microorganisms present in the soil can be explored towards the purpose of identifying salt tolerant strategies and crop cultivars. Current study was designed to elucidate the capabilities of salt stress tolerant plant growth promoting rhizobacteria (PGPR) Bacillus siamensis (PM13), Bacillus sp. (PM15) and Bacillus methylotrophicus (PM19) in undermining the effects of salt stress on wheat seedling. Strains were characterized for their IAA (81–113 μM/ml), ACC-deaminase (0.68–0.95 μM/mg protein/h) and exopolysaccharide (EPS) (0.63–0.97 mg/ml) producing activity both under normal and NaCl stressed conditions. Effects of bacterial inoculation on germination and seedling growth of wheat variety Pakistan 13 was observed under induced salinity stress levels (0, 4, 8, 16 dS/m). All the morpho-physiological characteristics of wheat seedlings were affected drastically by the NaCl stress and the growth parameters expressed a negative relationship with increased NaCl levels. PGPR application had a very positive influence on germination rate of wheat seedlings, root and shoot length, photosynthetic pigments etc. Elongated roots and enhanced vegetative shoot growth as well as seedling's fresh and dry weights were highest in plants treated with *B. methylotrophicus* PM19. Sequestration of Na^+ ion by EPS production and degradation of exuded ACC into α-ketobutyrate and ammonia by ACCD bacteria efficiently reduced the impact of salinity stress on wheat growth. Current findings suggested that the used PGPR strains are potential candidates for improving crop growth in salt stressed agricultural systems. However further research validation would be necessary before large scale/field application.

1. Introduction

Crop production is negatively affected by NaCl stress resulting in 65% loss of crop yield under saline conditions (Ansari and Ahmad, 2019). Human population is increasing at an exponential rate worldwide particularly in the developing countries (Kumaraswamy et al., 2009). Since the population is on the up rise and the cultivable land has consistently been reducing, there comes the dire need to increase the abiotic stress resistant varieties with high yield potential (Abbasanshor et al., 2018). Wheat is one of the most important crops in Pakistan grown by 80% of the farmers on an area of about 9 million hectares (40% of total cultivated land). Soils in Pakistan are mostly calcareous while the effect of salinity on crop yield has earlier been discussed and reported in numerous studies (Shahmansouri and Kumar 2018). Food and Agriculture Organization (FAO) has mentioned that about 6% of cultivable agricultural land has been affected by salinity (Hoagenwood and Smith, 2017). Till now > 20% of agricultural land around the globe is affected by high NaCl concentrations and prevailed conditions might reach the loss of 50% arability of agricultural land till the mid of 21st century. Under high NaCl conditions, stress-mediated ethylene production happens due to increased conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene. Increased ethylene level resulted

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Research article

Multi-stress tolerant PGPR *Bacillus xiamenensis* PM14 activating sugarcane (*Saccharum officinarum* L.) red rot disease resistance

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ARTICLE INFO

Keywords:
Antibiotic resistance
Bacillus xiamenensis
Biocontrol
Inhibition co-efficient
Phytopathogens
Red rot

ABSTRACT

Sustainability in crop production has emerged as one of the most important concerns of present era's agricultural systems. Plant growth promoting bacteria (PGPB) has been characterized as a set of microorganisms used for enhancing plant growth and a tool for biological control of phytopathogens. However, the inconsistent performance of these bacteria from laboratory/greenhouse to field level has emerged due to prevailing abiotic stresses in fields. Sugarcane crop encounters a combination of biotic and abiotic stresses during its long developmental stages. Nevertheless, the selection of antagonistic PGPB with abiotic stress tolerance would be beneficial for end-user by the successful establishment of product with required effects under field conditions. Stress tolerant *Bacillus xiamenensis* strain (PM14) isolated from the sugarcane rhizosphere grown in the fields was examined for various PGP activities, enzyme assays, and antibiotic resistance. Strain was screened for *in vitro* tolerance against drought, salinity, heat stress, and heavy metal toxicity. Inhibition co-efficient of *B. xiamenensis* PM14 was also calculated against six phyto-pathogenic fungi, including *Colletotrichum falcatum* (53.81), *Fusarium oxysporum* (68.24), *Fusarium monliforme* (69.70), *Rhizoconia solani* (71.62), *Macrophomina phaseolina* (67.50), and *Pythium splendens* (77.58). *B. xiamenensis* is reported here for the first time as the rhizospheric bacterium which possesses resistance against 12 antibiotics and positive results for all *in vitro* PGP traits except HCN production. Role of 1-aminocyclopropane-1-carboxylate deaminase in the amelioration of biotic and abiotic stress was also supported by the amplification of acds gene. Moreover, *in vitro* and *in vivo* experiments revealed *B. xiamenensis* as the potential antagonistic PGPB and bio-control agent. Results of greenhouse experiment against sugarcane red rot indicated that inoculation of *B. xiamenensis* to sugarcane plants could suppress the disease symptoms and enhance plant growth. Augmented production of antioxidative enzymes and proline content may lead to the induced systemic resistance against red rot disease of sugarcane. Thus, the future application of native multi-stress tolerant bacteria as bio-control agents in combination with current heat, drought, salinity, and heavy metal tolerance strategy could contribute towards the global food security.

1. Introduction

Crops are significantly affected by certain biotic and abiotic stresses. About, 20–40% of crop's yield reduction and estimated losses of 40 billion dollars are caused by phytopathogens worldwide (Rahman et al., 2018). Considering the rising concerns for global food security, food production must be increased by 70% till 2050 (Ingram, 2011). Among alternatives of disease control methods, biocontrol of plant diseases appears to be an efficient option for the development of eco-friendly, cost effective, and sustainable pathogens control program (Rahman

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RESEARCH ARTICLE

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Characterization of bio-fabricated silver nanoparticles for distinct anti-fungal activity against sugarcane phytopathogens

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Review Editor: Mingying Yang

Abstract

Advanced research, development, and application of silver nanoparticles is proceeding in recent times due to their incredible utilization in various fields. Present study was focused on the production, characterization, and antifungal activities of silver nanoparticles (AgNPs). An environment friendly extracellular biosynthetic approach was adopted to produce the AgNPs by using bacteria, fungi, and sugarcane husk. Agents used for reduction of silver to nanoparticles were taken from culture filtrate of plant growth promoting bacteria, *Fusarium oxysporum* and supernatant extract of sugarcane husk. Nanoparticles were also characterized by scanning electron microscopy (SEM). Synthesis of colloidal AgNPs was observed by UV-Visible diffused reflectance spectroscopy (UV-Vis DRS). Primary peak of surface plasmon resonance band was noticed around 339.782, 336.735, and 338.258 nm for bacterial, fungal, and sugarcane husk produced AgNPs. Structure of all biologically produced nanoparticles were crystalline cubic with nano size of 45.41, 49.06, and 42.75 nm for bacterial, fungal, and sugarcane husk-based nanoparticles, respectively as calculated by Debye–Scherrer equation using XRD. Fourier transform infrared spectroscopy (FTIR) analysis revealed the presence of various compounds that aid in the reduction, capping, and stability of AgNPs. The antifungal activity of AgNPs was also investigated for sugarcane fungal pathogens *Colletotricum falcatum* and *Fusarium moniliforme*. All nanoparticles exhibit prominent antifungal activities. Maximum zone of fungal inhibition was noticed about 18, 19, and 21 mm for *C. falcatum* while 21, 20, and 24 mm for *F. moniliforme* in case of bacterial, fungal, and plant-based nanoparticles (15 ppm), respectively. Best fungal inhibition was observed under application of sugarcane husk based AgNPs. Moreover, biologically produced AgNPs responded better towards the suppression of *F. moniliforme* in comparison to *C. falcatum*. Mentioned sources in present study can be ecofriendly nano-factories for biosynthesis of AgNPs and mankind should benefit from their commercial application.

KEYWORDS
antifungal, *Colletotricum falcatum*, *Fusarium moniliforme*, pathogens, silver nanoparticles

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