Mitigation of Heat Stress in *Solanum lycopersicum* L. by Using Heat Tolerant Plant Growth Promoting Bacteria

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Mitigation of Heat Stress in *Solanum lycopersicum* L. by Using Heat Tolerant Plant Growth Promoting Bacteria

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This is to certify that the research work presented in this thesis, entitled **"Mitigation of Heat Stress in *Solanum lycopersicum* L., by Using Heat Tolerant Plant Growth Promoting Bacteria"** was conducted by Miss.Tehmeena Mukhtar under the supervision of Dr. Hassan Javed Chaudhary.

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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DECLARATION

The research work presented in this thesis was carried out by me in the Plant Microbe Interactions 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.

Tehmeena Mukhtar

Dedication

I dedicated my dissertation work

To

My Brother

Syed Javed Mukhtar

Who taught me the first word to speak
First alphabet to write
&
Supported me throughout the educational life

Table of Contents

S.No ***Title*** ***Page No***

List of Figures

S. No ***Title*** ***Page No***

List of Tables

S.No ***Title*** ***Page No***

List of Appendices

S.No ***Title*** ***Page No***

LIST OF ABBREVIATIONS

ABA Abscisic acid

°C Celsius/Centigrade

µg Microgram

µl Microliter

O_{-2} Superoxide radical

ACC 1-aminocyclopropane-1-carboxylic acid

ANOVA Analysis of Variance

APX Ascorbate peroxidase

BLAST Basic local alignment search tool

BP Base pair

Ca Calcium

CAT Catalase

CFU g^{-1} Colony forming units per gram

chl.cont Chlorophyll content

CK Cytokinins

cm Centimeter

cm2 Square centimeter

cv. Variety

cvv. Varieties

Df Dworkin and Foster medium

DMSO Dimethyl sulfoxide

EC Electrical conductivity

ET Ethylene

F Forward

$FeCl_3$ Ferric chloride

$FePO_4$ Iron phosphate

G Gram

GA Gibberellic acid

IAA Indole acetic acid

gl-1 Gram per liter

H Hour

HSP Heat shock protein

H_2O_2 Hydrogen peroxide

H_3PO_4 Phosphoric Acid

Ha Hectares

$HClO_4$ Perchloric acid

HCN Hydrogen cyanide

ISR Induced systemic resistance

IAA Indole-3-acetic acid

LB Luria-Bertani medium

LSD Least significance difference

Min Minutes

Ml Milliliter

MBF1 Multiprotein bridging factor 1

NARC National Agriculture Research Centre

NB Nutrient broth

NCBI National Center for Biotechnology Information

OD Optical density

OM Organic matter

P Phosphorous

PCA Principal component analysis

PCR Polymerase chain reaction

PGPB Plant growth promoting bacteria

PGPR Plant growth promoting rhizobacteria

PGRs Plant growth regulators

POD Peroxidase

PSBs Phosphate solubilizing bacteria's

PVK Pickovskaya broth medium

PVP Polyvinylpyrrolidone

R Reverse

ROS Reactive oxygen species

RPM Revolution per minute

rRNA Ribosomal RNA

S Second

Sl Shoot lenght

SOD *Superoxide dismutase*

sp. *Specie*

spp. *Species*

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Tehmeena Mukhtar

Abstract

Bacteria can be evaluated for their capabilities of heat tolerance and plant growth promotion in sustainable agriculture. Three planned studies were piloted to assess the potential of heat tolerant plant growth promoting bacteria (PGPB) to mitigate heat stress in tomato plant. Samples were collected including tomato plants and rhizospheric soil from Larkana, Sindh, Pakistan during the year 2015. Seventy isolates were isolated, screened and characterized for plant growth promoting activities, extracellular enzymes activities and heat tolerance potential. These isolates were positively confirmed for indole acetic acid (IAA), phosphate, ammonia, siderophores, hydrogen cyanide, protease, amylase, pectinase, catalase, ACC-deaminase and exopolysaccharide production. The strains were screened at high temperature which was maintained at 60^0C. The Five promising potential heat tolerant isolates were identified through 16S rRNA gene sequencing technique. *Acds* gene was successfuly amplified from these promising bacterial strains. Morphological characterization revealed that four strains were Gram positive and one was Gram negative. ***Bacillus safensis* (SCAL1)** strain revealed maximum production of IAA (**0.52 µg/ml**) and ***Bacillus safensis* (T6) strain,** showed higher quantity of Giberellic acid **(8.73 µg/ml)** and Kinetin (**34.8 µg/ml)** under heat stress condition

Impact of plant growth promoting bacteria application on key physiological and biochemical analysis were studied under normal and high temperature stress conditions in green house. The results of morpho-physiological parameters revealed significant affect of heat on un-inoculated and inoculated tomato plants under high temperature stress. From all observation of experimental results, we found the best impact of inoculation of characterized bacteria *Bacillus safensis* **SCAL1**, significantly enhanced all agronomic parameters of both varities (Riogrande and Sweetie) including root length **(39.6** and **64.4%),** shoot length, **(37.1** and **61.4%)** fresh weight (**55.4** and **80.2**%) and dry weight **(22.1** and **60.04%)** and leaf surface area **(33.2** and **63.2%)** and number of flowers (**51.6** and **63.9**%) and fruit (**55.7** and **77.8**%) under normal and heat stress conditions.

A total of three field experiments were conducted at National Agriculture Research Centre, Islamabad, Pakistan, (NARC) during 2018, 2019 and climatically

important district Muzaffargarh, Punjab, Pakistan. As bacterial strains gave promising results in greenhouse experiment, multi-year/multi-location field trials were conducted to extend the heat mitigations effects at larger scale. In all three field experiments, plant growth promoting bacteria (PGPB) enhanced agronomic and yield parameters of tomato plants under heat stress conditions. First year 2018 field trial at National Agriculture Research Centre, Islamabad, Pakistan showed signifcant changes in different physiological and agronomic parameters. However, the parameters of number of flowers and number of fruits were of prime importance as it is main indicator of yield. *Bacillus safensis* **(SCAL1)** produced maximum number of flower's **(47%)** and fruits (**31.1**%), followed by *Bacillus safensis* **T6 (40.7 and 24.8%),** *Bacillus safensis* **BT (20.07 and 11.75%),** ***Bacillus cereus*** **KTES (30.01 and 8.56%) and** ***Klebsiella variicola*** **TR3 (17.1 and 9.48%)** respectively. All inoculated plants grown under heat stress condition enhanced the flowers and fruits per plant which can be regarded as major in terms of yield along with improvement in other parameters such as root and shoot length, fresh and dry weight, chlorophyll content, leaf area and number of flowers as compare to un-inoculated heat stressed plants.

In the second year, 2019, NARC, Islamabad, Pakistan field data expressed changes in results of inoculated compared with un-inoculated under heat stress. Among all the applied bacterial strains, ***Bacillus safensis*** (SCAL1) showed the best performance under field conditions as it was noticed to be more heat tolerant than *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3). *Bacillus safensis* **SCAL1** produced maximum number of flowers **(44.1%)** and fruits **(20.9%)**, followed by *Bacillus safensis* **T6 (43 and 11.03%),** *Bacillus safensis* **BT (24.5 and 6.27%),** ***Bacillus cereus*** **KTES (35.5 and 4.76%) and** ***Klebsiella variicola*** **TR3 (20.1 and 0.7%)** respectively.

The study year 2019, District Muzfargarh, Punjab, Pakistan field data revealed improvement in parameters under the inoculation of bacterial consortia. The important parameters of number of flower and fruits showed significant improvement as reported in the previous field studies of NARC, study years of 2018-19. The consortia improved the percentage of number of flowers and fruits by **16.9** and **52.1** % respectively.

The study comprehensively exhibited the role of plant growth promoting bacteria in the mitigation of heat stress. Up to the best of our knowledge, this is the very first study claiming the potential of isolated bacterial strains to mitigate the heat stress and plant growth promotion under greenhouse and field conditions from Pakistan. We also report that in our best of our knowledge for the **first-time, field studies** demonstrating the mitigation of effects of heat stress in tomato plant by inoculation of thermotolerant plant growth promoting bacteria.

Chapter. 1

General Introduction and Review of Literature

1.1. Introduction

Abiotic stress, such as high temperature can cause economic losses and provide proof of global warming (Ruelland & Zachowski, 2010). Based on several studies and crop modelling techniques, by the end of the 21st century (2081-2100), the worldwide temperature is expected to increase in the range of about 1 ^{0}C to 3.7^{0}C relative to their past levels of 1986-2005. Even though temperature rises may be favorable in some regions, but crop yield reductions are likely unless adaptation approaches are employed (Bita *et al*., 2013). Reduction in yield of agricultural has been directly related high temperature (Mingpeng *et al*., 2010). The growth and development of plant are affected due to high temperature, through morphological and physiological changes, delaying their developmental processes and ultimately resulting in yield loss (Grant *et al*., 2011). Sometimes, the productivity of agricultural crop is completely lost due to high-temperature condition. Heat stress is specific environmental conditions which are characterized by the temperature range, intensity and duration of heat. Meanwhile as the temperature increases above a threshold level a complex situation occurs which is resulted in yield loss (Grover *et al*., 2011). High temperature is a major environmental concern that constrains vital plant functions such as seed germination, seedling growth, and plant metabolism and reduces yield in various agro-ecological zones throughout the world (Fahad *et al*., 2017).

1.2. Tomato and heat

Tomatoes (*Solanum lycopersicum*) of Solanaceae family was originated in South America, brought to Europe and is consumed in various ways like fresh, part of a vegetable, with salad as well as cooked or processed as paste, sauces, ketchup, soups, and even pickled (Bauchet & Causse, 2012). Tomato fruits are a rich source of antioxidants like flavonoids and phenolics, vitamins, minerals, dietary fibers and carotenoids which are one of the beneficial nutraceutical molecules (Giovannetti *et al*., 2012). Tomato, being eaten either raw or in number of other cooked forms, has become part of our daily diet. Globally the annual production of tomatoes is about 159 million tonnes (http://www.agricorner.com). In Pakistan two crops of tomato are produced per year one in summer and second in autumn thus tomato is available throughout the year (Noorani *et al.,* 2018). Cultivated area for tomato in Pakistan is 63.20 thousand hectares and total production recorded as 601.098 thousand tones

during 2017. The mean tomato yields during that year was 9510.60 kilograms/hectare (FAO, 2017). Baluchistan was the largest tomato producing province with the production of 200 thousand tones on an area of 27 thousand hectares, followed by Sindh, Khyber Pakhtunkhwa and Punjab (GOP, 2018). According to a report on agriculture marketing information service, Pakistan produces 4.2 million tonnes of tomato annually (GOP, 2018).

The tomato crop is of subtropical regions and its production has been reduced than the highest standards and quality which is thought to be caused by unfavorable seasonal conditions including high temperatures (Bai *et al*., 2007). Tomato crop often has to face temperature stress in different areas of the world. It requires an optimum temperature of 20-26^{0}C at daytime and 15-20^{0}C at night for growth. Heat stress caused a worldwide reduction in tomato yield is one of the critical issues in agricultural sustainability. Reports have indicated that a wide range of economic losses in tomato production is caused mainly due to high temperatures resulting in fruit set reduction and declined tomato yields (Khan *et al*., 2015). More than 70% losses in the harvesting of cultivated tomato crops have been reported as a result of the hot summer season of many agricultural regions (Fahad *et al*., 2017).

1.3. Morphological and growth responses of plants to heat stress

High temperature is one of limiting factor that not only affects plant growth but also affects the crop yield. The first affected stage during plant growth is the germination stage. Abnormal growth of seedling, vigor, stunted radicle and stunted plumule growth during initial stages are the responses of the plant under heat stress conditions (Borriboon *et al*., 2018). Heat stress affects almost all tissues of the plant at all stages, however, the reproductive tissues and organs are the most sensitive. Even for a very short time of heat spell may result in damage of floral new buds and fruit abortion as well. During heat stress conditions the reproductive developmental stages may face less development, or no flower production and less fruit set some time occur (Grover *et al*., 2011). Sometimes under unusual and abrupt temperature rise may lead to reduce the size and area of leaves and it may lead to the fall of the young leaves. This eventually affects the plant photosynthetic efficiency (Greer & Weedon, 2012). The sunburns and scorching of leaves, branches, and stems are the other indications of high-temperature stress conditions. During the heat stress condition, leaf senescence, abscission, stunted shoot, and root inhibition, fruit damage and less pigmentation are

the promising problems (Guilioni *et al*., 2005). Heat stress adversely affects fertilization process, germination of pollen, tube growth of pollen, viability of ovule, stigma and style positions of the flower, pollination, the growth of the endosperm and poor fruit set (Wahid *et al*., 2007).

1.4. Anatomical changes in plants in response to heat stress

During the high-temperature stress conditions, the plant show various anatomical changes in the form of reduced cell area and size, enhanced the densities of stomata and reduced transpiration functions and affect the vascular system of plants (Golam *et al*., 2012). The chloroplast is the more sensitive and it is the main site of injury due to high temperatures. Major alterations occur in heat stress condition involve transformed thylakoids structural organization, grana stacking disturbance, physiochemical reactions changes in thylakoid the chloroplast and carbon metabolism processes in the stroma of chlorophyll (Wise *et al*., 2010). Mitochondria are very sensitive to heat and it is degenerated due to high temperature. Furth more protein expression profiles also are altered, decreased ATP accumulation and less uptake of oxygen has been observed in various plants under heat stress conditions. (Hampton *et al*., 2013). If the temperature of the environment increases above the upper threshold and the plants in this range are adapted by about 20°C within a few hours, there is definite evidence that the photosynthetic apparatus of chloroplasts is reversely damaged first and other plant parts functions diminishing after it (Efeoglu & Terzioglu, 2009).

1.5. Physiological responses of plants against heat stress

Under stress condition, mitochondria contribute to ROS signalling through the electron transport chain, chloroplast through Mehler reaction and peroxisomes through glycolate oxidase reaction (Mittler *et al*., 2006). The generation of ROS molecules causes oxidative stress which is detrimental to plants, but with the passage of time, plants developed scavenging mechanisms to bear oxidative stress and maintaining sensitive levels of ROS molecules.

1.6. Mechanism of heat tolerance in tomato plant

When temperature increases the plants show various mechanisms and metabolic processes for surviving under high-temperature environments and under these stressful conditions the plants show morphological, anatomical and phenological enduring changes and immediate changes occur in plants. Transpiration processes, changes in leaves intentions and change the cell membrane bilipid layers structure also occur. Under the heat stress condition, the plants at initial developmental stages are strictly linked to the reduction of crop yields, (Adams *et al*., 2001). The plants face different environmental stress conditions at various phases of the growth and their actions are different in various parts of the plants (Queitsch *et al*., 2000). The plant different metabolic processes response to heat stress condition and they assimilate to bear these high temperatures are countless applied and greatly influenced. Tolerable process are scavengers of free ions and their transport, compatibility of solutes, more protein during embryo development, forces of signals factors and control nutrients uptake are important to respond to the stress conditions (Wang *et al*., 2004). The plants change the metabolic processes and start with the absorption of heat and their response and the formation of chemicals substances that produce the capacity in plants to bear different high-temperature ranges. High-temperature effects are showing at various points that are cell membrane, in metabolic reactions in cytoplasmic fluids and in other cellular organs (Sung *et al*., 2003). The high temperature rapidly changes the processes of gene expression (Yang *et al*., 2006), production of HSP (heat shock protein) by expression of genes and it retard the other genes expression.

1.7. Mitigation remedies to heat stress in tomato

Today's technologies such as genetic, biotechnologies, and transgenic technologies have been developed to cope with heat stress issue. The thermo-resistance plants change the heat shock protein level in their body and handle the heat stress issue through heat shock proteins (HSPs) (Al-Whaib, 2011). Furth more phyto-hormones, tracing elements, signalling molecules and ions have a positive effect on plants development under heat stress through antioxidant capacity (Hassan et al., 2020). The heat-resistance variety plant selected through conventional strategies for breeding for growing heat-resistant crops in a targeted heat environment and with high crops yields (Ehlers & Hall, 1998). The cultural practices need to change and

manipulate to reduce adverse effects of heat stress to high range (Meiri *et al*., 2010). The most strategies and practices are cost-intensive and efficient in work, cheap, very easy methods for abiotic conditions such as high-temperature condition is a major challenge for the world. The microorganisms studies indicate that it copes the plants to stressed conditions, it's cheap and environmentally friendly strategies (Fahad *et al*., 2017).

There are many other currently using techniques to develop heat stress tolerance in the tomato plant. Advanced genetic editing and transformation (Brooks *et al*., 2014) along with techniques of engineering soluble metabolites like antioxidants and integral expression of HSPs have been proved as key tools in conferring heat tolerance in tomato crops (Li *et al*., 2015). Almost all currently using technologies to develop heat tolerance are costly and are cite specific as well. However, conferring microbial interactions with plants in order to mitigate abiotic stresses including high temperature is also being implemented as a promising and cheaper technique in agriculture (Guo *et al*., 2016). Such microbes are called as plant growth-promoting bacteria (PGPB) that not only to mitigate environmental stresses, but also enhance growth and yield of plants (Mukhtar *et al*., 2020). Up till now PGP strains of numerous genera have been identified out of which *Bacillus* and *Pseudomonas* are studied mostly (Gururani *et al*., 2012). Growth promoting capability of PGPBs has been investigated against a number of stresses like drought, waterlogging, metal toxicity and pathogenicity. Besides these stresses, PGPBs have also been tested for their ability to mitigate temperature stress in many crops (Nadeem *et al*., 2014).

Investigations have shown that these PGPBs are able to confer stress tolerance either by enhancing antioxidant efficiency in a plant (Yang *et al*.,2009) or by providing plants with substances that reduce growth inhibition under stressful environment (Mayak *et al*., 2004; Ahemad *et al*., 2014). A number of crop plants have been tested for heat stress tolerance induced by microorganisms (Ahemad & Kibret, 2014). Reports have indicated that a number of such plant growth promoting bacteria cause enhanced production of antioxidant enzymes like superoxide dismutate and catalase thereby removing toxic ROS produced under stressful environments (Wang *et al*., 2013). Investigations have also shown that inoculation of plants with thermotolerant PGPBs results in higher accumulation of antioxidants metabolites like proline (Mukhtar *et al*., 2020)

1.8. Plant growth promoting bacteria (PGPB)

Crop plants always are in a friendly relationship with microbes of soil like bacteria and fungus during their whole life cycle. These symbiotic soil microorganisms especially the bacteria living in the rhizosphere of almost every plant species have various beneficial effects on a plant (Raza *et al*., 2016). Plant growth is promoted with the application of bacterial strains through various mechanisms like phosphorous and potassium solubilization, nitrogen fixation, and nodulation etc. (Figure. 1.1). Besides improving plant growth, these bacteria defend plant health in an eco-friendly way (Akhtar *et al.,* 2012). PGPB and their friendly relations with plants have been studied commercially with wide scientific applications in sustainable agriculture (Gonzalez *et al*., 2015).

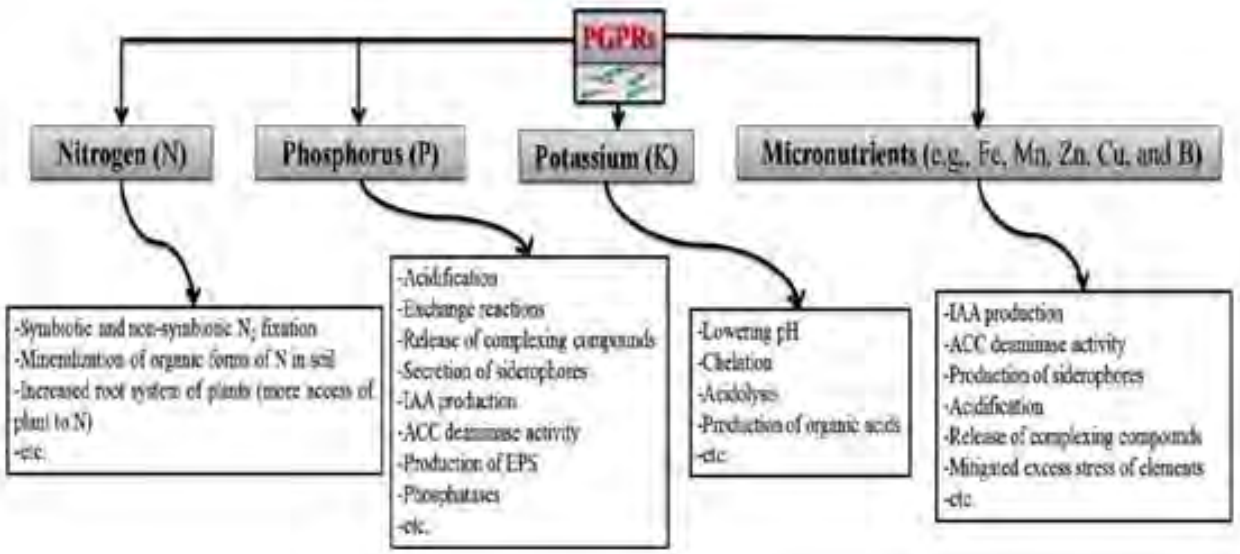

Figure. 1.1. Some of action mechanisms of PGPRs in alleviating nutritional Imbalance stress in plants

Furthermore, these strains have also been proved beneficial for plant growth under high temperature stress by producing PGP substances like ammonia, gibberellins and indole acetic acid along with improving physiological parameters like root and shoot length, dry and fresh biomass etc. (Ali *et al*., 2009).

1.9. Beneficial aspects of Plant growth promoting bacteria (PGPB)

The plant growth promoting bacteria inhabiting in the rhizosphere can affect on the growth of a plant and their development through regulatory hormones, enhanced the uptake of nutrients to plants (Nadeem, *et al*., 2014). Many PGPB also improve the heat stress, drought, flooding, salinity, heavy metals tolerance in the plant and enhance the capability of the plant to survive in unfavorable environmental (Prasad *et al*.,2015).

The Plant growth promoting bacteria help the plants in two different ways for the growth of the plant through direct ways and indirect ways (Glick, 2014). Through the indirect ways, the rhizobacteria prevent the plant from pathogen and reduce the pathogenic effect on plants in different ways (Glick & Bashan, 1997). The indirect mechanism inhibits the harmful substances produced by pathogens or it enhanced the plant resistance protect from pathogens (Persello *et al*., 2003). For example, the production of metabolites of rhizobacteria for reduction of pathogenic population and siderophores production reduced the availability of iron to pathogens because it reduces the growth and development of the plant (Bhattacharyya & Jha, 2012). The plant growth promoting bacteria also enhanced resistance of plants against diseases by altering plant host susceptibility by the various mechanism known as induced systemic resistance (ISR) and it protects the plant against pathogens (Garcia *et al*., 2012).

Direct growth promotion mechanisms include nitrogen fixation by bacteria. *Rhizobium* spp which are free-living bacteria with a capacity to fix atmospheric nitrogen, for example *Azospirillum* spp., (Bohlool *et al*., 1992). The gene responsible for nitrogen fixation in diazotroph is *nif* gene which is found in a cluster of around 20-24 kb with seven operons encoding 20 different proteins. Some scientists believed that if *nif* genes is isolated and characterized, then genetically engineer improvements in nitrogen fixation might be possible (Hardoim *et al*., 1997).

1.10. Mechanisms employed by plant growth promoting bacteria for the mitigation of adverse effects of stress on plants

In an optimal, and normal environment, the common mechanisms of plant growth promoting bacteria for augmentation of growth of plants. The growth improved by plant growth promoting bacteria by an assembly of different mechanisms such as reducing ethylene level, production of exopolysaccharides, induced systemic resistance, etc. (Upadhyay *et al*., 2011). Depressing of ethylene is one of the major mechanisms produced by PGPB for helping plant growth under stressful environment. Ethylene phytohormone improves growth plant at in low concentration (Glick, 2014). Level of ethylene are usually raised under stressconditions due to improved production of 1-aminocyclopropane-1-carboxylic acid (ACC), ethylene precursor biosynthetic pathway (Zapata, *et al.,* 2008). 1-Aminocyclopropane-1-carboxylate (ACC) is thought to cause an adversative effect on plant growth mostly on the elongation of roots that eventually affects whole body plant progressions including, in nutritional functions and physiological functions (Alarcón *et al*., 2012).

For maintaining a standard plant growth, it essential that ethylene concentration should be upto specific level that is suitable for the optimal growth of the plant. It is stated that certain PGPR bearing ACC-deaminase activity which can reduce ACC into ammonia and α-ketobutyrate (Glick*,* 2014). The ACC level reduce the ethylene level concentration in root locality that is helpful for enhancing growth of the root. According to the model designated by (Penrose *et al*., 2003). The occurrence of plant root exudates attracts PGPB to the root surface of plant (Lynch & Whipps, 1990). The PGPB produce the indole acetic acid (IAA) and their activity increase the ACC level in roots, and also endogenous IAA in plant encourages the ACC synthase activity and convert S-adenosylmethionine to ACC. Due to ACC-deaminase enzyme actions, the PGPB change it into ammonia compound and α-ketobutyrate and these protect the plant from lethal of ethylene production. By the degradation the root externally decrease ACC concentration, when roots produce more ACC it reduce the ethylene concentration in the root of the plant. The inhibitory effect of ethylene on the root elongation defeated by this mechanism (Glick *et al*., 2007). The ethylene production and concentration maintained by this model and describes efficiently that it increase plant growth under stress environment. The scientists work further proved and the use of this phenomenon for promoting growth and development of plant (Chen *et al.,* 2017).

Under the heat stress environments, the imbalance of nutrition also affects the plant. Plant growth promoting bacteria produce exopolysaccharides and it reduces the sodium ions uptake in plant and also form biofilm on the roots surface (Qurashi & Sabri, 2012). The decreased obtainability of sodium ions results in lowering the uptake of sodium ions and these sodium and potassium ions ratio able the plant to cope in salinity stressed surroundings (Ashraf & Harris, 2004; Han & Lee, 2005). The plant exopolysaccharides also play a vital role in plants to stand on water scarcity conditions. As drought stress injurious the plant and also negative effect on soil microbes and exopolysaccharides protect the bacteria and plants and allow them to continue the growth water stress environment (Sandhya *et al*., 2009).

Microbes in the soil affect the growth of the plant. The plant growth promoting bacteria increase the resistance of plant and also protect it from pathogens and plant combat against diseases. This is accomplished by a number of mechanisms including competition and parasitism from the above discussion, Plant growth promoting encourages plant growth and development by using different mechanisms and give protection the plant from harmful conditions by monitoring the accessibility of some specific biomolecules that affect plant growth and development. These agents can enhance plant resistance from stress surroundings.

The current study was aimed to isolate thermo-tolerant bacteria having plant growth promoting traits and evaluation of isolated strains under heat stress condition in greenhouse and field experiment.

1.11. Objectives

- Isolation, screening and characterization of bacterial isolates
- Evaluation of selected bacterial strain against heat stress conducting greenhouse
- Multi-year and multi-locational field trials

Chapter 2

Isolation, screening and characterization of bacteria

2.1. Introduction

Plant growth promoting bacteria benefit plants by stimulating growth and suppressing negative effects of environmental contaminants and disease are referred to as plant growth promoting bacteria (PGPB). PGPB have been tested not only as biocontrol agents for suppression of plant diseases but also have been used for bioremediation of various environmental contaminants (Sheng *et al*., 2012). Microorganisms particularly associated with rhizospheric region and endophytic bacteria play a vital role in minimizing various stress in plants which results in better crop production (Etesami & Beattie, 2017). Among these, bacteria, the most studied are plant growth promoting rhizobacteria (PGPR), with plant growth promoting traits have the potential to increase plant growth and yields under stress condition. Rhizospheric region is rich with microbes that surrounding the roots of plants where the biological and chemical properties of soils are affected by roots. Bacteria could make a symbiotic or non-symbiotic relationship with plants in the rhizosphere can be, which is determined by whether their mode of action is directly beneficial to the plant or not (Kundan *et al*., 2015).

Bacterial endophytes are bacteria that live inside plant tissues and have the potential to colonize plant inner tissues (Sturz *et al*., 2000). Just like plat growth promoting rhizobacteria, endophytic bacteria also play an important role in plant development under both normal and stress conditions (Yaish *et al*., 2015; Choudhary *et al*., 2016). PGPB facilitate plant nutrients uptake from nearby environments by various mechanisms including production of siderophores to sequester iron, by phosphorus solubilization and by nitrogen fixation (Etesami & Beattie, 2017).

Furthermore, PGPB can modify plant growth with production of phytohormones such as indole acetic acid (IAA) or sinking the ethylene production by the action of the 1-Aminocyclopropane-1-carboxylate (ACC) deaminase enzyme (Glick, 2014). In contrast, indirect plant growth promotion by PGPB occurs when they minimize or avoid the plant from damage caused by pathogenic agents (Compant *et al*., 2005). Multi genic and quantifiable tolerance to abiotic stress causes the accumulation of number of stress metabolites that includes proline, glycine-betaine, poly-sugars, and abscisic acid. These metabolites are also involved in up regulation of the synthesis of enzymatic and non-enzymatic antioxidant like superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione

reductase, α-tocopherol, ascorbic acid, glutathione (Agami *et al*., 2016). Comprehensive studies has been done on the application of PGPB in management of stress through various number of bacterial strains like *Pseudomonas fluorescens* and *Pseudomonas putida* having the potential to scavenge cadmium ions from soil and reduced the drastic effect of cadmium pollution in barley plants (Baharlouei *et al*., 2011). It has been documented that PGPB enhanced the water status of leaf under abiotic stresses (Naveed *et al*., 2014).

The isolation of rhizospheric and endophytic bacteria and the selection of promising plant growth promoting bacteria is a complex process because of the method for the isolation, selection and the organization of a huge data regarding the isolated bacterial strain (Barnett, *et al*., 2017). There are a lot of research methods that study the isolation and characterization of bacterial strains from rhizosphere and various plant parts. Usually, two methods are used in the isolation of bacteria. The main and widely used method is the culture-dependent method in which a large number of isolates are isolated, characterized and identified. At the end the best promising bacterial strains is selected for field application. This method is a step-by-step technique with various phases including at least three levels of investigation (isolation in laboratory, greenhouse and field experiment) (Yan *et al*., 2018). The second method was given by Kim *et al*., (2019). It is based on meta-genomic studies in which population diversity and phylogenetic analysis of each family group is focused (Petruzzi, *et al*., 2014). In the current study the rhizospheric and endophytic bacteria were isolated from tomato rhizosphere and various plant parts. Further characterization of bacterial isolates were characterized against plant growth promoting traits, heat stress and final selected isolates were identified. Overall objectives of this study are given below.

2.2. Objectives

 To perform the isolation, screening and characterization of bacterial strains

 To evaluate qualitative and quantitative analysis of ACC-deaminase and exo-polysaccharide production of isolated strains

 To perform the sequencing and identification of promising isolates

 The Quantification of growth regulators

2.3. Materials and methods

2.3.1. Sample collection

Soil and plant samples were collected from Larkana, Sindh Province, Pakistan (27.5570° N, 68.2028° E). Leaves, stem and roots from each collected plant sample were separated and kept in sterilized plastic bags. The samples were carefully transported to Plant Microbe Interactions Lab, Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan and stored in a refrigerator at 4ºC for further activities.

2.3.2. Isolation of plant growth promoting bacteria

2.3.2.1. Isolation of rhizospheric bacteria

2.3.2.1.1. Serial dilution method

The dilution plate method was used for isolation of bacteria from all these samples. The media was autoclaved for 30 mints. Pouring was done under a Laminar flow hood to avoid any contamination. Rhizosphere samples of tomato were washed with distilled water in flasks by continuous shaking. One ml aliquots from the suspension of the rhizosphere and rhizoplane were transferred separately in to 9 ml sterile distilled water to form 10^{-1} dilution and further dilutions were made up to 10^{-8} (8 times tenfold dilution). From each sample, 100 µl of each dilution was taken and spread on plates. Incubation of plates were done for 3-4 days at 32-35ºC (Amna *et al.,* 2019).

2.3.2.1.2. Isolation of endophytes

Soil and other debris from each stored sample were removed by thoroughly washing with tap and distill water.The washed samples were cut into small pieces and surface sterilization was performed with 1.25% NaOCl (10 min.) and 70% ethanol (for five min.), respectively. Each sample was further washed for two to three times with the sterilized distilled water (Mufti *et al*., 2015). Maceration of parts of plant were done in 1 ml sterile distilled water and shifted to the test tubes that contain the NFb and DN semisolid medium (5 ml). For the appearance of growth on surface of semisolid DN media, the test tubes were further kept in the incubator for 1 week at 32ºC. Isolates were streaked on solid DN media after growth appearance in test tubes following protocol of Mufti *et al*., (2006).

2.3.4. Plant growth potential of heat tolerant strains

2.3.4.1. Indole acetic acid (IAA)

Falcon tubes poured with LB broth that amended with tryptophan (precursor of IAA) were used for growth of bacterial culture. Addition of five drops of Kovac's reagent to each tube was carried out for confirmation of IAA (Hussain *et al*., 2019).

2.3.4.2. Phosphorus solubilization

Phosphorous solubilization test was performed following the procedure of Gupta *et al*., (2012). Sterilized Petri plates were used, and Pikovaskaya's media was poured into the Petri plates. Incubation of inoculated plates was done for 7 days at 28ºC.

2.3.4.3. Ammonia production

Peptone water was used to test bacterial isolates for ammonia production (Dinesh *et al*., 2015). Peptone water-10 ml (peptone 10 g and NaCl 5 g/L, pH 7±0.2) was used for the inoculation of freshly grown bacterial cultures in all tubes and incubated at 36±2ºC for 48-72 h. After that, mixing of Nessler's reagent (0.5 ml), was carried out in each tube. Ammonia production was confirmed by the appearance of brown to yellow color. Nessler's reagent: Potassium iodide (50 g) mixed in very small possible amount of water. A saturated mercuric chloride solution almost 22 g in 350 ml of H_2O, was added in solution of potassium iodide till the formation of precipitate. Addition of 200 (ml) of 5N NaOH was done and prepared up to 1liter, filteration was done after the settling of precipitation.

2.3.4.4. Hydrogen cyanide (HCN) determination

Bacterial isolates were screened out for hydrogen cyanide, (HCN) production through streaking of bacterial isolates on a plate containing LB, agar augmented with 4.4 g/L glycine. Filter papers were cut in round shape. A solution comprised of picric acid (0.5%) and sodium carbonate (2%) was used for dipping of filter paper. The dipped filter paper was placed in the upper lid of the plate. Parafilm was used to seal the plates in order to avoid gas discharge and incubated for 5 days at 30ºC. Positive indication of HCN is the change in color (from yellow to orange brown) of filter paper (Kumar *et al*., 2012).

2.3.4.5. Siderophore production

The proposed method of Louden *et al*., (2011) was conducted for siderophore production assay. A loop full of selected isolates was inoculated on selective media amended with CAS-substrate (without iron) and kept incubated for 7 days at 30ºC. The positive result of siderophore production was confirmed by appearance of halo zone (orange colored) around the isolated isolates.

2.5. Extracellular enzyme activities

2.5.1. Protease production

Agar medium with skim milk was used to evaluate the protease activity. Media was prepared by adding 1 g glucose, 2 g peptone, 5 g yeast extract, 1 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 5 g skimmed milk and 15 g agar in 1000 ml distilled water. Autoclaved the Media and plates were prepared. Bacterial strains were inoculated by spotting on skimmed milk media plates and incubated for 2-3 days at 30ºC. The appearance of halo zone around the bacterial colony is a indication of protease production (Chang & Hsieh, 2009).

2.5.2. Pectinase production

All isolates were screened for their pectinase producing capability by spot inoculation on agar plates containing 1 g yeast extract, 2 g NH_4SO_4. 6 g Na_2HPO_4, 3 g KH_2PO_4, 5 g pectin from citrus peer and 15 g of agar in 1 Liter of distilled water. After inoculation, parafilm was used to seal plates and incubated at 30ºC for 2 days. After completion of incubation period, plates are flooded with iodine solution and the formation of halo zone is a positive indication of pectinase production (Tiru *et al*., 2013).

2.5.3. Amylase production

Bacterial Isolates were tested for amylase production following the method of Ashwini *et al*., (2011). A loop full of the bacterial colony was spot inoculated in agar plate prepared by adding 1 g yeast extract, 0.1 g $MgSO_4$, 7 g K_2HPO_4, 2 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 5 g NaCl, 5 g starch and 15 g agar in one liter of distilled water. Incubation of plates were done for 2 days at 28ºC. After completion of the incubation period,

plates were saturated with iodine solution and formation of halo zone around bacterial colony was observed. Formation of halo zone is positive result of amylase production (Ashwini & Gaurav, 2011)

2.5.4. Catalase production

For testing catalase (CAT) enzyme activity, a single bacterial colony from 24 h old bacterial culture was placed on a clean glass slide and a drop of 30% (H_2O_2) hydrogen peroxide was added upon it. Production of gas bubbles indicates that CAT enzyme is present in the bacteria (Naseem & Bano, 2014).

2.6. 1-aminocyclopropane-1-carboxylic acid (ACC) Deaminase activity

The method of Pandey *et al*., (2019) was used to screen the bacterial isolates being able to utilize ACC as a nitrogen source. These bacterial cultures were inoculated by spotting on petri plates containing minimal DF salt media (Dworkin and Foster; media per liter: 4.0 g KH_2PO_4, 6.0 g Na_2HPO4, 0.2 g $MgSO_4.7H_2O$, 2.0 g glucose, 2.0 g gluconic acid and 2.0 g citric acid with trace elements: 10 mg H_3BO_3, 1 mg $FeSO_4.7H_2O$, 11.19 mg $MnSO4.H2O$, 124.6 mg $ZnSO_4.7H_2O$, 78.22 mg $CuSO_4.5H2O$, 10 mg MoO_3, pH 7.2) supplemented with and without ACC. Positive control of this experiment was Petri plates with ammonium sulfate. The growth on ACC supplemented plates were compared to positive and negative controls after three days of incubation at 28±2ºC and isolates using ACC as a nitrogen source were selected.

2.7. Screening of bacteria for heat tolerance potential

The isolates were screened for heat tolerance against different temperature ranges (32-60ºC) on LB solid media (Ali *et al*., 2009).

2.7.1. Growth curve under heat stress

Selected isolates were cultured on LB broth under temperature ranges of 32 - 60ºC to determine its growth potential. Bacterial cultures were kept on shaking at different temperatures for seven days. Spectrophotometer (Agilent 8453 UV-visible Spectroscopy System) was used to measure the optical density (OD) of each isolate at 600 nm wavelength after every 24 h up to seven days consecutively. The bacterial isolates having optical density value >0.2 (600 nm) at maximum temperature of

60°C were considered as thermo-tolerant and selected for further studies (Khan *et al*., 2020).

2.8. Morphological Characterization

Fresh colony of each isolates was streaked on LB agar plates and kept in incubator at 28ºC for 24 h.

2.8.1. Colony morphology

Observation of colony morphological characteristics (colony color, colony margins, colony surface texture, colony shape and elevation) was done by culturing of purified isolates on solid media: (Rohomania *et al*., 2015).

2.8.2. Gram staining and cell morphology

After complete purification, the isolates were further confirmed by using the Gram staining technique (Etesami *et al*., 2017).

2.9. Quantitative assay of ACC-deaminase activity

Bacterial isolates were further characterized for quantitative estimation of ACC-deaminase activity after qualitative screening. Selected isolates were grown in 5 ml of TSB under normal and heat stressed condition and centrifuged after 24 h to obtain cell pellets. The pellets were washed with 0.1MTris-HCl (pH 7.5). DF minimal medium was used for the augmentation of these cells and these cells were supplemented with 3mM ACC. Cells put in to incubator shaker for 72 h.

The α-ketobutyrate concentration in each sample was determined to measure ACC-deaminase activity. The induced cell pellets were labelled by adding 5% toluene (v/v). 50 µl aliquot from labelized cell suspension was incubated for 30 min after adding 0.3 M ACC (5 µl). Negative control did not contain ACC whereas the Tris-HCl and ACC were used for blank sample preparation. NH_4Cl (0.56) was added in each sample and later vortexed. After vortexing, centrifugation of cells was done at 12000 rpm for 5 min. The supernatant (500 µl) from each sample which was supplemented with DNF solution (150 µl) and 0.56 NH_4Cl (400 µl). After half an hour incubation, absorbance was recorded at 540 nm. At the time of absorbance one ml of 2N NaOH was added in each sample (Khan *et al*., 2020).

2.10. Characterization through QTS-24 kit

Strains were assessed for different secondary metabolites production (Yasmin & Bano, 2011). Freshly grown strains were inoculated to QTS tubes. These tubes were incubated at 37°C for 24 h. When incubation was done and reagents were added to QTS strips according to the instructions given in manual and results were noted.

2.11. Qualitative and quantitative assay of exopolysaccharide (EPS) production

Exopolysaccharide potential of selected isolates were carried out following protocol of Muminah *et al*. (2015). Quantitative assessment of exopolysaccharide was done under normal and high temperature.

2.12. DNA extraction

DNA of all selected bacterial strains was extracted by Phenol-chloroform method. Cell pellet was suspended in 450 µl TE buffer. Cells were incubated for an hour at 37^0C after adding 45 µl sodium dodecyl sulphate and 5 µl of Proteinase K (20mg/ml). Following incubation, 600 µl 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. The upper aqueous phase was collected in new Eppendorf tube. Samples were mixed until formation of DNA precipitates after adding 50 µl sodium acetate and 300 µl isopropanol. Samples were centrifuged at 10,000 rpm, for 5 mins for the removal of liquid phase, and washed with 70% chilled ethanol. Precipitated DNA was stored in 70 µl TE buffer at -20ºC (Satyanarayana *et al*., 2017).

2.13.1. Polymerase chain reaction for 16S rRNA genes

Amplification of extracted DNA was with 16S rRNA genes given below. 27F: 5-AGAGTTTGATC AC TGGCTCAG-3, 1492R: 5-CGG CTTACCTTGTTACGACTT-3. The PCR products sent to Macrogen, Korea for commercial sequencing with universal 785F 16S rRNA gene specific primers. The obtained sequences were blast on NCBI and aligned with the sequences of reference strains. These aligned sequences were used for the construction of phylogenetic tree through mega 6.0 software (Mufti *et al.,* 2015).

2.13.2. Amplification of *acds* gene

Universal primers described by Duan *et al.,* (2009) were used to amplify *acds* gene responsible for ACC deaminase enzyme. Primer sequences were, Forward: 5'-GGCAAGGTCGACATCTATGC-3', Reverse: 5'- GGCTTGCCATTCAGCTATG-3'.The reaction conditions of PCR which are, initial denaturation for 180 sec at 94ºC, subsequent denaturation at 94ºC for 60 sec (30 cycles), annealing at 58ºC for 60 sec, extension at 72ºC for 180 sec. A final primer extension at 72ºC for 300 sec (Singh *et al.,* 2015).

2.13.3. Agarose gel electrophoresis

Observation of PCR products were done in 2% agarose gel made in 1X TAE buffer. Samples were run at 85V for 35 min in a horizontal electrophoresis unit and were visualized in a gel doc system (Satyanarayana *et al*., 2017).

2.14. Evaluation of plant growth regulators under normal and high temperature

2.14.1. Extraction and purification

Extraction, purification and quantification of growth regulator from bacterial cultures were made to understand the mechanism of growth promotion of these microbes used as inoculants. Growth media (100 ml) were inoculated with 24h old bacterial cultures and incubated at 30°C on a shaker at 100 rpm for 7 days till the OD of cultures turn in to equivalent to 1 at 600nm. Thereafter the bacterial cells were harvested, centrifuged for 10 mins at 10,000 rpm. The supernatant was used for extraction of growth hormones. Supernatant pH was adjusted to 2.8 with 1N HCl. Extraction of growth regulators was performed by following the method as described by Saber *et al*. (2015). Ethyl acetate (an equal volume) was added to the cell-free culture and mixed thoroughly in a separating funnel. This extraction procedure was repeated three times and the ethyl acetate phase was evaporated at 35°C under vacuum. The residues were dissolved in 1000µl of methanol.

2.14.2. Quantification of growth regulators

Quantification of growth regulators was carried out on HPLC (Agilent 1100) by using a UV detector and C18 column (39 x 300mm). The IAA, GA3 and Kinetin (Commercially grade, Sigma chemical company the USA) were used as a standard for

identification and quantification of IAA, GA3 and Kinetin produced by bacterial isolates. Methanol: acetic acid: water (30:1:70; v/v) was used as the mobile phase at the rate of 1500µl/min with a run time of 20min/sample. Samples (100 µl) were filtered through a 0.45 Millipore filter and injected into the column. The growth regulators were identified based on the retention time of the standard IAA using a UV detector at 280 nm wavelength and GA3 and Kinetin at 254 nm respectively (Saber *et al*., 2015).

2.4. Results

2.4.1. Sample collection and isolation

Rhizospheric and endophytic strains were isolated from rhizosphere and different plant parts which were root, stem and leaves. Collection of samples was done from Larkana, province, Sindh, Pakistan. Seventy isolates were obtained from samples. These seventy isolates were purified, preserved and characterized. Screening of these isolates were done against different level of temperature and after screening five most promising isolates were obtained that were survived against high temperature and have plant growth promoting and extracellular enzymes activities.

2.4.1.1. IAA production

All Seventy bacterial strains showed positive results for IAA production by the formation of the cherry red ring on the top of the test tube (Table 2.1, Figure 2.1).

Figure 2.1. Indole acetic acid production by isolated bacteria

2.4.1.2. Phosphate solubilization

All seventy strains showed a positive result of P-solubilization (Table 2.1, Figure 2.2).

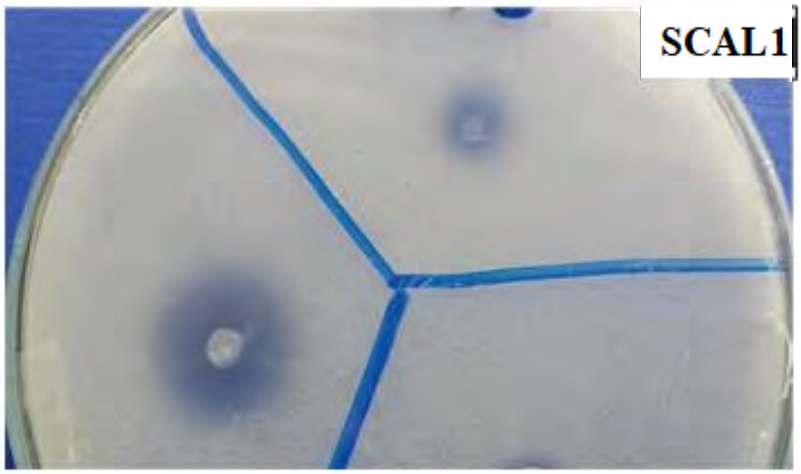

Figure 2.2. Phosphate solubilizing activity of isolated bacterial strain

2.4.1.3. Ammonia production

All isolates showed positive result by the formation of brown to yellow color (Table 2.1) (Figure 2.3).

Figure 2.3. Ammonia production in isolated strain. C: control

2.4.1.4. Siderophore production

Two bacterial isolates (SCAL1 and KTES) out of seventy showed positive results for siderophores production (Figure 2.4, Table 2.1).

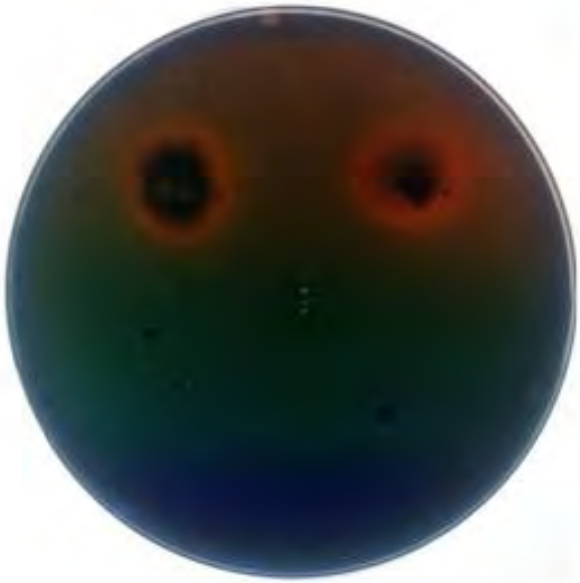

Figure 2.4. Siderophore production

2.4.1.5. HCN production

Two bacterial isolates (T6 and SCAL1) out of seventy, showed the positive result for HCN production. (Table 2.1, Figure 2.5).

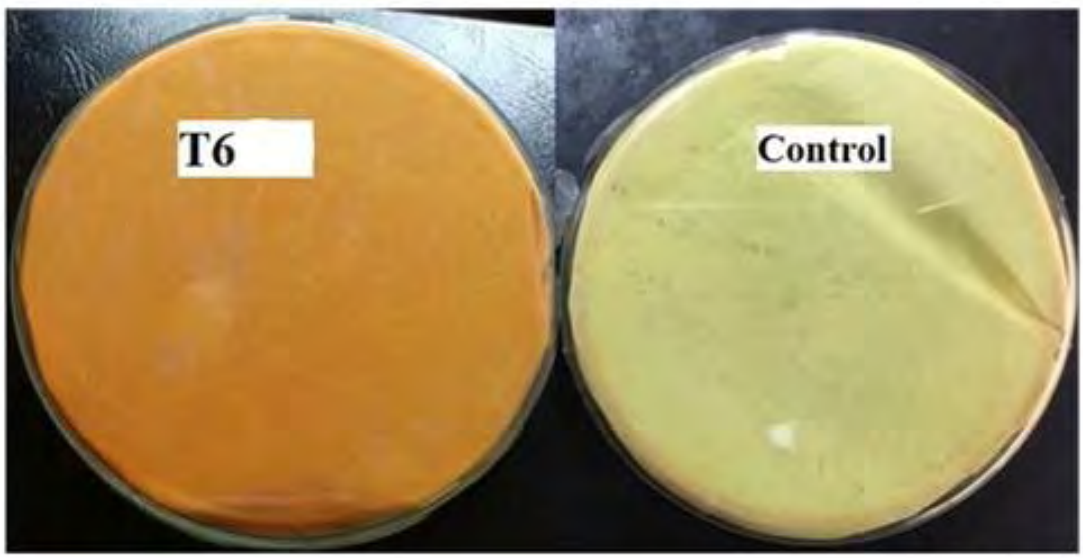

Figure 2.5. HCN determination of isolated strain.

Table: 2.1. Plant growth promoting characterization of bacterial isolates

Sr.no	Code of strain	IAA	Phosphorous solubilization	HCN	Ammonia production	Siderophore
1	T6	+	+	+	+	-
2	KTES	+	+	-	+	+
3	SCAL	+	+	-	+	-
4	SCAL1	+	+	+	+	+
5	SCALT	+	+	-	+	-
6	TR3.1	+	+	-	+	-
7	Tr3	+	+	-	+	-
8	Tm24	+	+	-	+	-
9	Tm2	+	+	-	+	-
10	Mk2	+	+	-	+	-
11	T6w	+	+	-	+	-
12	T6Y	+	+	-	+	-
13	V3	+	+	-	+	-
14	Scal(0)	+	+	-	+	-
15	VR	+	+	-	+	-
16	SR	+	+	-	+	-
17	M107	+	+	-	+	-
18	Sca	+	+	-	+	-
19	$KTES^{3}p$	+	+	-	+	-
20	TR3(3.1)	+	+	-	+	-
21	M105(b)	+	+	-	+	-
22	$BTRP^{4}$	+	+	-	+	-
23	CUX10y	+	+	-	+	-
24	$Btrs10^{5}$	+	+	-	+	-
25	KTE63D	+	+	-	+	-
26	ScaR	+	+	-	+	-
27	T6(2)	+	+	-	+	-
28	$Scal10^{7}$	+	+	-	+	-
29	Ktrp(1)	+	+	-	+	-
30	$Scal^{2}$	+	+	-	+	-
31	TR3ve	+	+	-	+	-
32	$Tr^{3}C^{3}$	+	+	-	+	-

33	Ktes	+	+	-	+	-
34	Ktes^{3}p	+	+	-	+	-
35	Ts1	+	+	-	+	-
36	VL1	+	+	-	+	-
37	TM37	+	+	-	+	-
38	M10	+	+	-	+	-
39	TR1	+	+	-	+	-
40	JAR3	+	+	-	+	-
41	TM41	+	+	-	+	-
42	TM42	+	+	-	+	-
43	TM43	+	+	-	+	-
44	TM44	+	+	-	+	-
45	V5	+	+	-	+	-
46	TM46	+	+	-	+	-
47	KP1	+	+	-	+	-
48	TM48	+	+	-	+	-
48	TM49	+	+	-	+	-
50	Ca(1)	+	+	-	+	-
51	TM51	+	+	-	+	-
52	TM52	+	+	-	+	-
53	TM53	+	+	-	+	-
54	TM54	+	+	-	+	-
55	TM55	+	+	-	+	-
56	TM56	+	+	-	+	-
57	TM57	+	+	-	+	-
58	TM58	+	+	-	+	-
59	TM59	+	+	-	+	-
60	TM60	+	+	-	+	-
61	TM61	+	+	-	-	-
62	TM62	+	+	-	-	-
63	TM63	+	+	-	-	-
64	TM64	+	+	-	-	-
65	TM65	+	+	-	-	-

66	TM66	+	+	-	-	-
67	TM67	+	+	-	-	-
68	TM68	+	+	-	-	-
69	TM69	+	+	-	-	-
70	TM70	+	+	-	-	-

(+): present, **(-):** absent

2.4.5. Extracellular enzyme tests

2.4.5.1. Protease production

Forty-nine strain showed positive results for protease production by the formation of halo zone around the bacterial colony (Table 2.2, Figure 2.6).

Figure 2.6. Protease production by bacterial isolates

2.4.5.2. Pectinase production

Thirty- five bacterial isolates showed a positive result for pectinase production by the formation of halo zone around bacterial colony after flooding with 50 mM iodine solution (Table 2.2, Figure 2.7).

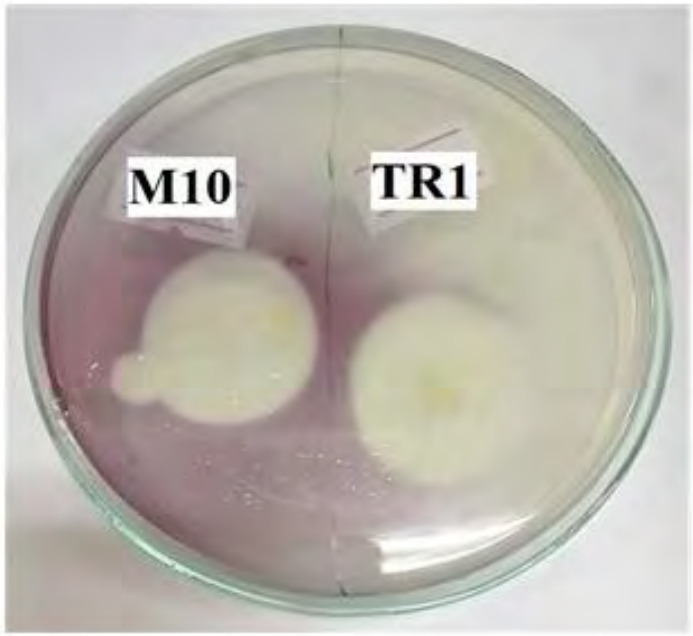

Figure 2.7. Pectinase test of the bacterial isolates.

2.4.5.3. Amylase production

Forty-One isolate showed positive results for amylase production (Table 2.2, (Figure 2.8).

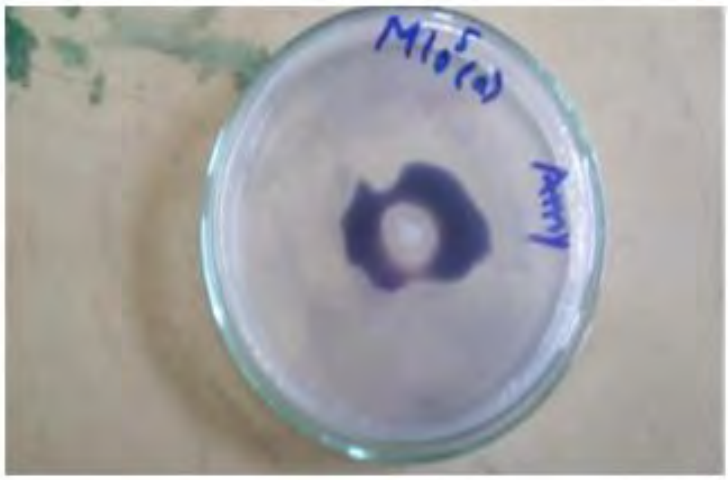

Figure 2.8. Amylase production of bacterial isolates

2.4.5.4. Catalase production

All isolates showed bubble formation demonstrating that those have catalase producing ability (Table. 2.2, Figure 2.9).

Figure 2.9. Catalase activity of bacterial isolates

Table.2.2: List of the extracellular enzyme tests of plant growth promoting bacterial isolates.

Sr.no	Code of isolates	Protease	Amylase	Pectinase	Catalase
1	T6	+	+	+	+
2	KTES	+	+	-	+
3	SCAL	+	+	-	+
4	SCAL1	+	+	+	+
5	SCALT	+	-	+	+
6	TR3.1	+	+	+	+
7	Tr3	+	+	-	+
8	Tm24	+	-	-	+
9	Tm2	+	-	+	+
10	Mk2	+	+	-	+
11	T6w	+	+	+	+
12	T6Y	+	+	-	+
13	V3	-	+	-	+
14	Scal (0)	+	+	+	+
15	VR	+	+	-	+
16	SR	-	+	+	+
17	M107	+	+	-	+
18	Sca	+	+	+	+
19	KTES3p	+	-	+	+
20	TR3 (3.1)	+	+	+	+
21	M105 (b)	+	+	-	+

22	BTRP4	-	-	+	+
23	CUX10y	_	+	-	+
24	Btrs105	+	+	+	+
25	KTE63D	+	-	-	+
26	ScaR	+	+	+	+
27	T6 (2)	+	_	+	+
28	Scal107	+	+	-	+
29	Ktrp (1)	+	-	+	+
30	Scal2	+	+	-	+
31	TR3ve	+	+	+	+
32	Tr3C3	+	-	-	+
33	Ktes	-	+	+	+
34	Ktes3p	-	-	-	+
35	Ts1	-	+	+	+
36	VL1	+	-	-	+
37	TM37	+	+	+	+
38	M10	+	-	+	+
39	TR1	+	-	+	+
40	JAR3	+	+	+	+
41	TM41	-	+	-	+
42	TM42	+	+	+	+
43	TM43	+	-	-	+
44	TM44	-	+	-	+
45	V5	-	-	+	+

46	TM46	+	-	+	+
47	KP1	_	+	-	+
48	TM48	+	-	+	+
49	TM49	+	-	+	+
50	Ca (1)	+	+	-	+
51	TM51	-	-	+	+
52	TM52	+	+	-	+
53	TM53	+	-	+	+
54	TM54	-	+	-	+
55	TM55	-	-	-	+
56	TM56	+	+	+	+
57	TM57	-	+	+	+
58	TM58	+	-	-	+
59	TM59	_	-	+	+
60	TM60	+	+	+	+
61	TM61	-	-	-	+
62	TM62	-	-	-	+
63	TM63	-	-	-	+
64	TM64	-	-	-	+
65	TM65	-	-	-	+
66	TM66	-	-	-	+
67	TM67	-	-	-	+
68	TM68	-	-	-	+
69	TM69	-	-	-	+

70	TM70	-	-	-	+

(+): present, **(-):** absent

2.4.5.5. ACC-Deaminase Activity

An efficient growth was observed on plates with Ammonium sulfate serving as a positive control compared to growth on plates with only DF medium. However, variation and growth pattern of all isolates at agar plates supplemented with ACC was absorbed. Thirty seven out of seventy isolates showed positive results for ACC (Table 2.3, Figure 2.10).

Negative Control **Positive Control** **ACC-deaminase**

Figure 2.10. ACC-deaminase activity of bacterial isolates

Table: 2.3. List of the Isolates along with codes and ACC deaminase

S. No	Code of isolates	ACC Deaminase
1.	T6	✔
2.	KTES	✔
3.	SCAL	✔
4.	SCAL1	✔
5.	SCALT	✔
6.	TR3.1	✔
7.	Tr3	✔
8.	Tm24	✔
9.	Tm2	✔
10.	Mk2	✔
11.	T6w	✔
12.	T6Y	×
13.	V3	×
14.	Scal (0)	×
15.	VR	✔
16.	SR	×
17.	M107	×
18.	Sca	✔
19.	KTES3p	×
20.	TR3 (3.1)	×
21.	M105 (b)	✔
22.	BTRP4	×
23.	CUX10y	✔
24.	Btrs105	×
25.	KTE63D	×
26.	ScaR	✔
27.	T6 (2)	✔
28.	Scal107	✔
29.	Ktrp(1)	×
30.	Scal2	✔

31.	TR3ve	×
32.	Tr3C3	✔
33.	Ktes	✔
34.	Ktes3p	×
35.	Ts1	×
36.	VL1	×
37.	TM37	✔
38.	M10	✔
39.	TR1	✔
40.	JAR3	✔
41.	TM41	✔
42.	TM42	✔
43.	TM43	×
44.	TM44	✔
45.	V5	×
46.	TM46	✔
47.	KP1	✔
48.	TM48	×
49.	TM49	✔
50.	Ca (1)	×
51.	TM51	✔
52.	TM52	×
53.	TM53	✔
54.	TM54	×
55.	TM55	×
56.	TM56	✔
57.	TM57	×
58.	TM58	×
59.	TM59	✔
60.	TM60	×
61.	TM61	×
62.	TM62	×
63.	TM63	×

64.	TM64	✔
65.	TM65	✔
66.	TM66	×
67.	TM67	×
68.	TM68	×
69.	TM69	×
70.	TM70	×

(✔): presence of ACC deaminase activity **(x):** Absence of ACC deaminase activity

2.4.5.6. Screening of isolates against heat stress

Only 5 bacterial isolates out of 70 were survived till 60ºC (Figure 2.11, Table 2.4).

Table: 2.4. Number of heat tolerant bacterial isolates

Temperature range		Number of isolates
1.	60ºC	5
2.	55 to 59ºC	17
3.	51 to 55ºC	23
4.	41 to 50ºC	62
5.	32 to 40ºC	70

Number of isolates against different temperature

Figure 2.11. Growth of bacterial isolates on LB solid media against different temperature

2.4.5.7. Growth curve analysis of selected bacterial isolates

Analysis of growth curve of selected bacterial isolates against different temperature ranges for 7 days is shown in Figure 2.12. Growth curve analysis of isolate SCAL1 revealed peak growth at the third day and started to decline at optimum temperature of selected bacterial isolates. Peak growth at 2nd and 3rd day was noted at 50ºC and 60ºC, respectively (Figure 2.12 a). Whereas the growth curve analysis of bacterial isolate T6 revealed peak growth at 2nd day and started decline at an optimum temperature of the selected bacterial isolate. Peak growth at 2nd and 3rd was noted at 50ºC and 60ºC, respectively as shown in Figure 2.12 (B). The growth curve analysis of bacterial isolate BT showed the peak growth at 2nd day and started decline at the optimum temperature of the selected bacterial isolate. Peak growth at 3rd day was noted at 50ºC and 60ºC, respectively as shown in Figure 2.12 (C). Whereas the growth curve analysis of bacterial strain KTES revealed peak growth at 3rd day and started decline at the optimum temperature of selected bacterial strain. Peak growth at 3rd day was noted at 50ºC and 60ºC, respectively as shown in Figure 2.12 (D). Whereas the growth curve analysis of bacterial strain TR3 showed peak growth at 3rd day and started decline at optimum temperature of selected bacterial strain. Peak growth at 2nd day was noted at 50ºC and 60ºC, respectively as shown in Figure 2.12 (E).

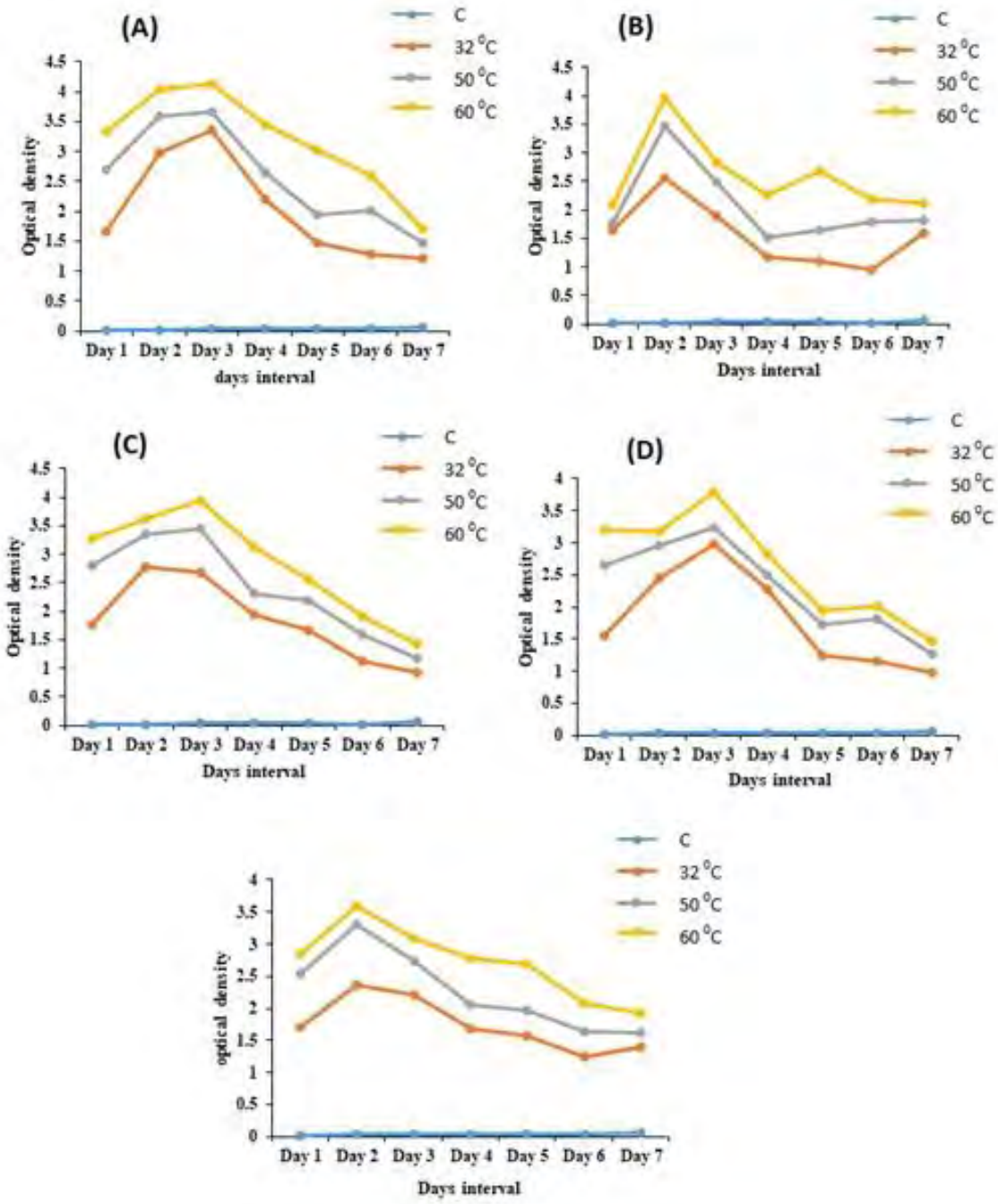

Figure 2.12. Growth curve analysis of of bacterial isolate SCAL1 (A), T6 (B), BT (C), KTES (D) and TR3 (E) under different temperature level for its tolerance with control

2.4.5.8. Morphological characterization

Bacterial isolates showed their characteristics through cell morphology, color, and shape and Gram staining reaction (Table 2.5, Figure 2.13).

Table: 2.5. Morphological characterization of selected isolated strains

Sr. No	Isolate Name	Gram staining	Shape	Colony color	Form	Elevation	Margin	Opacity
1	KTES	+	rod	yellow	circular	umbonate	erose	translucent
2	SCAL1	+	rod	off white	circular	convex	lobate	translucent
3	BT	+	rod	off white	irregular	flat	entire	translucent
4	T6	+	rod	off white	circular	convex	erose	translucent
5	TR3	-	rod	off white	circular	convex	entire	translucent

(+): present, **(-):** absent

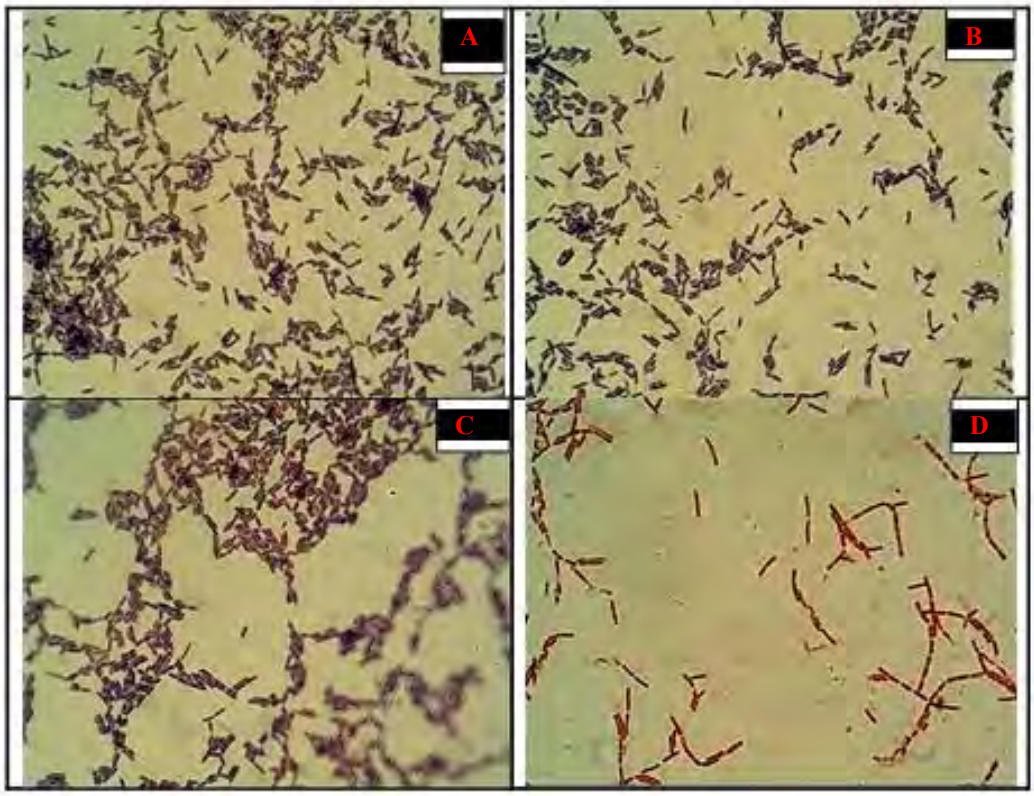

Figure 2.13. Gram staining of bacterial isolates, SCAL1 (A), T6 (B), BT (C), and TR3 (D)

2.4.5.9. Quantitative assay of ACC-deaminase activity

Bacterial Isolate SCAL1 produced maximum quantity of enzyme activity 0.96 µM/mg protein/h under the stress condition while the ACC-deaminase production was noted in isolate T6 was 0.95 µM/mg protein/h under heat stress. ACC-deaminase production in isolate BT was 0.93 µM/mg. ACC- deaminase production in isolate KTES was 0.9 µM/mg protein/h against the heat stress. Quantitative estimation of ACC-deaminase production (0.81 µM/mg protein/h) was observed in isolate TR3 under heat stress condition. Among all bacterial isolates, SCAL1 exhibited higher production of ACC-deaminase under high temperature condition against control (Figure 2.14).

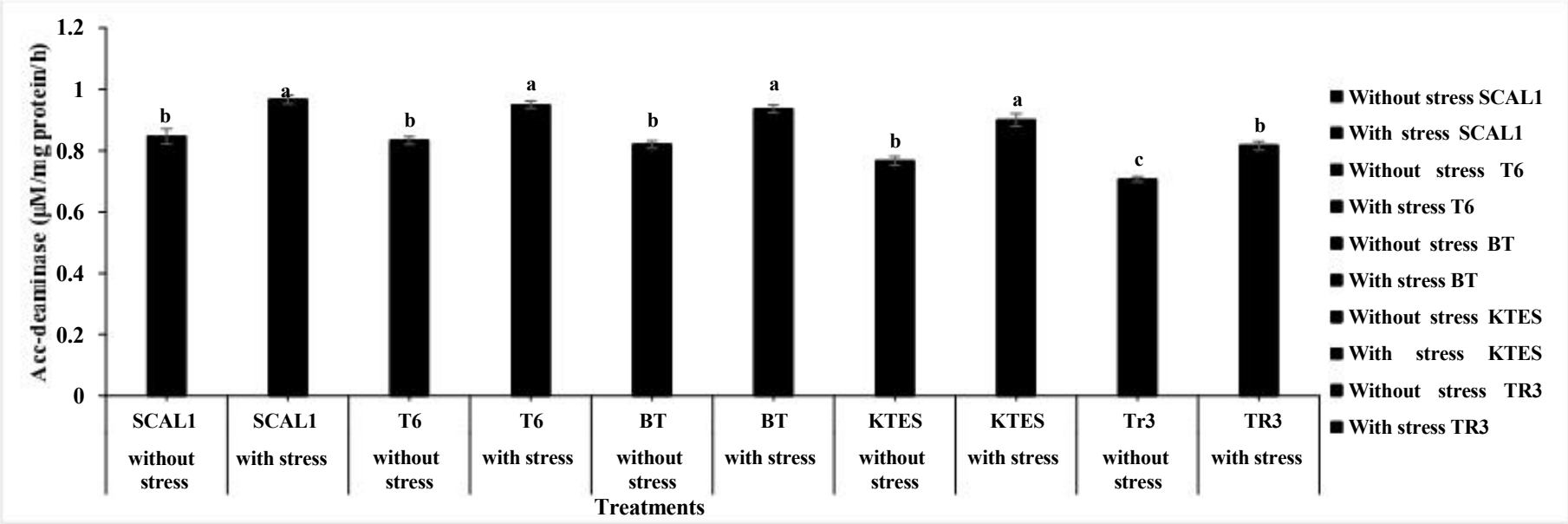

Figure 2.14. Quantitifcation of ACC-deaminase: SCAL1 (*Bacillus safensis*), T6 (*Bacillus safensis*), BT (*Bacillus Safensis*), KTES (*Bacillus cereus*) and TR3 (*Klebsiella variicola*)

2.4.5.10. Acds gene amplification

The PCR mediated amplification of *acds* gene of five strains was performed using a universal set of primers (Figure. 2.15). Although, ACC deaminase enzyme activity was confirmed quantitatively in all strains but by using the set of primers its activity was further verified by the amplification of gene in the selected strains. The separated bands were noticed by agarose gel electrophoresis.

Figure2.15. Gel imaging picture of amplification and presence of acdc gene in the selected bacterial Isolates (SCAL1, T6, BT, Kt and TR3)

2.4.5.11. Characterization of selected bacterial strain through QTS-24 kit

The selected bacterial strains exhibited positive results for different tests were carried out through microbial identification kits QTS-24 (Table 2.6). Whereas SCAL1, T6 and BT showed positive for all test of microbial identification kit. On the other hand KTES showed positive results except CIT, ADH, H_2S and GEL. While the strain TR3 exhibited positive results for all tests except CIT, LDC, ADH, H_2S and VP.

Table: 2.6. Characterization of selected bacterial isolates with QTS-24 kit

QTS Test	SCAL1	T6	BT	KTES	TR3
ONPG	+	+	+	+	+
CIT	+	+	+	-	-
MALO	+	+	+	+	+
LDC	+	+	+	+	-
ADH	+	+	+	-	-
ODC	+	+	+	+	-
H_2S	+	+	+	-	-
UREA	+	+	+	+	+
TDA	+	+	+	+	+
IND	+	+	+	+	+
VP	+	+	+	+	-
GEL	+	+	+	-	+
GLU	+	+	+	+	+
MALT	+	+	+	+	+
SUC	+	+	+	+	+
MANN	+	+	+	+	+
ARAB	+	+	+	+	+
RHAM	+	+	+	+	+
SORB	+	+	+	+	+
INOS	+	+	-	+	-
ADO	+	+	+	+	+
MEL	+	-	+	+	+
RAF	+	+	-	+	-

+ (Presence), - (Absent).). Orthro-nitrophenyl-β-galactoside (ONPG), Sodium citrate (CIT), Sodium malonate (Malo), Lysine decarboxylase (LDC), Arginine dihydrolase (ADH),Ornithine decarboxylse (ODC), Tryptophane deaminase (TDA), Acetion (VP), Gelatin hydrolysis (GEL), Acid from glucose (GLU, (NO3), Acid from maltose (MALT), Acid from sucrose (SUC) and Acid from mannitol (Mann), Arabinose (ARAB), Acid from rhamnose (RHAM), Acid from sorbitol (SORB), Inositol (INOS), Adonitol (ADO), Melibiose (MEL)

2.4.5.12. Qualitative and quantitative assay of exopolysaccharide (EPS) production

In the case of qualitative EPS production, selected bacterial isolates (SCAL1, T6, BT, KTES and TR3) showed positive result by the formation of mucoid transparent colonies as shown in Figure 2.16. After the confirmation of qualitative production, then the quantitative estimation of EPS was conducted for tested bacterial isolates under the normal and heat stress conditions. The bacterial isolates SCAL1 showed highest EPS production 0.92mg/ml under heat stress condition and 0.73mg/ml under normal condition than other isolates. While the EPS production in isolate, T6 was 0.9 and 0.71 mg/ml under heat stress and non-heat stress conditions respectively. On the other hand 0.88 mg/ml EPS was produced in heat stress condition while the 0.66mg/ml EPS in isolate BT. The isolate KTES produced 0.91mg/ml EPS under the heat stress condition and 0.66 mg/ml was observed in non-heat stress condition. Quantitative estimation of EPS production was 0.73 and 0.66 mg/ml in Isolate TR3 under heat stress and non-heat stress condition respectively as shown in Figure 2.17.

Figure 2.16. Qualitative analysis of exopolysaccharide production of selected isolate

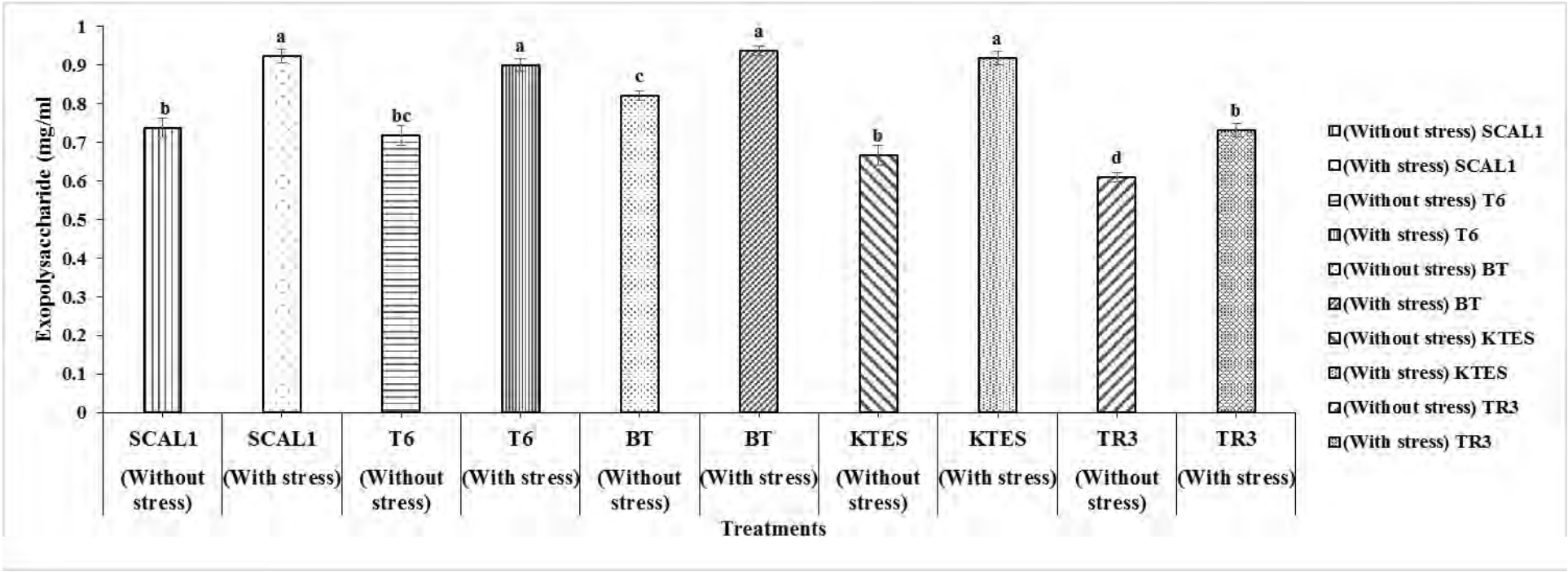

Figure 2. 17. Quantitative analysis of EPS of bacterial isolates (SCAL1

, T6, BT, KTES and TR3) under heat and normal condition

2.4.5.13. Identification of selected bacterial strains

Molecular identification of all five bacterial isolates (KTES, BT, TR3, T6 and SCAL1) were carried through 16S rRNA gene sequencing. Among five bacterial Isolates; three were *Bacillus safensis* and one was *Bacillus cereus* and one was *Klebsiella variicola* (Table 2.7, Figure 2.18).

Table 2.7: Identification of five bacterial isolates by 16S rRNA gene technique.

Sr. No.	Isolates Code	Scientific name	Accession numbers
1.	SCAL1	*Bacillus Safensis*	PRJNA286914
2.	KTES	*Bacillus cereus*	MK784894
3.	BT	*Bacillus Safensis*	MK910212
4.	T6	*Bacillus Safensis*	MK910213
5.	TR3	*Klebsiella Variicola*	MT704811

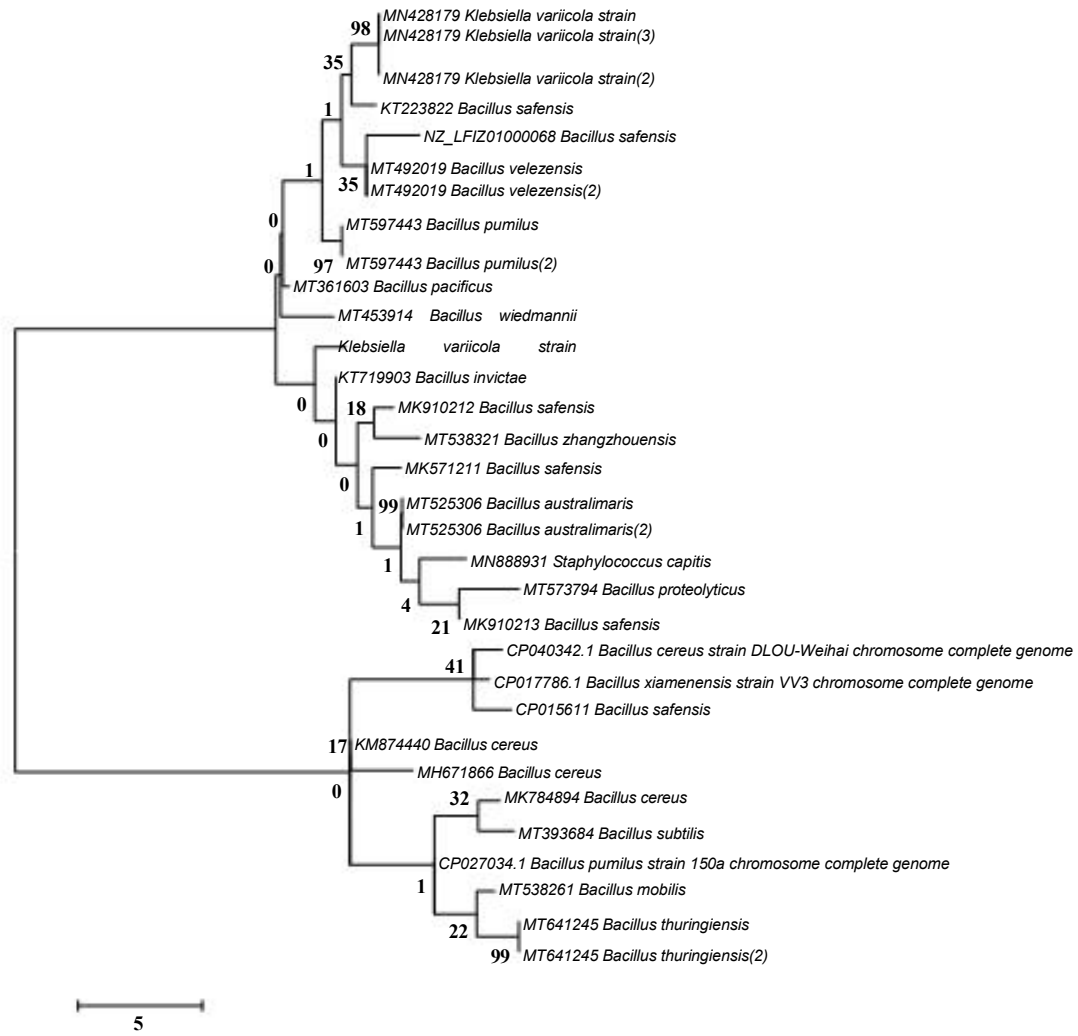

Figure 2.18. Molecular phylogenetic analysis by maximum likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model, Tamura & Nei (1993). The tree with the highest log likelihood (-21138.0769) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 32 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 591 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2013).

2.4.5.14. Growth regulators

Bacterial strain Bacillus safensis (SCAL1) showed maximum IAA (0.52 ug/ml), GA3 (15.4 ug/ml) and kinetin production (27ug/ml) under heat stress condition (Table. 2.8). On the other hand, minimum IAA (0.24 ug/ml) and GA3 (15.9ug/ml) production was shown by bacterial strain BT (B. safensis), while strain KTES (*B. cereus*) showed minimum quantity of kinetin (19.6 ug/ml) as compared to non-stress condition.

Table: 2.8. Quantitative estimation of growth regulators through High Performance Liquid Chromatography (HPLC)

Bacterial strains	IAA (ug/ml))		GA_3 (ug/ml))		Kinetin (ug/ml))	
	Without stress	**With heat stress**	**Without stress**	**With heat stress**	**Without stress**	**With heat stress**
SCAL1	0.59 ± 0.03^{c}	0.52 ± 0.02^{c}	19.84 ± 1.46^{b}	15.4 ± 0.12^{b}	30.78 ± 1.81^{a}	27 ± 2.51^{a}
T6	0.48 ± 0.05^{d}	0.46 ± 0.05^{d}	17.09 ± 1.19^{c}	18.73 ± 1.14^{c}	29.59 ± 1.17^{b}	34.8 ± 1.31^{a}
BT	0.29 ± 0.03^{b}	0.24 ± 0.02^{b}	16.1 ± 1.53^{a}	15.9 ± 0.04^{a}	20.33 ± 4.09^{a}	20.6 ± 1.76^{a}
KTES	0.38 ± 0.06^{c}	0.3 ± 0.02^{c}	20.74 ± 1.10^{a}	16.5 ± 0.12^{b}	19.09 ± 3.46^{ab}	19.6 ± 2.40
TR3	0.47 ± 0.01^{c}	0.44 ± 0.02^{c}	17.54 ± 0.12^{b}	17.1 ± 0.05^{b}	18.6 ± 4.43^{b}	33.06 ± 2.02^{a}

Quantitative analysis of growth regulators of bacterial strains, IAA (Indole acetic acid), GA_3 (Gibberellic acid) and kinetin under normal heat stress condition. *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3)

2.5. Discussion

Plant growth promoting bacteria (PGPB) have a prominent role in sustainable agriculture by providing the stratigies of tolerance to several abiotic stresses (Mukhtar *et al*., 2020). The samples were collected from the high-temperature area (Larkana Sindh, Pakistan) by keeping in mind, the effects of ecology on microbial genetics (Patel *et al.,* 2017). Bacterial strains were isolated from endophytic and rhizospheric zones. In the current study, bacterial strains were screened to determine their heat tolerance capability and growth promoting potential of plant (Ali *et al*., 2011). We have observed the traits in isolated strains for indole acetic acid production, phosphorus solubilization, Ammonia, siderophore and HCN. The biochemical traits of plants growth promoting bacteria have been reported based on the ability of phosphate solubilization and indole acetic acid (Rajkumar *et al*., 2008), Siderophore (Kamran *et al*., 2017), HCN, Ammonia production (Wani *et al*., 2007) and enzymatic activity such as proteas which is involved in plant growth promotion (Pandey *et al*., 2013), Pectinase, amylase and catalase (Islam *et al*., 2014).These all enzymes activities are involved in plant growth promotion and support the plant in tolerance of abiotic stress (Mukhtar *et al*., 2020).

Maximum ACC-deaminase production was shown with *Bacillus safensis* (SCAL1) under heat stress condition. Up to the best of our knowledge, this is the very first attempt reporting the evidence for minimizing the effect of high temperature stress in tomato with the application of bacterial strains from Pakistan. In the current investigation, biochemical characterization of the isolated heat stress tolerant PGPB was done on the basis of phosphate solubilization (PSB), siderophore, indole acetic acid (IAA), Hydrogen cyanide (HCN) , ammonia production, protease, catalase, amylase, pectinase and ACC- deaminase activity.

Ethylene concentration increased in plant tissues due to various abiotic stresses (Ali *et al*., 2014, Barnawal *et al*., 2012). Ethylene level increased beyond the threshold level during abiotic stress which leads to reduced the seed germination, root development and stunted growth of plant's. ACC-deaminase producing PGPB helped to alleviate ethylene production which cleaved the ACC to α-ketobutyrate and ammonia, and decreased the adverse effects of ethylene on plant growth under heat stress. The inoculation of ACC-deaminase-producing bacterial strains in plants has been linked

with abiotic stress tolerance (Chen *et al*., 2017). It also enhanced nutrient uptake and root growth (Shahzad *et al*., 2013). SCAL1 produced the maximum amount of ACC-deaminase activity (0.96 µM/mg protein/h) under heat stress condition while the strain TR3 produced minimum amount of ACC-deaminase activity (0.81 µM/mg protein/h) under heat stress. Very recently, Misra *et al*, (2020) also tested *B. safensis* and obtained an ACC-deaminase production of 4.12 nM/mg protein/h under abiotic stress which is far less than our current results. It clearly reflected that our tested strain had the promising potential for ACC- deaminase production against heat stress.

The selected bacterial strains showed significant potential for exopolysaccharides (EPS) production under stress conditions. SCAL1 (0.92 mg/ml) revealed the maximum amount of EPS production, while the strain TR3 (0.73 mg/ml) produced minimum EPS under heat stress. The current pattern of enhanced EPS production is in agreement with Misra *et al*, (2020), as they also obtained an enhanced EPS production with *B. safensis* under abiotic stress. Sandhya *et al.,* (2009b) reported that EPS-producing bacteria can provide resistance in plants against abiotic stress. EPS enhance water uptake surrounding the roots area's by the formation of biofilm that also aids in the stabilization of soil aggregates, regulation of nutrients and organic carbon sources. These characteristics enhanced plants growth in both (under normal and stress) conditions.

The current study involved the quantification of plant growth regulators and obtained the maximum quantity of IAA (0.52 µg/ml) with SCAL1, showed the increased value in comparison with the study Misra *et al*, (2020). *B. safensis* tested by Misra *et al*, (2020) and obtained an IAA production of 16.19 nM/mg protein/h under abiotic stress which is far less than our current results. Production of GA_3 (18.73 µg/ml) with T6 showed higher values as compared with the studies of (Sunera *et al*., 2020). Bacterial strain T6 exhibited maximum Kinetin production (34 µg/ml) under heat stress as compared with findings of Sunera *et al*, (2020). Our findings were supported by the results of Iqbal *et al,* (2013) and Spaepen *et al,* (2014) in which different bacterial strains (*Aeromonas punctata*, *Serratia marcescens* and *Azospirillum brasilense*) has induced growth and morphological alterations in *Arabidopsis thaliana* due to enhanced synthesis of IAA, GA_3 and kinetin under heat stress condition. The IAA producing ability of bacteria in rhizosphere depends on uptake of microbial IAA by plant and presence of precursors. Growth promotion may be attributed to other

mechanisms such as production of plant growth promoting hormones in the rhizosphere (Arshad & Frankenberger, 1993; Glick, 1995).

Production of gibberellin by PGPB strains and its effects on tomato varieties was supported with the results of Kang *et al.,* (2014), as they stated that inoculation of gibberellins-producing strain *Promicromonospora* sp. (SE188) increased gibberellins concentration in plant shoots. Demonstration of Barea *et al.,* (1974), Azcon *et al.,* (1975) supported our findings that the inoculation of cytokinin- producing bacteria enhanced the shoot growth, fruit formation and increased the resistance of plants to abiotic stress. Moreover, our findings were strengthened with the results of Liu *et al.,* (2013) that the resistance of *Platycladus orientalis* to abiotic stress increased with inoculation of cytokinin-producing *Bacillus subtilis*. PGPB inoculation has been observed to minimize the adverse effects of heat stress on plant growth and its productivity (Ali *et al.,* 2011).

Morphological characterization of selected bacterial strains were observed with compound microscope and gram staining technique. Furthermore, genotypic characterization of promising bacterial strains was carried out by 16S rRNA gene sequencing which is a common method used to identify bacterial species. Sequence analysis of the PCR-amplified 16S rRNA gene sequencing identified three bacterial strains as *Bacillus safensis* (SCAL1, *Bacillus safensis* (T6) and *Bacillus safensis* (BT) Bacterial strains KTES and TR3 were identified as *Bacillus cereus* and *Klebsiella variicola* respectively (Coelho *et al.,* 2011). This is the first report indicating the presence of heat tolerant plant growth promoting bacterial strains from rhizosphere and different parts of tomato from genus *Bacillus* and *Klebsiella* in Pakistan.

Plant growth promoting bacteria are known to influence plant growth by various direct or indirect mechanisms. The five promising bacterial isolates revealed a higher potential for heat tolerant during the current investigation and might be suggested to be tested under greenhouse and field conditions. Selected bacterial strains exhibiting more than two PGP traits, might be helpful to the plant growth either directly or indirectly (Joseph *et al.,* 2007). Plant growth promoting bacteria (PGPB) has been functioning as a co-evolution between plants and microbes showing synergistic interactions with microorganisms and the soil. (Gouda *et al.,* 2018). Application of plant growth promoting bacteria (PGPB) is a useful option to minimize various abiotic stresses and is now widely in practice.

2.6. Conclusion

Seventy bacterial strains were isolated, characterized and screened for heat stress tolerance and plant growth promoting activities. Five out of 70 strains were selected, based on heat tolerance potential. Plant growth promoting bacterial strains have abilities that linked to plant growth promotion such as IAA, solubilization of phosphate, ammonia production, HCN, siderophores, protease, amylase, pectinase and catalase. These bacterial strains were characterized for ACC-deaminase, exopolysacharide production and quantification of growth regulators. These selected strains were identified through 16S rRNA gene sequencing as with their closely related species, strain code along with gene bank accession number in brackets i.e. *Bacillus safensis* **SCAL1** (PRJNA286914), *Bacillus safensis* **T6** (MK910213), and *Bacillus safensis* **BT** (MK910212), *Bacillus cereus* **KTES** (MK784894) and *Klebsiella variicola* **TR3** (MK410214). The study provides data of potential plant growth promoting bacterial isolates *Bacillus safensis* **SCAL1**, (PRJNA286914), *Bacillus safensis* **T6** (MK910213) and *Bacillus safensis* **BT** (MK910212), *Bacillus cereus* **KTES** (MK784894) and *Klebsiella variicola* **TR3** (MK410214) for further evaluation under greenhouse and field conditions.

Chapter 3

Evaluation of selected isolates in green house experiment

3.1. Introduction

Occurrences of heat stress due to the fluctuating global climate can adversely affect the growth and yield of temperature-sensitive crops like tomato maize and rice etc (Mukhtar *et al.,* 2020). Increased temperatures decrease crop productivity by affecting biochemical, physiological, molecular, and morphological phenomenon either individually or in combination with other abiotic stresses. High temperature is a main environmental issue that constrains vital plant functions such as seed germination, seedling growth, plant metabolism, and reduces its yield in various agroecological zones throughout the world (Fahad, *et al.,* 2017; Khan, *et al.,* 2019). However, raised temperature has a sturdy impact on crop yield that varies with different severity levels and period of heat stress (Barnabas, *et al.,* 2008; Hedhly, *et al.,* 2009). Seed germination may be delayed or inhibited due to high temperatures at 30 to 38 ◦C (Prasad, *et al.,* 2014). Particularly, reproductive stage of plants has been found to be more sensitive for heat stress, as reported in many crops, such as chickpea, lentil, mung bean, wheat and sorghum (Farooq *et al.,* 2011; Prasad *et al.,* 2015; Mattioli, *et al.,* 2008).

Proline is an amino acid that accumulates in plants under various stress condition like heat, heavy metals, drought, cold and salt stress; and it can play a beneficial role in growth and flowering of plants (Siddique *et al.,* 2018). Disruption of proline transport and sugar metabolism occurs during the narrow window of male reproductive processes under elevated temperature that cause the failure of fruit setting in tomato plants (Sato *et al.,* 2006). The application of plant growth-promoting rhizospheric and endophytic bacteria offers an ecofriendly approach for improving agriculture crop production and counteracting the negative effects of heat stress (Wahid *et al.,* 2007). In agricultural practices, the application of beneficial microbes is an integral component which should be validated to enhance crop productivity in a defensible way under different abiotic stresses (Gill *et al.,* 2016). Plant growth promoting bacteria (PGPB) assist the plant growth either by direct mechanisms which include the production of plant growth regulators, enhanced nutrient availability or by indirect mechanisms which encompasses the suppression of pathogens by antibiosis, induced systemic resistance (ISR) and synthesis of lytic enzymes (Glick *et al.,* 2014). During abiotic stress, plant growth promotion activities have been reported in cucumber, maize, tomato, mung bean, white clover and wheat (Tiwari *et al.,* 2011).

PGPB improved growth of plants by increasing the uptake of nutrients, particularly mineral phosphorus (Rafique *et al.,* 2019). Phytohormones production like gibberellic acid, indole-3-acetic acid, cytokinins, abscisic acid, antibiotics and siderophore play vital roles in this regard (Gill *et al.,* 2010; Warnita *et al.,* 2019). PGPB produce antioxidants that enhance the abscisic acid (ABA) accumulation and degradation of reactive oxygen species. Bacteria such as *Pseudomonas* survive under stress conditions due to exopolysaccharides production (Sandhya *et al.,* 2009). This mechanism provides a defence to microorganisms under abiotic stress conditions (Bramhachari *et al.,* 2006). ACC producing bacteria have the ability to supply the nitrogen and energy to plants. (Zahir *et al.,* 2009). Inoculation with ACC-deaminase producing bacteria induced longer roots and provided help in the taking up of more amounts of water under stress conditions that, in turn, increased the efficacy of the plants under abiotic stress conditions (Glick *et al.,* 1998). Tomato is one of the most economically significant and widespread horticultural crops ranked 7th position in the world (Dell-Amico *et al.,* 2002) and its production was 34 million tons in 2018 worldwide (Ronga *et al.,* 2019).

Several bacterial strains isolated from the tomato microbiome were found to stimulate significant plant growth, and also prime plant defence against certain stresses. Therefore, the current study was conducted to screen out the isolated indigenous heat tolerant bacteria with multiple plant growth promoting activities, and evaluate the role of heat tolerant bacterial strains that minimized the deleterious effects of heat stress condition on growth of tomato under greenhouse condition.

3.2. Objectives

Application of selected PGP bacterial strains against heat stress in tomato varieties in greenhouse

3.3. Materials and methods

3.3.1. Innocula preparation

Luria Bertani media was used to make fresh bacterial cultures. Broth was Centrifuged for 10 min at 3000 rpm. After collection of pellet, Pellet was dispersed in autoclaved double distilled water (dH_2O). Optical density was adjusted to 10^9 colony forming unit (CFU) at 660 nm. Tomato seeds were soaked in bacterial culture for 2-4 hours before sowing in pots (Amna *et al*., 2019).

3.3.2. Experimental Design

Experiment was carried out under greenhouse conditions at McDonald campus, Sainte-Anne-de-Bellevue, McGill University, Canada (45° 24′ 27″ N, 73° 56′ 18″ W) with completely randomized design having three replicates. Each replicate of the conducted experiment comprise of four treatments including; C= control (without bacterial inoculation/ without heat stress), T1= Plants inoculated with bacterial strains, T2= un-inoculated plant with heat stress and T3= bacterial inoculated plants with heat stress. Tomato seedlings were sown in plug trays (53.5×25.5 cm) filled with autoclaved media (G-10: sand: manure, 2:1:1) having five seeds per cell. Seedlings were transplanted into 6-inch pots filled with autoclaved media. After two weeks of sowing, seedlings were transferred to greenhouse under semi controlled conditions (70-80% humidity, 25±2°C temperature and 14 h photoperiod: PAR 300 µmol m−2 s−1). Irrigation was supplied manually on daily basis and each pot was watered with 20 mL Hoagland solution (1.6 g/L) twice a week. Heat stress (42°C) was applied at flowering stage to plants growing with and without bacterial inoculation. Plants were exposed to heat stress for six h/day in growth chamber till the fruiting stage. After exposure to heat stress, plants were placed again in greenhouse for recovery (temp. 25 ± 2°C). Plants were harvested after 96 days of seed sowing. Harvested plants were washed thoroughly with sterile distilled water to remove the debrisfrom roots.

Harvested plants were preserved for further agronomic and biochemical analysis (Ali *et al*., 2011).

3.3.3. Agronomical, photosynthetic and biochemical analysis of plants

3.3.3.1. Length of shoot and root

Shoots and roots length of freshly harvested plants were measured with measuring tape (Hussain *et al*., 2019).

3.3.3.2. Fresh weight

Digital balance was used to measure the fresh weight of harvested plants (Hussain *et al*., 2019).

3.3.3.3. Dry weight

After drying the plants in paper bags in oven for 2 days at 70°C, dry weight of plants was measured. Digital balance was used to measue the weight of fully dried plants (Hussain *et al*., 2019)

3.3.3.4. Leaf surface area

Leaf surface area of leaf of each treatment was measured with leaf area meter (Afridi *et al*., 2019).

3.3.3.5. Number of flowers and fruits

Number of flowers and fruits were counted in every treatment with naked eye (Mukhtar *et al*., 2020).

3.3.4. Physiological parameters

3.3.4.1. Chlorophyll *a, b* and carotenoid contents

Leaf material (0.1 g) from each treatment was used for the estimation of chlorophyll contents. Fresh leaves (small pieces) were putted in 4 ml $DMSO_4$ (Dimethylsulfoxide) and then incubated (Hussain *et al*.,2019). After 4 h of incubation at 65ºC, absorbance of extracts was recorded at wavelengths (663, 645 and 480 nm). Formulas which were used for calculaton of chlorophyll *a*, chlorophyll *b* and carotenoid contents are following

Chl a (mg/g) = [1.07 (OD 663) - 0.09 (OD 645)]

Chl b (mg/g) = [1.77 (OD 645) - 0.280 (OD 663)]

Carotenoid (mg/g) = (O.D.480 nm) - 0.144(O.D.663 nm) - 0.6308(O.D.645 nm)

3.3.5. Biochemical parameters

3.3.5.1. Protein Estimation

The concentration of protein was calculated following the protocol of Afridi *et al*, (2010). Construction of a standard curve was done with bovin serum albumin for protein estimation (Glick *et al*., 2014).

3.3.5.2. Proline estimation

Proline content was quantified in shoot materials following the method of Ali *et al*, (2019). Shoot fresh material of 0.5 g was grounded in 3% sulphosalicylic acid (4 ml) and placed at 5°C for night. Suspension material were centrifuge at 3000 rpm for 5 min. Supernatant (2 ml) was mixed with acidic ninhydrin after centrifugation. Preparation of acid ninhydrin reagent was done with agitation by using ninhydrin (1.25 g) in 20ml of phosphoric acid (6 M) and 30 ml of glacial acetic acid (1M H_3PO_4=3N H_3PO_4), until it dissolved properly. The contents in tubes were heated at 100°C for 1 h in water bath. When cooling of mixture was compeleted, then it was extracted by using separate funnel with toluene (4ml). The optical density (OD) was set against toluene as blank at 520 nm with help of spectrophotometer. Determination of Proline concentration was carried out with the help of standard curve. Proline µg/g = k value*dilution factor*absorbance/fresh sample weight K value = 17.52

Dilution factor = 2

Wt. of sample = 0.5 g

3.3.5.3. Superoxide dismutase (SOD) activity measurement

Mixing of buffer solutions was carried out by taking monosodium dihydrogen phosphate (117 ml) and of disodium mono hydrogen phosphate (183 ml). The total volume (300 ml) was raised to the final volume of 600 ml and pH was setted up to 7. Buffers which were prepared for SOD activity described in following lines.

(a) Preparattion of Monosodium dihydrogen phosphate was carried out by addition of 15.6 g into 500 ml of distilled water. (b) Disodium hydrogen phosphate was prepared by taking 53.65 g into 600 mL of distilled water.

Both the solutions were prepared by mixing of monosodium dihydrogen phosphate (25.5 ml) and disodium mono hydrogen phosphate (275.5 ml). The total volume (300 mL) was raised to the final volume (600 ml) and pH 7.8 was maintained. SOD assay was conducted by following the protocol of Afridi *et al*. (2019) method. The plant tissue (0.5 g) was grounded in a solution formed by adding of PVP (1 g) + Na_2EDTA (0.028 g) in phosphate buffer (100 ml) and pH was kept at 7. The mixture was centrifuged for 10 min at 4°C and the supernatant was collected. Volume of the supernatant was raised to 8 mL by adding phosphate buffer and pH was 7. The preparation of reaction mixture (3ml) was carried out by adding EDTA (0.0278 g), methionine (1.5 g) and Nitro blue tetrazolium chloride (0.04 g). 10 ml of above solution was taken, and its volume is raised to 50 ml with phosphate buffer of pH 7.8. After that 0.00113 g of Riboflavin was dissolved in 100 ml phosphate buffer of pH 7.8. From above solution, 20mL was taken and its volume is raised to 50 ml with DH_2O. The reference samples were placed in complete dark condition. While the samples were placed in light chamber for reaction mixture for 20 min. Spectrophotometer was used to record the absorbance at 560 nm. SOD activity, the amount of enzyme which deceased the reading of absorbance by 50% against lacking enzyme material (control). Expression of superoxide dismutase activity was described as units/100 g F.W. SOD activity was calculated by using the following formula

R1 = O.D of reference, R2= O.D of blank,

R3= O.D of sample, R4= R3-R2

Final= R4/A

3.3.5.4. Peroxidase (POD) activity

Peroxidase activity was carried out following the method of Afridi *et al.,* (2019). Preparation of phosphate buffer (0.1M) was done by taking the Monosodium dihydrogen phosphate (3.9 g) and Disodium monohydrogen phosphate (4.45 g) in distilled water (500 ml) and pH was set at 6.5. The 1% H_2O_2 (hydrogen peroxide) prepared in 0.1M phosphate buffer At pH 6.5.

Fresh shoot materials (1 g) were grinded in 10 ml of phosphate buffer (pH 6.5) and centrifuged for the collection of supernatant. The spectrophotometer was adjusted to read zero at 430 nm. H_2O_2 (0.5 ml) was added to test cuvette and mixed. Absorbance change was recorded for 3 min through the spectrophotometer. One unit of peroxidase was taken as the change in absorbance per minute at 430 nm.

Change in A430 = Af - Ai

3.3.5.5. Catalase activity.

The catalase activity was performed by using the protocol of Afridi *et al*. (2019) with minor modifications. Preparation of phosphate buffer (0.067 M) and pH was set at 7.0. The activity was carried out by using (5.963 g- Disodium monohydrogen phosphate and (5.226 g- Monosodium dihydrogen phosphate) in 500 ml. Hydrogen peroxide 2 mM (12.6 µl) was prepared using this buffer (100 ml). Plant materials (0.5 g) was grinded in 8 ml in solution buffer (phosphate buffer). The centrifugation of extract was carried out for estimation of enzyme activity. For this assay, phosphate buffer was added to test tube with H_2O_2 (3.0 ml) and added 40 µl of enzyme extract. The mixture was mixed thoroughly. The decrease of 0.05 units in absorbance was noted with the spectrophotometer (at 240 nm). For the control, the enzyme extract was combined with phosphate buffer. The decrease in absorbance of enzyme (0.05) units at 240 nm is considered as one enzyme unit.

3.3.5.6. Relative water content and electrolyte leakage

Fresh leaves (0.5 g) were taken and kept in water bath for 4 h at 4°C to determine turgid weigh. Dry weight was obtained by drying the plants in oven at 80°C. Fresh weight, turgid weight and dry weight of taken leaves were used for estimation of relative water content. It was calculated by implementing the formula, given below (Ahmad *et al.,* 2015).

$$RWC = \frac{(FW-DW) \times 100}{(TW-DW)}$$

Whereas, FW=fresh weight, DW=dry weight and TW=turgid weight

Electrolyte leakage (ELL) was computed with formula suggested by Ahmad *et al*. (2015).

$$\textit{Electrolyte Leakage (ELL)} = \frac{\textit{(Electrical conductivity 1)}}{\textit{(Electrical conductivity 2)}} \times 100$$

3.3.6. Statistical analysis

Two-way ANOVA was performed using Statistixs software (Version 8.1) for both varieties based on bacterial (control or inoculated) and temperature treatments (non-heat and heat stress). Adjustment for multiple comparisons were made using the LSD test, keeping significant level at $p>0.05$. The application of bi-plots correlation analysis was performed on mean values of all variables using XL-STAT 2015.

3.4. Results

3.4.1. Shoot length

Bacterial inoculation enhanced shoot length significantly in both varieties (VI-Riogrande and V2- Sweetie) under normal (T1) and heat stress conditions (T3) with respective control (C and T2). Significant increase in shoot length was observed with the inoculation of ***Bacillus safensis*** **(SCAL1)**, under normal **(37.1%)** and heat stress conditions **(61.4%)** in V1 as compared to respective control (C and T2). Same findings were observed in V2 with the maximum significant increase in shoot length with inoculation of ***B. safensis*** **(SCAL1)**, under normal **(35.9%)** and heat stress **(55.8%)** conditions as compared to respective control (C and T2). Heat stress dramatically decreased shoot length of un-inoculated tomato plant in both varieties. Decrease (65.34%) in shoot length was observed in V2 under un-culated heat stress condition (T2) as compared to respective control, as shown in Figure 3.1 and Appendix.

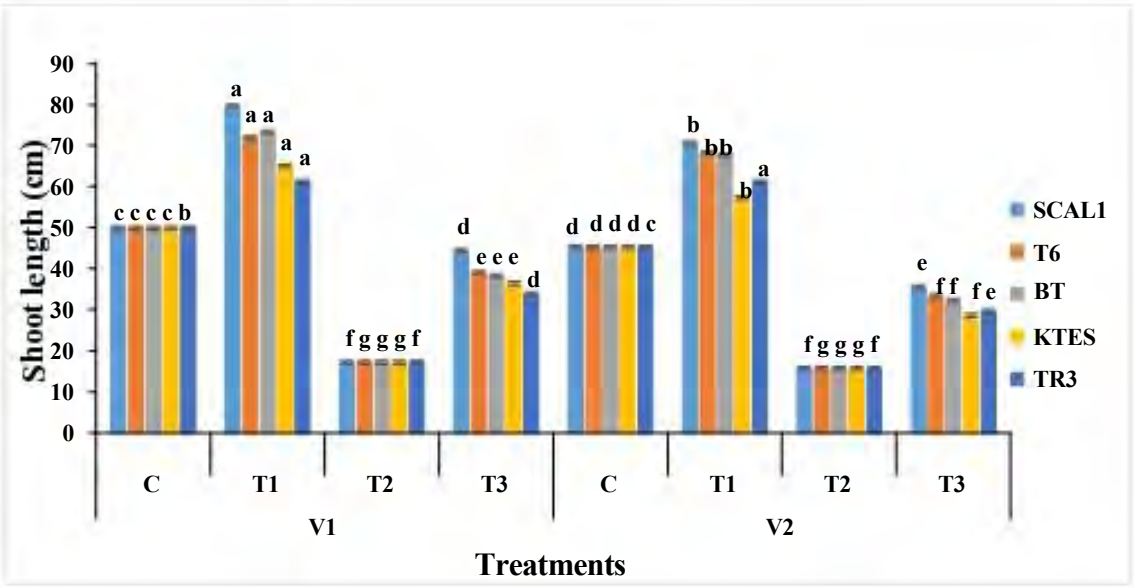

Figure 3.1. Effect of therm-tolerant strains, *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on shoot length two tomato varieties (V1- Riogrande and V2- Sweetie). C: control, T1- Inoculated plants under normal condition, T2- Uninoculated under heat condition and T3- inoculated plants under heat condition at $p>0.05$

3.4.2. Root length

Bacterial inoculation increased the root length significantly in both varieties (VI-Riogrande and V2- Sweetie) under normal (T1) and heat stress conditions (T3) with respective control (C and T2). Significant increase in root length was observed with the inoculation of ***Bacillus safensis*** **(SCAL1)** under normal **(39.6%)** and heat stress **(60.4%)** condition in V1 as compared to respective control (C and T2). Similar findings were observed in V2 with the maximum significant increase in root length with inoculation of ***B. safensis*** **(SCAL1)** under normal **(36.8%)** and heat stress **(44.6%)** conditions in variety 2 as compared to respective control (C and T2). Heat stress showed the remarkable decrease in root length of un-inoculated tomato plant in both varieties. Decreased root length (38.22%) was observed in V2 under un-inoculated heat stress condition (T2) as compared to respective control, as shown in Figure 3.2.

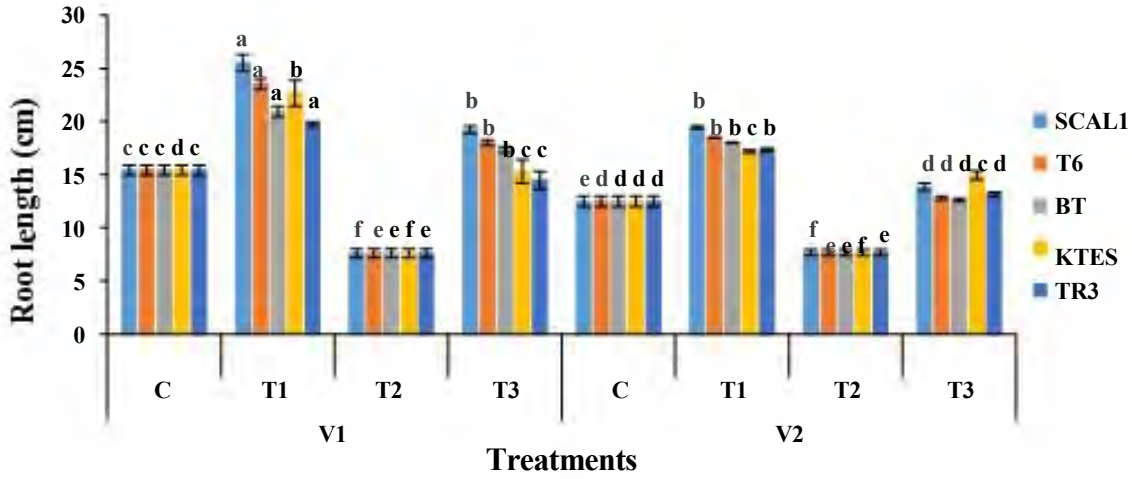

Figure 3.2. Effect of therm-tolerant strains, SCAL1 *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on root length of two tomato varieties (V1- Riogrande and V2- Sweetie). C: control, T1- Inoculated plants under normal condition, T2- Uninoculated under heat condition and T3- inoculated plants under heat condition at $p>0.05$

3.4.3. Fresh weight

Inoculation of bacterial enhanced the fresh weight significantly in both varieties (VI-Riogrande and V2- Sweetie) under normal (T1) and heat stress conditions (T3) with respective control (C and T2). Highest quantity of fresh weight was observed with inoculation of ***Bacillus safensis*** **(SCAL1)** under normal **(55.4%)** and heat stress **(80.2%)** conditions inV1 with respect to control (C and T2). Same findings was observed in V2 with the maximum significant increase in fresh weight with inoculation of ***B. safensis*** **(SCAL1)** under normal **(50.1%)** and heat stress **(74.1%)** conditions as compared to respective control (C and T2). Heat stress showed the decreased fresh weight of un-inoculated tomato plant in both varieties. Decrease in fresh weight (62.68%) was observed in V2 under un-inoculated heat stress condition (T2) as compared to respective control, as shown in Figure 3.3.

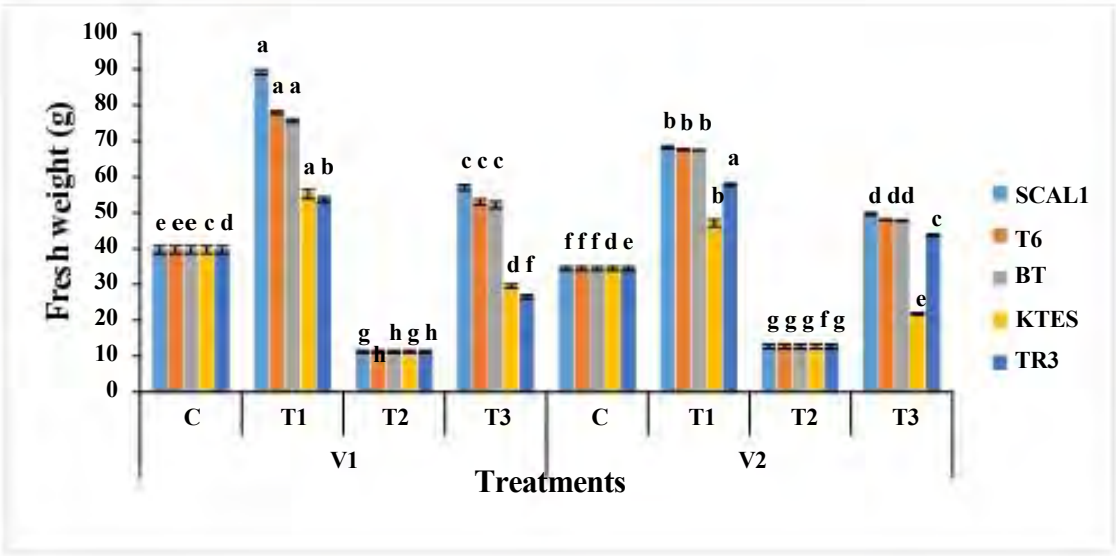

Figure 3.3. Effect of therm-tolerant strains, *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on fresh weight of two tomato varieties (V1- Riogrande and V2- Sweetie). C: control, T1- Inoculated plants under normal condition, T2- Uninoculated under heat condition and T3- inoculated plants under heat conditions *at p>0.05*

3.4.4. Dry weight

Plant growth promoting bacterial inoculation enhanced the dry weight significantly in both varieties (VI-Riogrande and V2- Sweetie) under normal (T1) and heat stress conditions (T3) with respective control (C and T2). Heighest increase in dry weight was observed with inoculation of ***Bacillus safensis*** **(T6)** under normal **(42.2%)** and heat stress **(66.1%)** conditions inV1 as compared to respective control (C and T2). Similar findings were noted in V2 with the maximum significant increase in dry weight with inoculation of ***B. safensis*** **(T6)** under normal **(70.4%)** and heat stress **(39.4%)** conditions in comparision to respective control (C and T2). Heat stress dramatically showed the decreased dry weight of un-inoculated tomato plant in both varieties. Decrease in dry weight (27.2%) was observed in V2 under un-inoculated heat stress condition (T2) as compared to respective control, as shown in Figure 3.4.

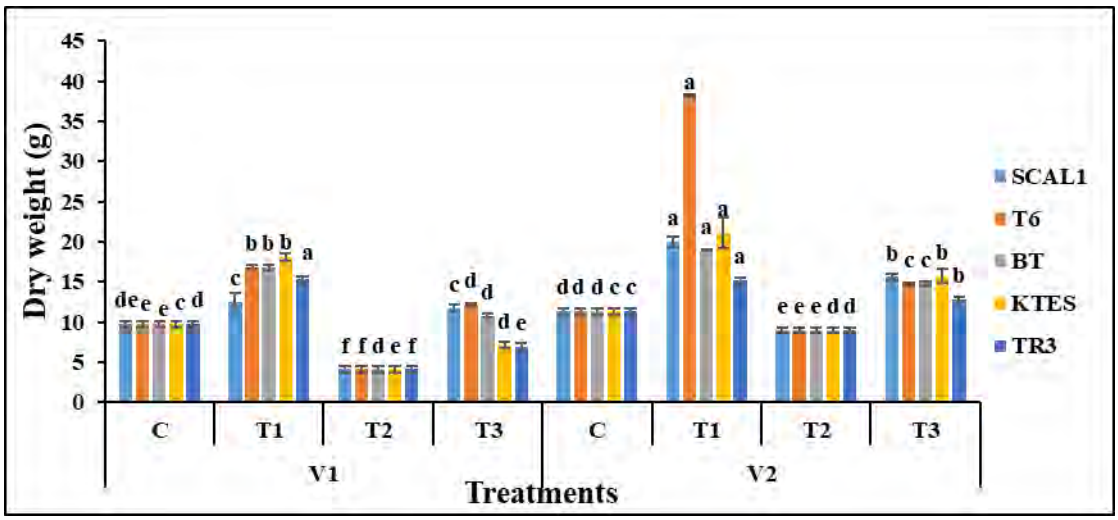

Figure 3.4. Effect of therm-tolerant strains, *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on dry weight of two tomato varieties (V1- Riogrande and V2- Sweetie). C: control, T1- Inoculated plants under normal condition, T2- Uninoculated under heat condition and T3- inoculated plants under heat condition at $p>0.05$

3.4.5. Leaf surface area

Bacterial inoculation enhanced leaf surface area significantly in both varieties (VI-Riogrande and V2- Sweetie) under normal (T1) and heat stress conditions (T3) with respective control (C and T2). Significant rise in leaf surface area was observed with inoculation of ***Bacillus safensis*** **(SCAL1)** under normal **(33.2 %)** and heat stress **(60.6%)** conditions in V1 as compared to respective control (C and T2). Similar findings was observed in V2 with the maximum significant increase in leaf surface area with inoculation of ***B. safensis*** **(SCAL1)** under normal (11.1 %) and heat stress (52.2%) conditions as compared to respective control (C and T2). Heat stress showed a remarkable decrease in leaf surface area of un-inoculated tomato plant in both varieties. Decreased leaf surface area (64.07%) was observed in V2 under un-inoculated heat stress condition (T2) as compared to respective control, as shown in Figure 3.5.

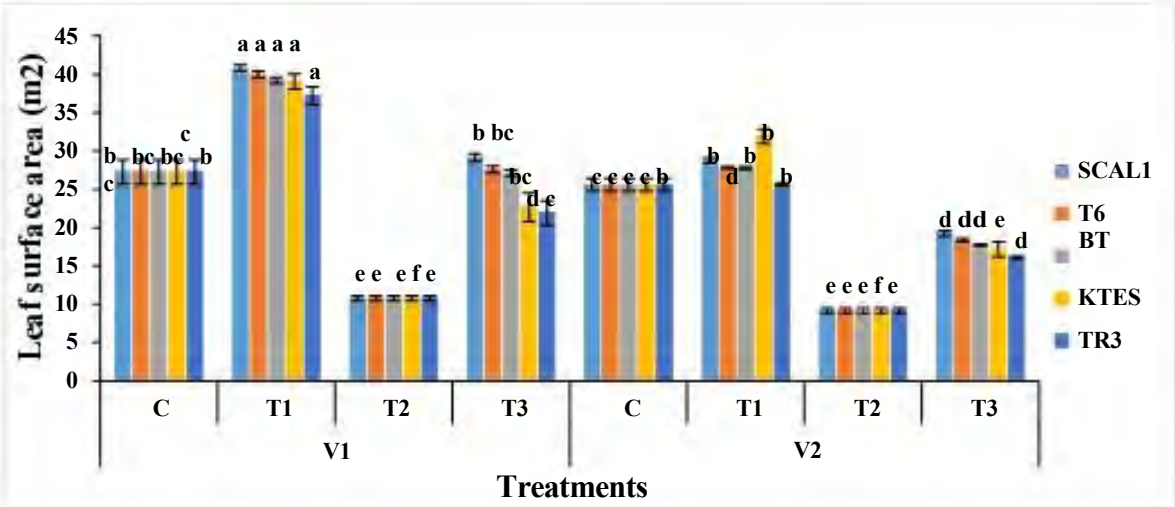

Figure 3. 5. Effect of therm-tolerant strains, *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on leaf surface area of two tomato varieties (V1- Riogrande and V2- Sweetie). C: control, T1- Inoculated plants under normal condition, T2- Uninoculated under heat condition and T3- inoculated plants under heat condition at *p>0.05*

3.4.6. Number of flowers

Bacterial inoculation increased the flowers numbers significantly in both varieties (VI-Riogrande and V2- Sweetie) under normal (T1) and heat stress conditions (T3) with respective control (C and T2). Significant rise in flowers numbers was observed with inoculation of ***Bacillus safensis*** **(SCAL1)** under normal **(51.6 %)** and heat stress **(63.9%)** conditions in V1 as compared to respective control (C and T2). On the other hand in V2 the inoculation of ***Bacillus safensis*** **(T6)** showed the maximum increase under normal **(58.4 %) and heat stress (77.8 %)** conditions as compared to respective control (C and T2). Heat stress (T2) showed a remarkable decrease in the number of flowers of un-inoculated tomato plant in both varieties. The decrease in the number of flowers (62.4%) was observed in V2 under un-inoculated heat stress condition (T2) as compared to respective control, Figure 3.6.

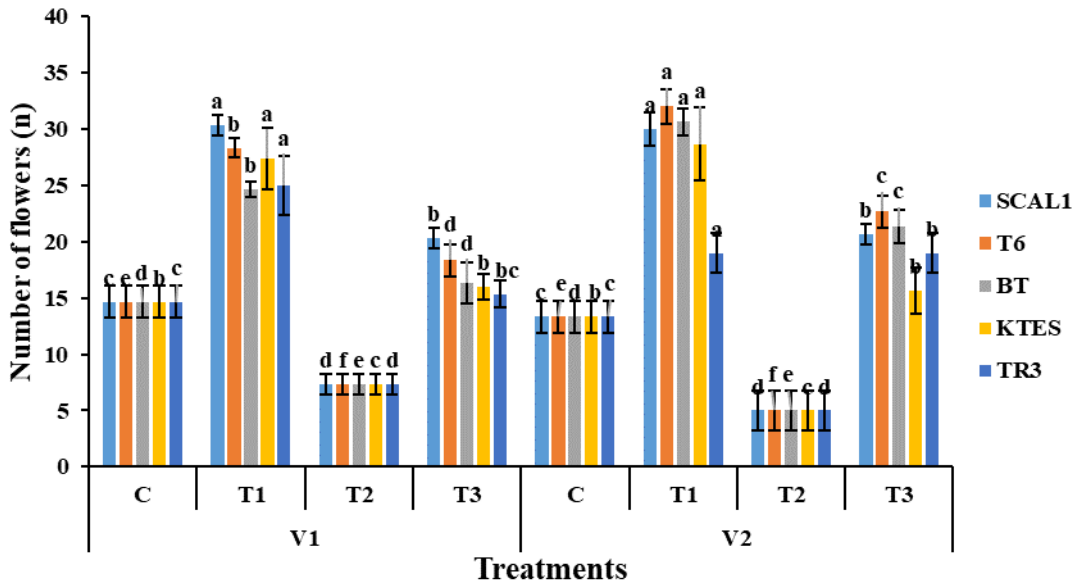

Figure 3.6. Effect of therm-tolerant strains, *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on number of flowers of two tomato varieties (V1- Riograndi and V2- Sweetie). C: control, T1- Inoculated plants under normal condition, T2- Uninoculated under heat condition and T3- inoculated plants under heat condition at *p>0.05*

3.4.7. Number of fruits

Bacterial inoculation increased the fruits numbers significantly in both varieties (VI-Riogrande and V2- Sweetie) under normal (T1) and heat stress conditions (T3) with respective control (C and T2). Significant rise in fruits numbers was observed with inoculation of ***Bacillus safensis*** **(T6)** under normal (58.4 %) and heat stress **(80.1 %)** conditions in V1 as compared to respective control (C and T2). On the other hand, inoculation of ***Bacillus safensis*** **(T6)** in V2 showed maximum significant increase in number of fruits under normal **(71.6 %)** and heat stress (77.5 %) conditions inV2 as compared to respective control (C and T2). Heat stress showed a remarkable decrease in the number of fruits of un-inoculated tomato plant in both varieties. Decrease number of fruits (50.06%) was observed in V2 under un-inoculated heat stress condition (T2) as compared to respective control, as shown in Figure 3.7 and appendix 7-11.

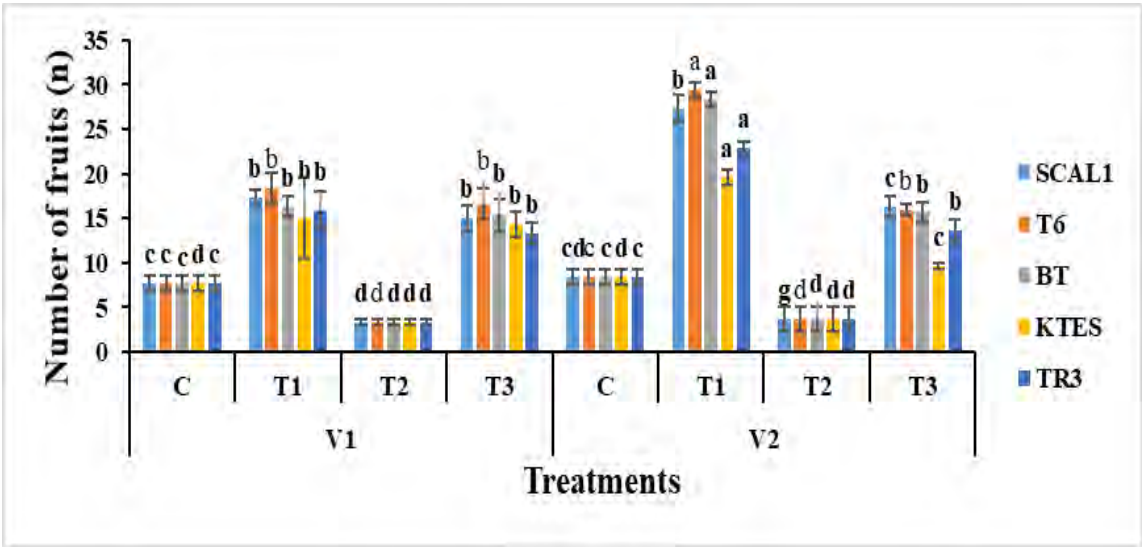

Figure 3.7. Effect of therm-tolerant strains SCAL1 (*Bacillus safensis*), *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on number of fruits of two tomato varieties (V1-Riograndi and V2- Sweetie). C: control, T1-Inoculated plants under normal condition, T2-Uninoculated under heat condition and T3- inoculated plants under heat condition at $p>0.05$.

3.4.8. Photosynthetic pigments

3.4.8. 1. Chlorophyll *a*

Bacterial inoculation enhanced chlorophyll *a* significantly in both varieties (VI-Riogrande and V2- Sweetie) under normal (T1) and heat stress conditions (T3) with respective control (C). The significant increase in chlorophyll *a* was observed with inoculation of ***Bacillus safensis*** **(SCAL1)** under normal (36.6%) and heat stress **(91%)** conditions in V1 as compared to respective control (C and T2). Same findings was observed in V2 (with the maximum significant increase in chlorophyll ***a*** with inoculation of ***B. safensis*** **SCAL1** under normal **(55.7%)** and heat stress (79.04%) conditions as compared to respective control (C and T2). Heat stressdramatically decreased chlorophyll *a* of un-inoculated tomato plant in both varieties. The decrease in chlorophyll *a* (68.11%) was observed in V2 under un-inoculated heat stress condition (T2) as compared to respective control, as shown in Figure 3.8.

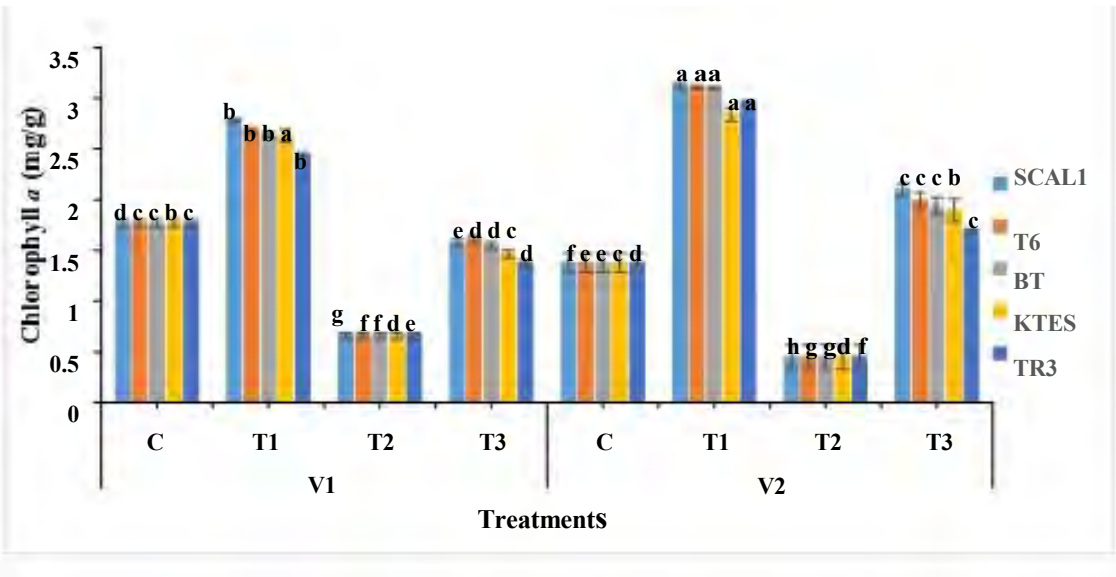

Figure 3.8. Effect of therm-tolerant strains, *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on chlorophyll *a* of two tomato varieties (V1- Riograndi and V2- Sweetie). C: control, T1- Inoculated plants under normal condition, T2- Uninoculated under heat condition and T3- inoculated plants under heat condition at $p>0.05$

3.4.8.2. Chlorophyll *b*

Bacterial inoculation enhanced chlorophyll *b* significantly in both varieties (VI-Riogrande and V2- Sweetie) under normal (T1) and heat stress conditions (T3) with respective control (C). The significant increase in chlorophyll *b* was observed with inoculation of ***Bacillus safensis*** **(BT)** under normal **(29.1%)** and heat stress (68.5 %) conditions in V1 as compared to respective control (C and T2). Same findings were observed in V2 with the maximum significant increase in chlorophyll *b* with inoculation of ***B. safensis*** **(T6)** under normal (19.5 %) and heat stress **(68.6 %)** conditions as compared to respective control (C and T2). Heat stress dramatically decreased chlorophyll *b* of un-inoculated tomato plant in both varieties (Riogrande and Sweetie). Drastic decrease in chlorophyll *b* (70.83%) was observed in V2 under un-inoculated heat stress condition (T2) as compared to respective control, as shown in Figure. 3.9

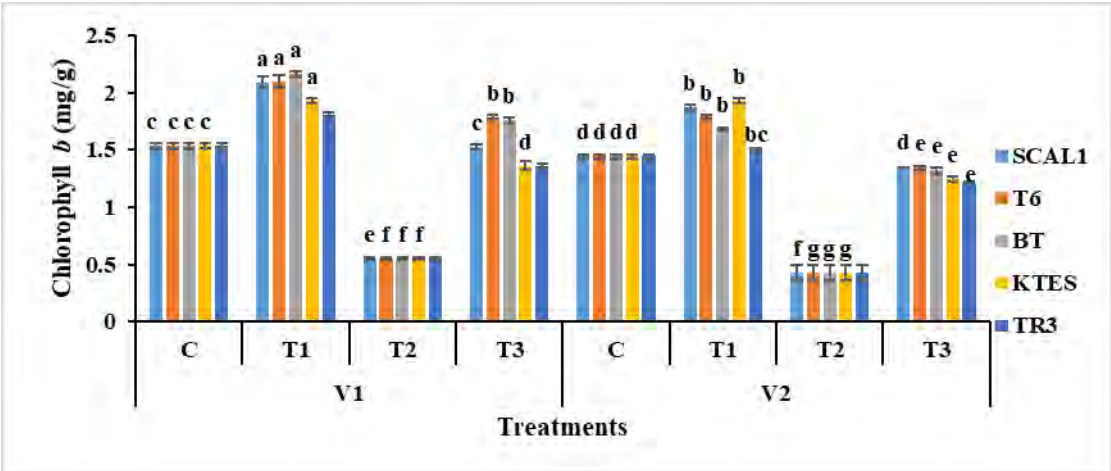

Figure 3. 9. Effect of therm-tolerant strains, *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on chlorophyll *b* of two tomato varieties (V1- Riograndi and V2- Sweetie). C: control, T1- Inoculated plants under normal condition, T2- Uninoculated under heat condition and T3- inoculated plants under heat condition at $p>0.05$

3.4.8.3. Carotenoid

Inoculation of bacteria increased the carotenoid content significantly in both varieties (VI-Riogrande and V2- Sweetie) under normal (T1) and heat stress conditions (T3) with respective control (C and T2). The heighest increase in carotenoid content was observed with inoculation of ***Bacillus safensis*** **(SCAL1)** under normal **(62.5 %)** and heat stress **(69.3 %)** conditions in V1 as compared to respective control (C and T2). On the other hand, with the inoculation of ***Bacillus safensis*** **(SCAL1)** in V2 exhibited a maximum significant increase in carotenoid content under normal (61.5 %) and under the heat stress (75.5%) condition as compared to respective control. Inoculation of ***Bacillus cereus*** **(KTES)** increased the carotenoid content **(79.5%)** under heat stress condition (T3) in V2 as compared to respective control (T2). Heat stress showed a remarkable decrease in carotenoid content of un-inoculated tomato plant in both varieties. Decreased carotenoid content (71.3%) was observed in V2 under heat stress condition (T2) as compared to respective control, as shown in Figure. 3.10.

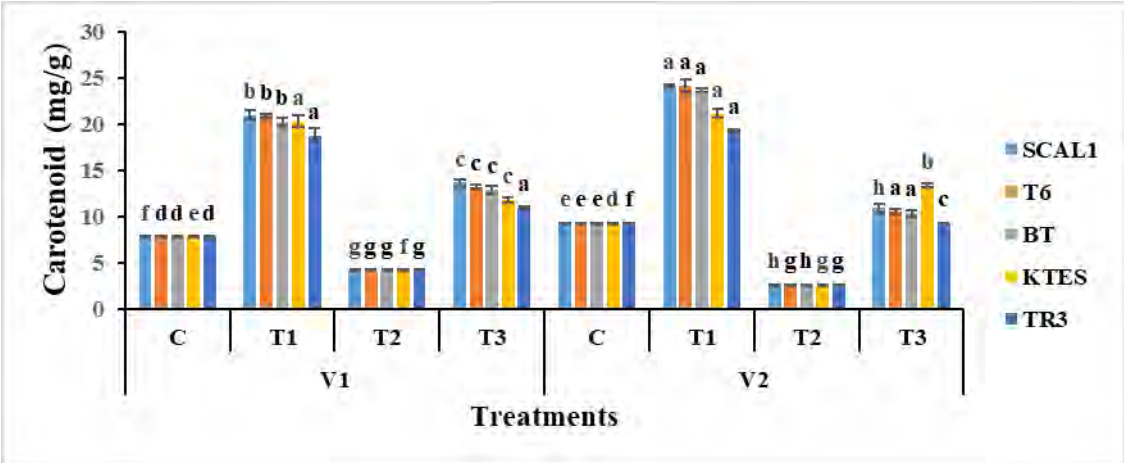

Figure 3.10. Effect of therm-tolerant strains**,** *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on carotenoid of two tomato varieties (V1- Riograndi and V2- Sweetie). C: control, T1- Inoculated plants under normal condition, T2- Uninoculated under heat condition and T3- inoculated plants under heat condition at $p>0.05$

3.4.9. Protein

Bacterial inoculation increased the protein content significantly in both varieties (VI-Riogrande and V2- Sweetie) under normal (T1) and heat stress conditions (T3) with respective control (C and T2). The significant rise in protein content was observed with inoculation of ***Bacillus safensis*** **(SCAL1)** under normal **(38.9%)** and heat stress **(66.9%)** conditions in V1 as compared to respective control (C and T2). Meanwhile the inoculation of ***Bacillus safensis*** **(SCAL1)** increased the protein content in V2 with the maximum significant increase in protein content was observed under normal **(61.3%)** and heat stress (62.1 %) conditions as compared to respective control (C and T2). Heat stress showed a remarkable decrease in the protein content of un-inoculated tomato plant in both varieties. Decreased protein content (27.8%) was observed in V2 under un-inoculated heat stress condition (T2) as compared to respective control, as shown in Figure 3.11.

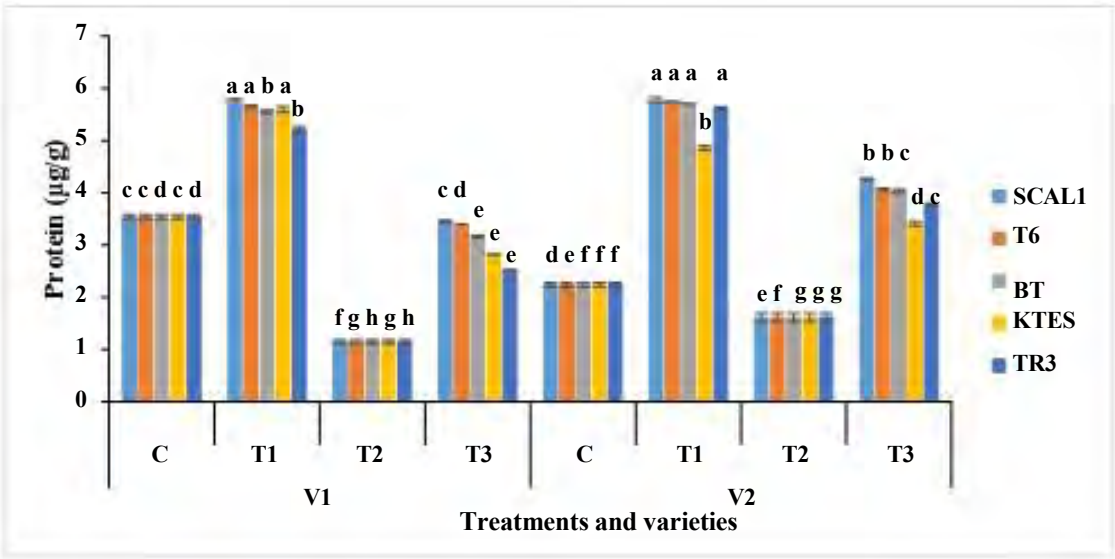

Figure 3.11. Effect of therm-tolerant strains, *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on protein of two tomato varieties (V1- Riogrande and V2- Sweetie). C: control, T1- Inoculated plants under normal condition, T2- Uninoculated under heat condition and T3- inoculated plants under heat condition at $p>0.05$

3.4.10. Proline

Bacterial inoculation increased the proline content significantly in both varieties (VI-Riogrande and V2- Sweetie) under normal (T1) and heat stress conditions (T3) with respective control (C and T2). The significant increase in proline content was observed with inoculation of Bacillus safensis (SCAL1) under normal (42.7 %) and heat stress (42.08%) conditions in V1 as compared to respective control (C and T2). Meanwhile the inoculation of ***Bacillus safensis*** **(SCAL1)** increased the proline content in V2 with the maximum significant increase in proline content was observed under normal **(57.2%)** and heat stress **(47.9 %)** conditions as compared to respective control (C and T2). Heat stress showed a remarkable decrease in the proline content of un-inoculated tomato plant in both varieties. Proline content (27.70%) was observed in low quantity in V2 under heat stress (T2) condition as compared to respective control, as shown in Figure 3.12.

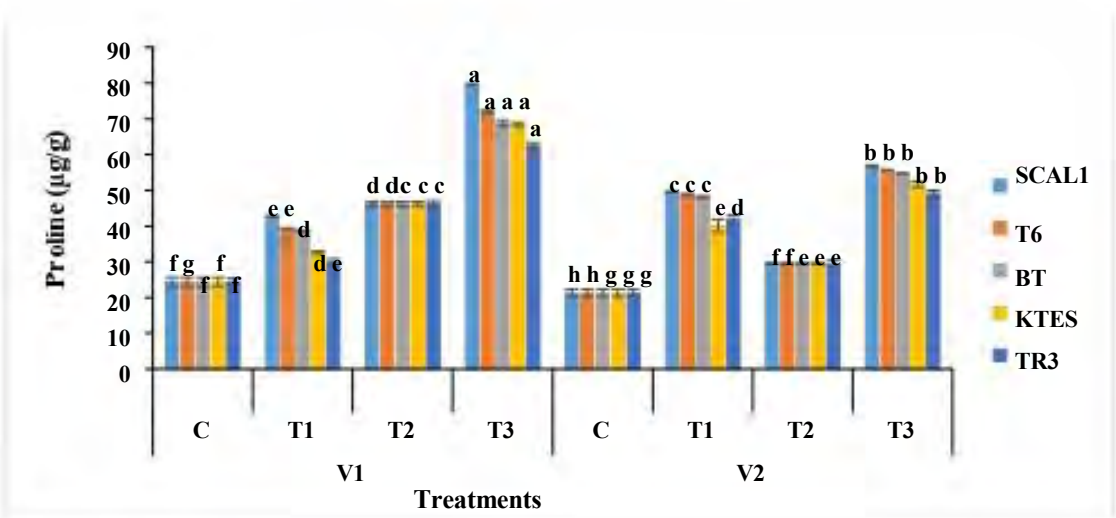

Figure 3.12. Effect of therm-tolerant strains, *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on proline of two tomato varieties (V1- Riogrande and V2- Sweetie). C: control, T1- Inoculated plants under normal condition, T2- Uninoculated under heat condition and T3- inoculated plants under heat condition at *p>0.05*

3.4.11. SOD

Bacterial inoculation increased the SOD activity significantly in both varieties (VI-Riogrande and V2- Sweetie) under normal (T1) and heat stress conditions (T3) with respective control (C and T2). The significant increase in SOD activity was observed with inoculation of Bacillus safensis (SCAL1) under normal (5.16 %) and increased under heat stress (40.3 %) conditions in V1 as compared to respective control (C and T2). On the other hand, inoculation of Bacillus safensis (SCAL1) in V2 showed a maximum significant increase in SOD activity under normal (63.3%) and under the heat stress (74.6%). Decreased SOD activity (23.3%) was observed in V1 under un-inoculated heat stress condition (T2) as compared to respective control, as shown in Figure 3.13.

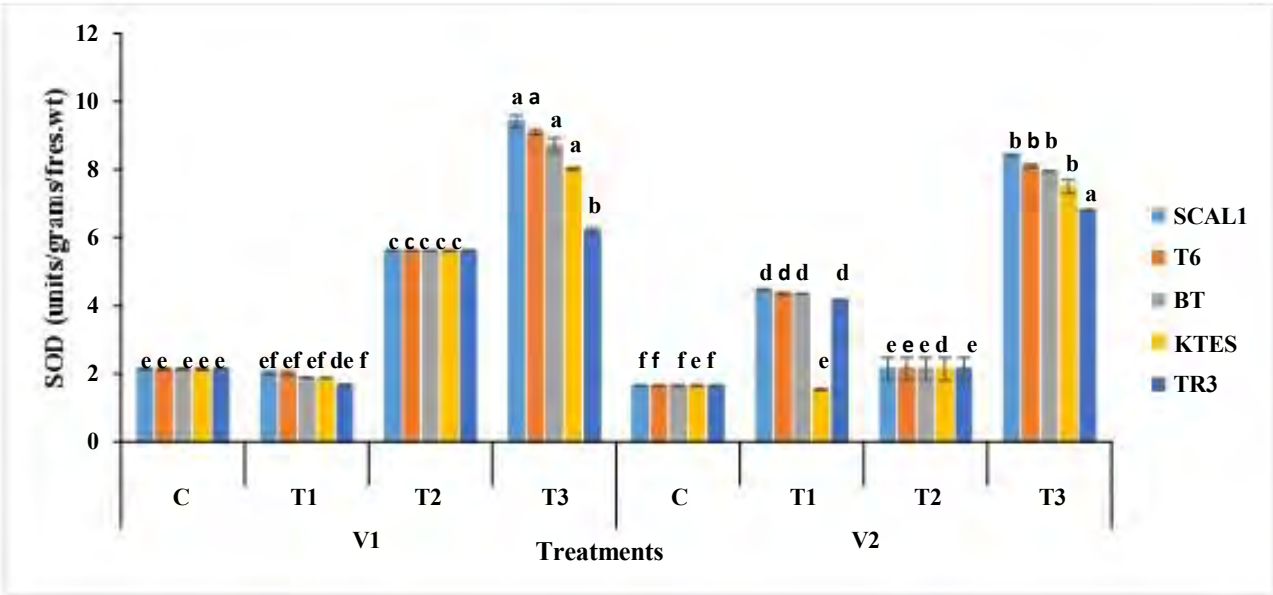

Figure 3.13. Effect of therm-tolerant strains**,** *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on SOD of two tomato varieties (V1- Riogrande and V2- Sweetie). C: control, T1- Inoculated plants under normal condition, T2- Uninoculated under heat condition and T3- inoculated plants under heat condition at $p>0.05$

3.4.12. POD

Bacterial inoculation increased the POD activity significantly in both varieties (VI-Riogrande and V2- Sweetie) under normal (T1) and heat stress conditions (T3) with respective control (C and T2). The significant increase in POD activity was observed with inoculation of ***Bacillus safensis*** **(SCAL1)** under normal **(97.6%)** and heat stress **(97.8 %)** conditions in V1 as compared to respective control (C and T2). Meanwhile the inoculation of ***Bacillus cereus*** **(KTES)** increased the POD content in V2 under normal (96.2 %) and heat stress (95.7 %) conditions as compared to respective control (C and T2) Heat stress showed the increase in POD activity (58.3%) was observed in V 2 under heat stress condition (T2) as compared to respective control, as shown in Figure 3.14.

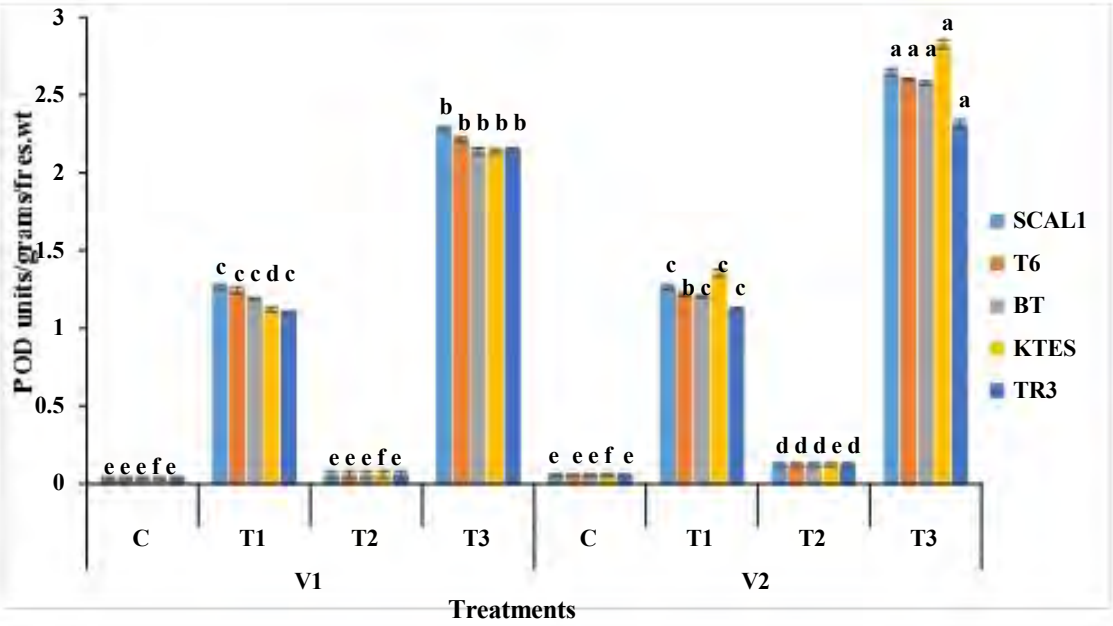

Figure 3.14. Effect of therm-tolerant strains, *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on POD of two tomato varieties (V1- Riogrande and V2- Sweetie). C: control, T1- Inoculated plants under normal condition, T2- Uninoculated under heat condition and T3- inoculated plants under heat condition at *p>0.05*

3.4.13. Catalase activity

Bacterial inoculation increased the catalase activity significantly in both varieties (VI-Riogrande and V2- Sweetie) under normal (T1) and heat stress conditions (T3) with respective control (C and T2). The significant increase in catalase activity was observed with inoculation of ***Bacillus safensis*** **(T6)** under normal **(91.6 %)** and increased in heat stress (7.03 %) conditions in V1 as compared to respective control (C and T2). On the other hand inoculation of ***Bacillus safensis*** **(SCAL1)** in V2 with the maximum significant increase in catalase activity was observed under normal **(86.7%)** and heat stress condition (63.4%) as compared to respective control (C and T2). Heat stress also showed the increase in catalase activity of un-inoculated tomato plant in both varieties. Increased catalase activity (84.04%) was observed in V1 under heat stress condition (T2) as compared to respective control, as shown in Figure 3.15.

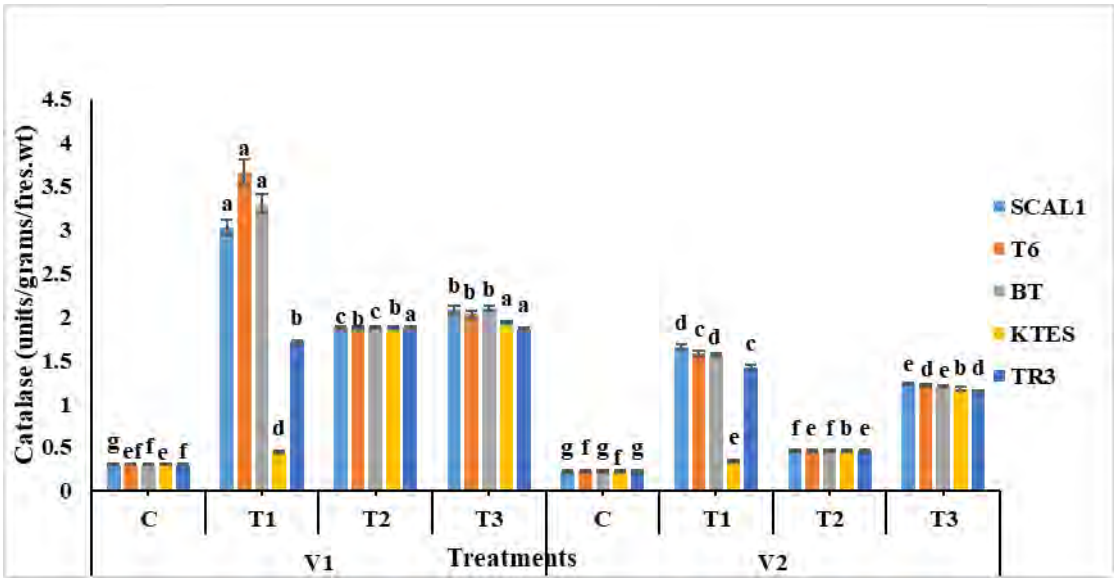

Figure 3.15. Effect of therm-tolerant strains, *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on catalase of two tomato varieties (V1-Riogrande and V2-Sweetie). C: control, T1- Inoculated plants under normal condition, T2- Un-inoculated under heat condition and T3- inoculated plants under heat condition at $p>0.05$

3.4.14. Relative water content

Inoculation of bacteria increased the relative water content significantly in both varieties (VI-Riogrande and V2- Sweetie) under normal (T1) and heat stress conditions (T3) with respective control (C and T2). The significant Increase in relative water content was observed with inoculation of ***Bacillus safensis*** **(SCAL1)** under normal (33.4 %) and heat stress (35.1%) conditions in V1 as compared to respective control (C and T2). On the other hand, inoculation of ***Bacillus safensis*** **(SCAL1)** in V2 showed significant increase in relative water content under normal **(41.8%)** and under the heat stress **(39.7%)** as compared to respective control (C and T2). Inoculation of ***Bacillus cereus*** **(KTES)** increased the relative water content under normal (37.3 %) and heat stress (41.4%) conditions in V2 as compared to respective control (C and T2). Heat stress showed a remarkable decrease in relative water content of un-inoculated tomato plant in both varieties. Decrease in relative water content (19.03) was observed in V2 under heat stress condition (T2) as compared to respective control, as shown in Figure 3.16.

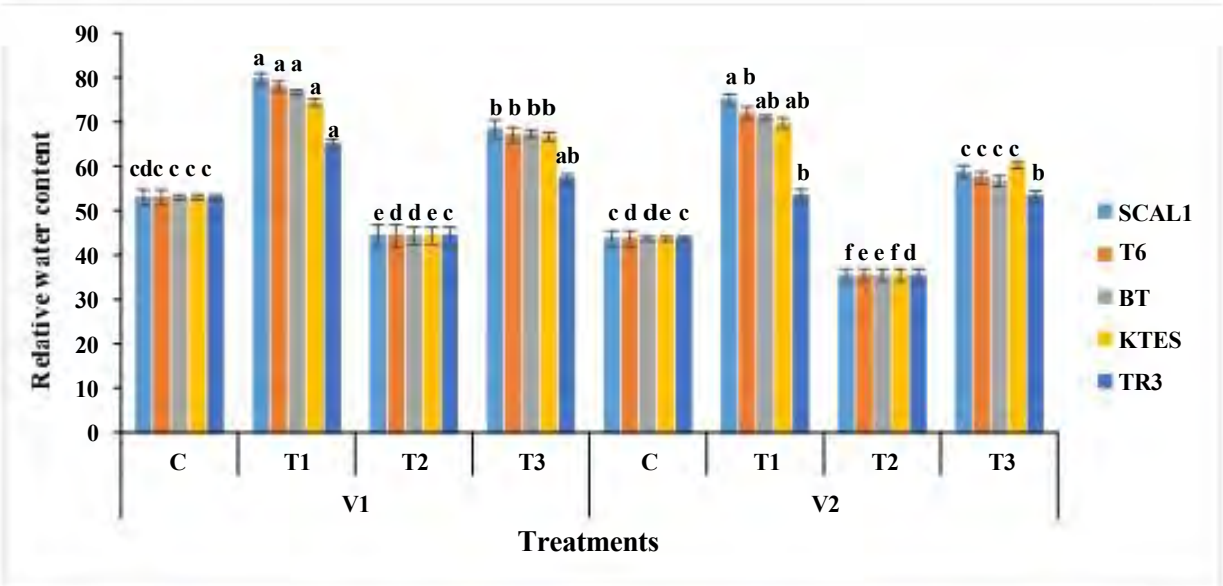

Figure 3. 16. Effect of therm-tolerant strains, *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on relative water content of two tomato varieties (V1- Riogrande and V2- Sweetie). C: control, T1- Inoculated plants under normal condition, T2- Un-inoculated under heat condition and T3- inoculated plants under heat condition at $p>0.05$.

3.4.15. Electrolyte leakage

Inoculation of bacteria decreased the electrolyte leakage significantly in both varieties (VI-Riogrande and V2-Sweetie) under normal (T1) and heat stress conditions (T3) with respective control (C and T2). The significant increase in electrolyte leakage activity was observed with inoculation of ***Bacillus safensis*** **(SCAL1)** under normal (2.99%) and decreased under heat stress (9.09 %) conditions in V1 as compared to respective control (C and T2). Meanwhile the inoculation of ***Bacillus cereus*** **(KTES)** decreased the electrolyte leakage in V2 was observed under normal **(27.9 %)** and heat stress (53.8 %) conditions as compared to respective control (C and T2) as shown in Figure 3.17.

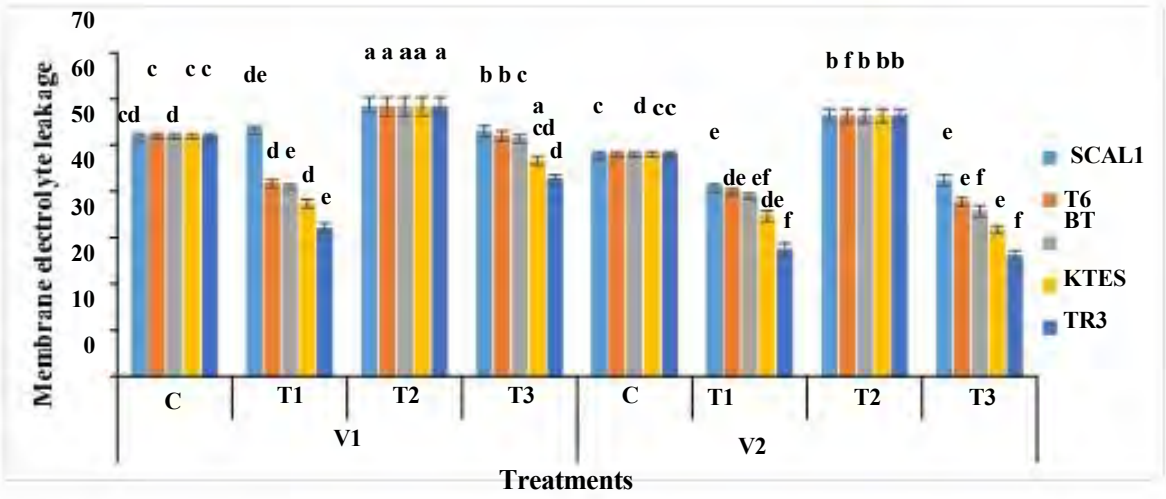

Figure 3.17. Effect of therm-tolerant strains, *Bacillus safensis* (SCAL1), *Bacillus safensis* T6), (*Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on membrane electrolyte leakage of two tomato varieties (V1-Riogrande and V2- Sweetie). C: control, T1- Inoculated plants under normal condition, T2- Un-inoculated under heat condition and T3- inoculated plants under heat condition at $p>0.05$.

3.4.16. Pearson correlation analysis *Bacillus safensis* (SCAL1)

Pearson correlation bi-plot analysis revealed 85.07% of the total variation between the plotted data F1 and F2. F1 and F2 individually contributed about 69.24% and 15.83% variation, respectively. Closely present variables in the same quadrant exhibited a strong association with each. Furthermore, the correlation between parameters displayed by red dots while blue dots demonstrated a correlation between the studied

treatments. **Bacterial strain (*Bacillus safensis*)** exhibited a strong positive response towards various plant growth parameters under heat stress condition as revealed from principal component analysis. All studied parameters revealed the significantly positive correlation among each other except membrane electrolyte leakage that showed negative correlation as shown in bi-plot analysis (Figure 3.18).

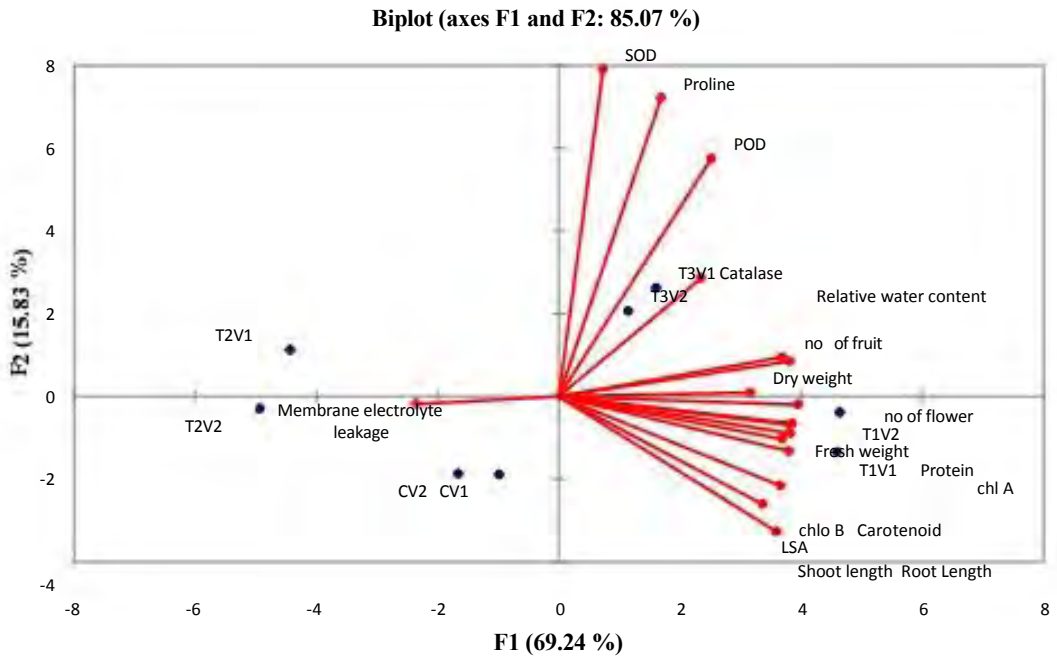

Figure 3.18. The correlation bi-plot analysis between different treatments, tomato varieties, growth traits and biochemical analysis for *Bacillus safensis* (SCAL1).

3.4.17. Pearson correlation analysis of *Bacillus safensis* (T6)

Pearson correlation bi-plot analysis revealed 85.91% of the total variation between the plotted data F1 and F2. F1 and F2 individually contributed about 69.78% and 16.12% variation, respectively. Closely present variables in the same quadrant exhibited a strong association with each other. Furthermore, the correlation between parameters by red dots while blue dots demonstrated a correlation between the studied treatments. Bacterial strain ***Bacillus safensis* (T6)** exhibited a strong positive response towards various plant growth parameters under heat stress condition as

revealed from principal component analysis. All studied parameters revealed the significantly positive correlation among each other except membrane electrolyte leakage that showed negative correlation as shown in the bi-plot analysis (Figure 3.19).

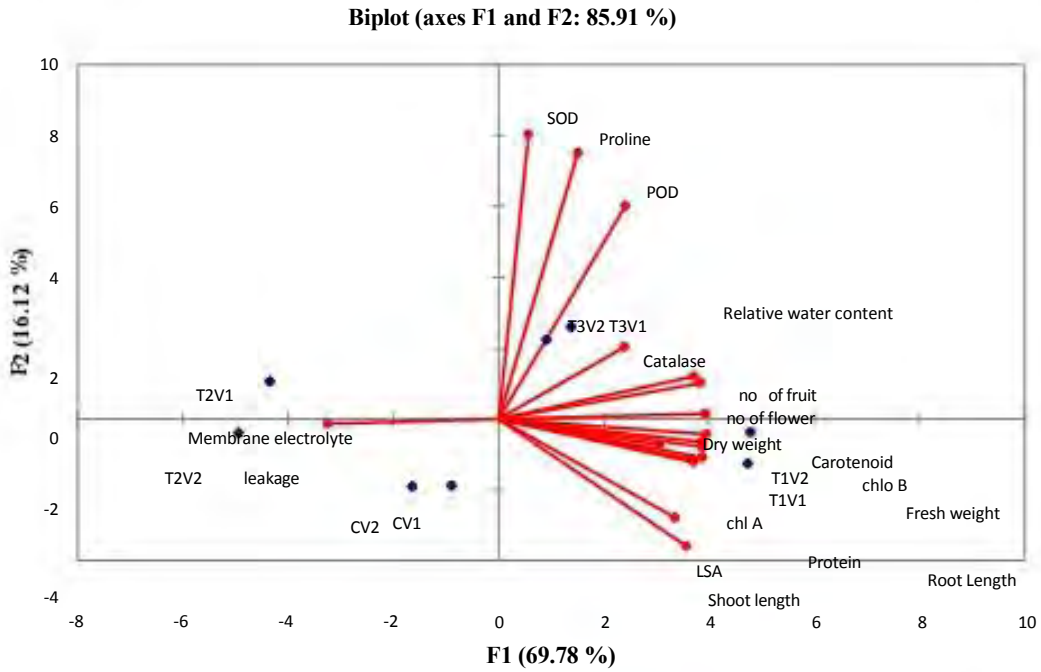

Figure 3.19. The correlation bi-plot analysis between different treatments, tomato varieties, growth traits and biochemical analysis for *Bacillus safensis* (T6).

3.4.18. Pearson correlation analysis of *Bacillus safensis* (BT)

Pearson correlation bi-plot analysis revealed 86.80% of the total variation between the plotted data F1 and F2. F1 and F2 individually contributed about 70.02% and 16.78% variation, respectively. Closely present variables in the same quadrant exhibited a strong association with each. Furthermore, the correlation between parameters displayed by red dots while blue dots demonstrated a correlation between the studied treatments. Bacterial strain ***Bacillus safensis*** **(BT)** exhibited a strong positive response towards various plant growth parameters under heat stress condition as revealed from principal component analysis. All studied parameters revealed the significantly positive correlation among each other except membrane electrolyte leakage that showed negative correlation as shown in the bi-plot analysis (Figure 3.20).

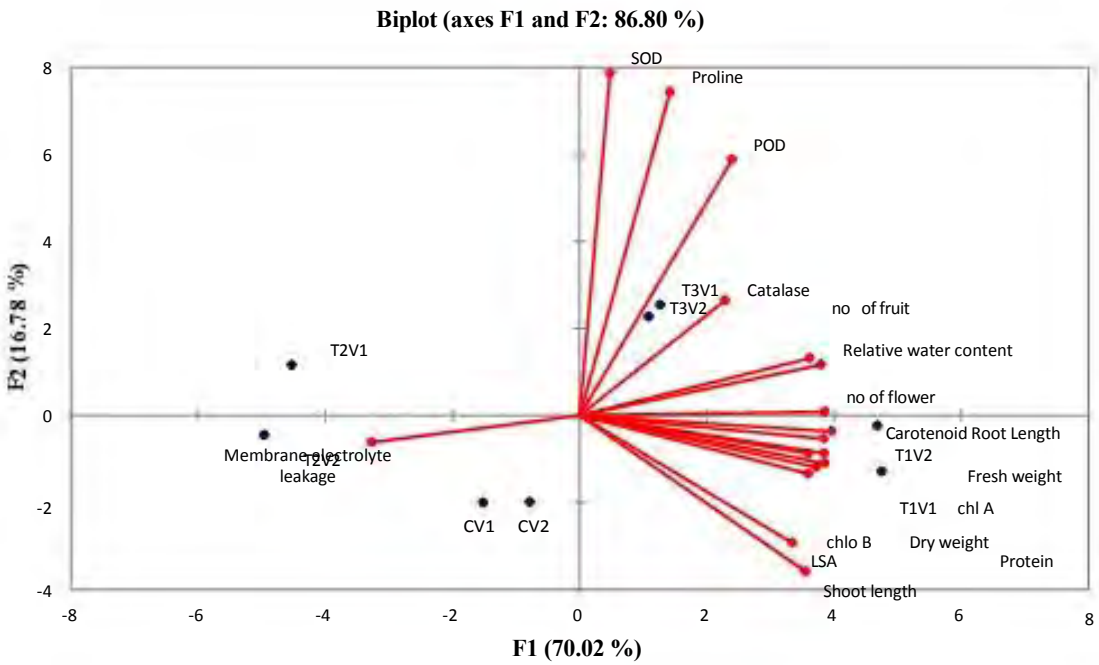

Figure 3.20. The correlation bi-plot analysis between different treatments, tomato varieties, growth traits and biochemical analysis for *Bacillus safensis* (BT).

3.4.19. Pearson correlation analysis of *Bacillus cereus* (KTES)

Pearson correlation bi-plot analysis revealed 91.81% of the total variation between the plotted data F1 and F2. F1 and F2 individually contributed about 71.89% and 19.92% variation respectively. Closely present variables in the same quadrant exhibited strong association with each. Furthermore, the correlation between parameters revealed by red dots while blue dots demonstrated a correlation between the studied treatments. Bacterial strain ***Bacillus cereus* (KTES)** exhibited a strong positive response towards various plant growth parameters under heat stress condition as revealed from principal component analysis. All studied parameters revealed the significantly positive correlation among each other except SOD, proline, catalase and membrane electrolyte leakage that showed negative correlation as shown in the bi-plot analysis (Figure 3.21).

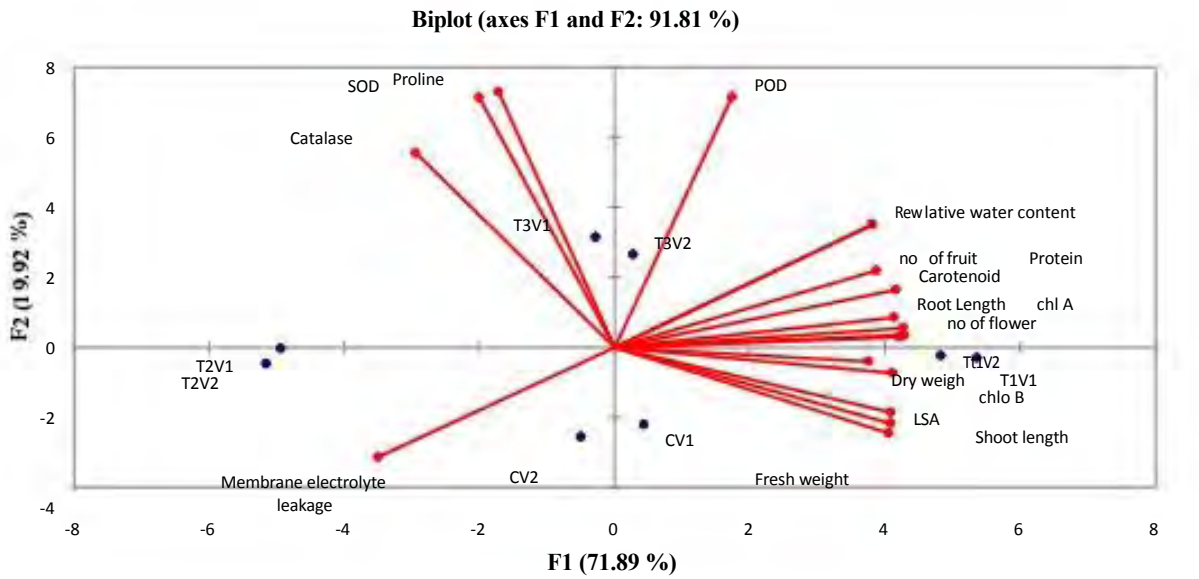

Figure 3.21. The correlation bi-plot analysis between different treatments, tomato varieties, growth traits and biochemical analysis for *bacillus cereus* (KTES).

3.4.20. Pearson correlation analysis of *Klebsiella variicola* (TR3)

Pearson correlation bi-plot analysis revealed 87.37% of the total variation between the plotted data F1 and F2. F1 and F2 individually contributed about 65.68 % and 21.69% variation respectively. Closely present variables in the same quadrant exhibited a strong association with each. Furthermore, the correlation between parameters shown by red dots while blue dots demonstrated a correlation between the studied treatments. Bacterial strain ***Klebsiella variicola*** **(TR3)** exhibited a strong positive response towards various plant growth parameters under heat stress condition as revealed from principal component analysis. All studied parameters revealed the significantly positive correlation among each other except SOD and membrane electrolyte leakage that showed negative correlation as shown in the bi-plot analysis (Figure 3.22).

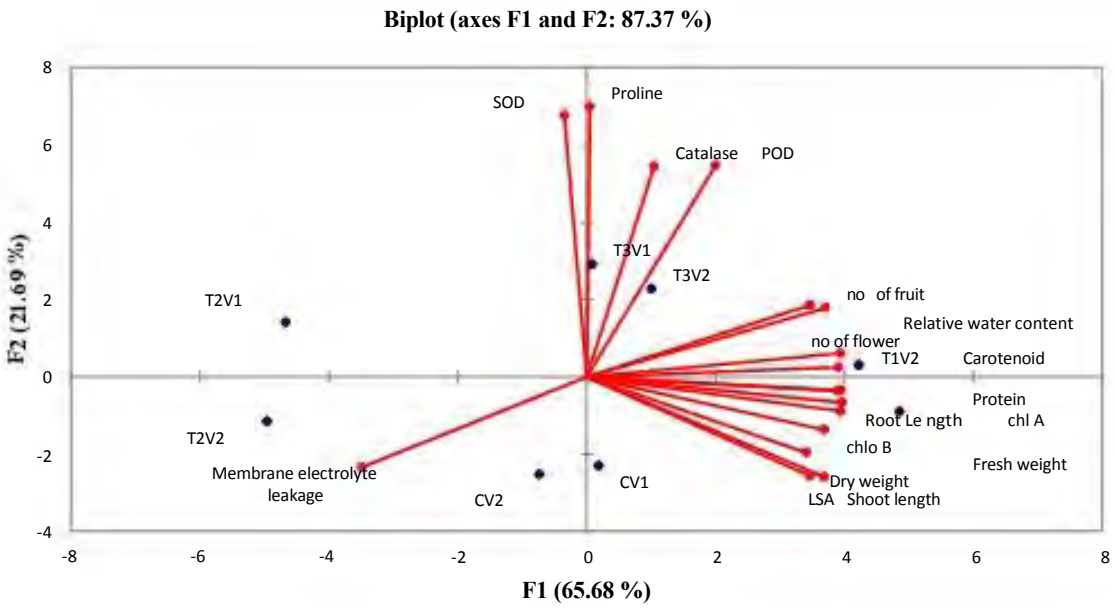

Figure 3.22. The correlation bi-plot analysis between different treatments, tomato varieties, growth traits and biochemical analysis for *Klebsiella variicola* (TR3).

3.5. Discussion

Agricultural production is significantly influenced by the different environmental stresses specifically heat stress and is become alarming with the passage of time. Utilization of advanced, cost-effective and environment friendly technologies is utmost need to overcome heat stress (Mukhtar *et al.,* 2020). The plant growth promoting bacteria is well documented to confer the tolerance to different abiotic stresses and improved plant health (Amna *et al.,* 2019). In the ongoing investigation the applications of plant growth promoting bacteria (PGPB) showed enhanced agronomic variables, shoot length and root length, fresh weight and dry weight, leaf surface area, flowers and fruits number, chlorophyll content, membrane electrolyte leakage, relative water content and antioxidant activities (Afridi *et al.,* 2019).

Plant growth promoting bacteria is microbial flora residing asymptomatically in all plant parts enhance nutrients uptake and counteract adverse effect of heat stress to host plants by the production of plant growth regulators (Mukhtar *et al.,* 2020).

In the recent investigation, enhanced shoot length and root length under normal (T1) and heat stress (T3) compare to control (C) and un-inoculated heat treatment (T2)

were observed. Also, Riogrande revealed higher shoot and root length compare to Sweetie. Our findings are supported by Khan *et al.,* (2016) as they obtained enhanced shoot length and root length and chlorophyll content with application of bacterial strain *bacillus subtilus* (MPB 2.1). The IAA production helps in plants growth and also aid to cope with different environmental stresses (Sarma *et al.,* 2014; FAOSTAT. 2013). Moreover, *Klebsiella* sp. (SBP-8) significantly improved plant growth under saline and heat stress (Afridi *et al.,* 2019). Moreover, our findings were strengthened with the results of Liu *et al.,* (2013) in which they demonstrated that the resistance of *Platycladus orientalis* to abiotic stress increased with cytokinin-producing *Bacillus spp*.

Plant biomass (fresh and dry weight) was enhanced with inoculation of plant growth promoting bacteria (PGPB) under heat stress (T3) and normal (T1) as compared to control (C) and un-inoculated heat stress treatment (T2). Our results are strongly supported with outcomes of Khan *et al.,* (2014) as they reported that, bacterial inoculation enhanced the plant growth attributes of wild-type and Got-3 tomato cultivars. Similar trends were observed in the study of Ali *et al.* (2011). Inoculation of growth promoting bacteria reported to minimize adverse effects of heat stress and increased the growth of plant and productivity of crop (Ali *et al.,* 2011). Tomato variety Sweetie was comparatively more heat sensitive than Riogrande under inoculated conditions. Similar trend was stated by Daim *et al.* (2014), for plants under heat stress conditions and revealed the application of Plant growth promoting bacteria improved plant growth.

Bacterial inoculation under heat stress increased the biomass of both varieties in contrast to un-inoculated plants. Heat stress mitigation was also observed in a similar trend in the study of Ali *et al.,*(2009). Chandra *et al.*, (2018) found that inoculation of the plant with a *Pseudomonas* sp increased growth trait, fresh weight, dry weight and shoot and root length under abiotic stress and normal conditions. Previous studies demonstrated that growth of plants increased in response to PGPB application because of the production of plant growth regulators inside roots which stimulates root development and maximizes water and nutrient absorption from soil (Ali *et al.,* 2011).

High temperature influenced flowering and fruit setting of tomato crop especially in tropical and temperate regions throughout the world. Previous studies reported reduction in number of flowers of tomato crop due to the heat stress condition (Wahid *et al.,* 2007).

The decrease in the level of carbohydrates and growth regulators due to elevated temperature is also responsible for poor tomato fruit setting (Mukhtar *et al.,* 2020). The number of flowers and fruits significantly enhanced with bacterial inoculation in tomato plants under greenhouse conditions in contrast to un-inoculated control under non-heat condition. ***Bacillus safensis* (SCAL1)** showed the maximum number of flower **(42.3%)** under normal and heat stress (**49.1%)** condition as compared to respective control (C and T2). ***Bacillus safensis* (SCAL1)** produced the maximum number of fruits among all bacterial strains under normal **(39.3%)** and under **heat (32.4 %)** condition. Current findings are found in better trend as compared to results of Aini *et al.* (2019).

Leaf surface area was observed in maximum quantities with the bacterial inoculation under heat stress and normal conditions and this results are in line with outcomes of Namasivayam *et al.,* (2011) as they reported that inoculation of bacterium sp. enhanced IAA production which might be responsible for the enhancement of leaf surface area.

Our results showed that chlorophyll b content was maximum in heat stress against normal conditions in Riogrande. Increased chlorophyll contents could be due to higher photosynthetic leaf area that results from inoculation with PGPB, which was significantly reduced under un-inoculated plants exposed to heat stress compared to un-inoculated plants grown under normal conditions (Rehman *et al*., 2019). Fahad *et al.,* (1992), reported that heat stress significantly declined plant photosynthesis which might be possibly due to alteration in photosynthetic mechanisms balance. Furthermore, our current results are strongly supported by the findings of Ali *et al.,* (2018), as they reported that application of *P. putida* enhanced the chlorophyll content in shoots of the plant. Similarly (Ansari *et al.,* 2018, Ahamd *et al.,* 2017 ; Ahmadi *et al.,* 2015) also documented that the *Brevibacterium sp* (FAB3) application aided to minimize the abiotic stress conditions via enhanced chlorophyll content and improved plant yield attributes.

Current study resulted in enhance protein and proline concentration with bacterial inoculation under heat stress (T3) and normal (T1) as compared to control (C) and un-inoculated heat treatment (T2). It reflected that bacterial inoculation under heat stress and normal, enhanced plant capabilities in accumulation of protein, proline and carbohydrates. Ali *et al.* (2009), studied plant seedlings subjecting elevated temperature and reported higher accumulation of cellular metabolites. Further,

inoculation of *P. putida* strain (AKMP7) enhanced proline accumulation under heat stress as compared to un-inoculated treatment in plant (Ali *et al.,* 2011). Bano and Fatima (2009) also observed enhance proline concentration in plant due to *Rhizobium* and *Pseudomonas* inoculation. Proline accumulation is considered as an important adaptive mechanism as they bind to membranes, regulate cells permeability and affect the water transportation among tissues.

Our results revealed increased relative water content with bacterial inoculation under heat stress (T3) and normal (T1) as compared to control (C) and un-inoculated heat treatment (T2). Increase in relative water content was also observed in inoculated plant under abiotic stress as compared to un-inoculated plants (Afridi *et al.,* 2019). Similarly, higher membrane electrolyte leakage was observed under heat stress conditions, however membrane electrolyte leakage was significantly decreased with bacterial inoculation under heat stress. Our results are in agreement with the findings of Ali *et al.,* (2011), as they demonstrated that lower electrolyte leakage in inoculated plants are responsible to protection of membrane integrity with bacterial inoculation and might be due to alterations of plant lipid metabolism.

Reactive oxygen species (ROS) induced under abiotic stress conditions including heat stress and cause injury to the cell membrane. In response to ROS induction under heat stress, plant tries to modulate heat stress through antioxidant enzymes system including superoxide dismutase (SOD), Peroxidase (POD) and catalase (CAT) expression against oxidative damage in the cell membrane (Ahamd *et al.,* 2017). These antioxidant enzymes are used as a substantial pathway to tolerate adverse conditions (Chakraborty *et al.,* 2011; Garbero *et al.,* 2011). The SOD represented as the most important enzyme in ROS scavenging followed by CAT and POD for the protection of cellular membrane degradation (Gill *et al.,* 2010; Liu *et al.,* 2012). Our results indicated significantly elevated activities of SOD, POD and CAT with bacterial inoculation under normal (T1) and heat stress (T3) as compared to control (C) and un-inoculated heat stress treatment (T2). Ali *et al.,* (2011), documented that inoculation of thermo-tolerant *P. putida* strain (AKMP7) to plant under heat stress reduce the level of ROS as compared to un-inoculated plants under heat stress and resulted in lower damage to cellular components.

3.6. Conclusion

Plant-microbe interaction can facilitate plant growth under various stress conditions. This study investigated the five thermo-tolerant bacterial strains on tomato growth, physiology, antioxidants under greenhouse conditions. The findings of this study revealed adverse effects of heat stress on studied parameters. However, plants inoculated with Plant growth promoting bacteria (PGPB) alleviate heat stress possibly through improved physiological machinery and antioxidants. Inoculation of ***Bacillus safensis*** **SCAL1** showed the maximum number of flower under normal (51.6%) and heat stress condition **(63.9%)** and fruits under normal **(55.7%)** heat stress **(77.8%)** in variety Sweetie and this strain could be a strong candidate for field trial. It is therefore, suggested that the application of PGPB can be a feasible approach to ameliorate of heat stress in the tomato plant. Knowing that the natural conditions of ecosystems are not possible to simulate in the laboratory further trials will be needed, especially in the field, thereby confirming the behavior of plant microbe interaction in its natural environment.

CHAPTER: 4

Multi -year and multi-locational field trials of selected isolates

4.1. Introduction

Abiotic stresses including high temperature, droughts, flash floods, cold waves, elevated carbon dioxide (CO_2) and cyclones are natural disasters which can cause economic losses and provide the proof of global warming (Grover *et al.,* 2011; Carbonel *et al.,* 2019). Global circulation models gave the prediction that greenhouse gases will become the major reason for steadily increasing the average ambient temperatures around the world, and mean temperature per decade of the world will rise by 0.3^0C resulting in temperature increases of approximately 1 and 3^0C in 2025 and 2100, respectively. Heat stress is a problem to the agriculture field and there is an imperative need to tackle this problem for sustaining high productivity of crop plants under high temperatures (Wahid *et al.,* 2009).

Biofertilizers are considered as an alternative or complement to chemical fertilization to increase the production of crops in low input agricultural systems and under various stress conditions as well. There are some PGPB that can fix nitrogen, solubilize mineral nutrients and mineralize organic compounds. The well-studied PGPB considered biofertilizers correspond to nitrogen fixation and utilization of insoluble forms of phosphorus (Egamberdieva & Kucharova, 2009). PGPB not only increase the growth of plant under normal condition but also help the plant by impartingtolerance to various abiotic stress conditions (Fahad *et al.,* 2019). In last few years PGPB of various genera including *Bacillus, Rhizobium, Pseudomonas*, *Pantoea*, *Burkholderia, Paenibacillus*, *Azospirillum, Achromobacter*, *Microbacterium*, *Methylobacterium*, *Enterobacter and variovorax*, etc. have been well documented for imparting the tolerance to host plant against abiotic stress conditions (Dixit *et al.,* 2020).

Various mechanisms have been proposed by many scientists about elicited stress tolerance in crop plants by bacteria. PGPB produce many plant growth promoting compounds like indole acetic acid, gibberellins and some other unknown determinants which improved agronomic parameters of crop plants leading to well plant health under heat stress and normal conditions (Egamberdieva & Kucharova, 2009). Many studies have confirmed the plant growth promoting activities in tomato, canola, bean, lettuce and pepper against the abiotic stress conditions (Barassi *et al.,* 2006).

As the studied bacterial strain gave promising results in green house conditions so further the current study was designed to check the effects of plant growth

promoting bacterial strains with multiple PGP activities on tomato growth and physiology in field conditions.

4.2. Objective

To evaluate the selected isolates in multi-year and multi locational field trials

4.3. Materials and Methods

A total of three field trials were conducted at National Agriculture Research Centre (NARC), Islamabad (during 2018 and 2019) and district Muzaffargarh (during 2019) systematically. Overall strains were analyzed in two consequtive years along with two different locations i.e. National Agriculture research centre (NARC), Islamabad and district Muzaffargarh, Punjab, Pakistan.

4.3.1. National Agriculture Research Centre (NARC), Islamabad during 2018 an d 2019

The current experiment comprises of four treatments i.e. C, control, T1, plants with bacterial inoculation, T2, plants under heat stress without inoculation of bacteria and T3, plants inoculated with bacteria. The field experiment was conducted with completely randomized block design.

Table: 4.1 List of the treatments used in the field experiments conducted at NARC during 2018 and 2019.

Sr. No.	Treatments
1.	C = Control of Variety Riogrande
2.	SCAL1 + Variety Riogrande
3.	T6 + Variety Riogrande
4.	BT + Variety Riogrande
5.	KTES + Variety Riogrande
6.	TR3 + Variety Riogrande
7.	Control of Variety Riogrande + Heat Stress
8.	SCAL1+ Variety Riogrande+ Heat Stress
9.	T6+ Variety Riogrande + Heat Stress
10.	BT + Variety Riogrande + Heat Stress
11.	KTES + Variety Riogrande + Heat Stress
12.	TR3 + Variety Riogrande+ Heat Stress

Bacillus safensis (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3)

4.3.1.2. District Muzaffargarh, Punjab, Pakistan (during 2019)

Experiment comprises of two treatments which include C, control and consortia (Plants treated with bacterial consortia). Bacterial consortia consist of five bacterial strains that was *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3).

Table: 4.2 List of the treatments used in the field experiment conducted at District Muzaffargarh during 2019.

Sr. No	Treatments
1.	Control plants without bacterial treatments
2.	Plants treated with bacterial consortia

4.3.2. Collection of data

The number of flowers and fruits were observed after days of the experiments. Plants were randomly selected from field experiments and were analyzed for important agronomic and biochemical parameters after 120 days of sowing (Khan *et al*., 2020).

4.3.3. Agronomic and photosynthetic analysis of plants

4.3.3.1. Length of shoot and root

Shoots and roots length of freshly harvested plants were measured with measuring tape (Hussain *et al*., 2019).

4.3.3.2. Fresh weight

Digital balance was used to measure the fresh weight of harvested plants (Hussain *et al*., 2019).

4.3.3.3. Dry weight

After drying the plants in paper bags in oven for 2 days at 70°C, dry weight of plants was measured. Digital balance was used to measue the weight of fully dried plants (Hussain *et al*., 2019)

4.3.3.4. Number of flowers and of fruits

The number of flowers and fruits were counted with the naked eye in every treatment (Muktar *et al*., 2020).

4.3.4. Physiological parameters

4.3.4.1. Photosynthetic contents

Leaf material (0.1 g) from each treatment was used for estimation of chlorophyll contents. Fresh leaves (small pieces) were putted in 4 ml $DMSO_4$ (Dimethylsulfoxide) and then incubated (Hussain *et al*.,2019). After 4 h of incubation at 65°C, absorbance of extracts was recorded at wavelengths (663, 645 and 480 nm). Formulas which were used for the calculation of chlorophyll *a*, chlorophyll *b* and carotenoid contents are following

Chl a (mg/g f.w) = [1.07 (OD 663) - 0.09 (OD 645)]

Chl b (mg/g f.w) = [1.77 (OD 645) - 0.280 (OD 663)]

Carotenoid (mg/g) = (O.D.480 nm) - 0.144(O.D.663 nm) - 0.6308(O.D.645 nm)

4.3.5. Statistical analysis

The two-way ANOVA was performed by using Statistixs software (Version 8.1) for variety (Riogrande) based on bacterial (control or inoculated) and temperature treatments (non-heat and heat stress). Adjustment for multiple comparisons were made using LSD test, keeping significant level at $p \geq 0.05$. The application of biplots correlation analysis was performed on mean values of all variables using XLSTAT 2015.

4.4. Results of field study conducted in National Agriculture Research Centre (year 2018)

4.4.1. Shoot length

Inoculation of heat tolerant plant growth promoting bacteria (PGPB) enhanced shoot length under heat stress and normal condition. The applied PGPB increased shoot length of tomato plant under heat stress and normal condition. Inoculation *of* ***Bacillus safensis*** **(SCAL1)** revealed a maximum significant increase (**56%)** in fresh weight under normal and heat stress **(57.2%)** condition as compared to respective control (C and T2). Shoot length of tomato plant without bacterial inoculation was decreased (26%) under heat stress condition as compared to control condition (Figure 4.1).

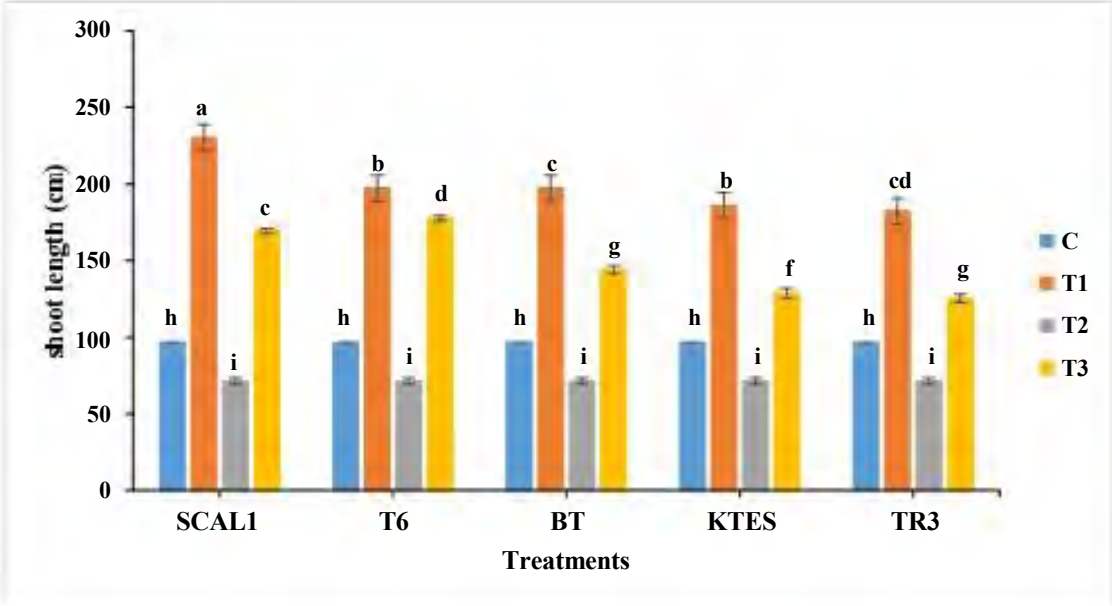

Figure 4.1. Effects of thermo-tolerant strains *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on shoot length of tomato plant variety (Riogrande) under non-heat and heat condition. C: control, T1-Inoculated plants under normal condition, T2-Un-inoculated under heat condition and T3- inoculated plants under heat condition at *p>0.05.*

4.4.2. Root length

Inoculation of heat tolerant plant growth promoting bacteria (PGPB) enhanced root length under heat stress and normal condition. The applied PGPB increased root

length of tomato plant under heat stress and normal condition. Inoculation of ***Bacillus safensis*** **(SCAL1)** revealed the maximum significant increase **(38.2%)** in root length under normal and heat stress condition **(56.5%)** as compared to respective control. The root length of un-inoculated tomato plant was decreased **(44.1%)** under heat stress condition, as shown in Figure 4.2.

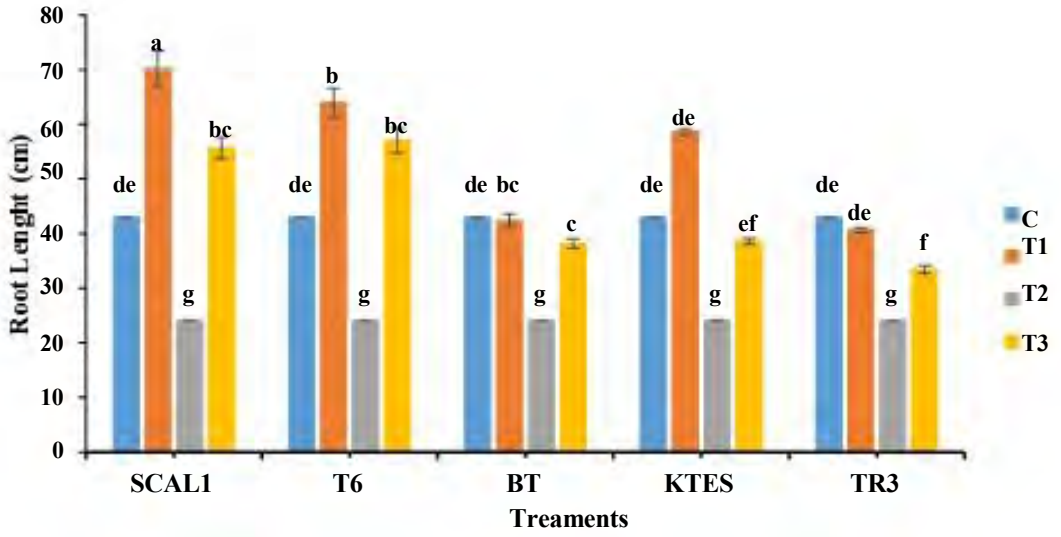

Figure 4.2. Effects of thermo-tolerant strains *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on root length of tomato plant variety (Riogrande) under non-heat and heat condition. C: control, T1- Inoculated plants under normal condition, T2- Un-inoculated under heat condition and T3- inoculated plants under heat condition at $p>0.05$.

4.4.3. Fresh weight

Inoculation of heat tolerant plant growth promoting bacteria (PGPB) enhanced fresh weight under heat stress and normal condition. The applied PGPB, increased fresh weight of tomato plant under heat stress and normal condition. Inoculation of ***Bacillus safensis*** **(SCAL1)** revealed the maximum significant increase **(78.2%)** in fresh weight under normal and heat stress condition (11%) as compared to respective control (C and T2) as shown in Figure 4.3.

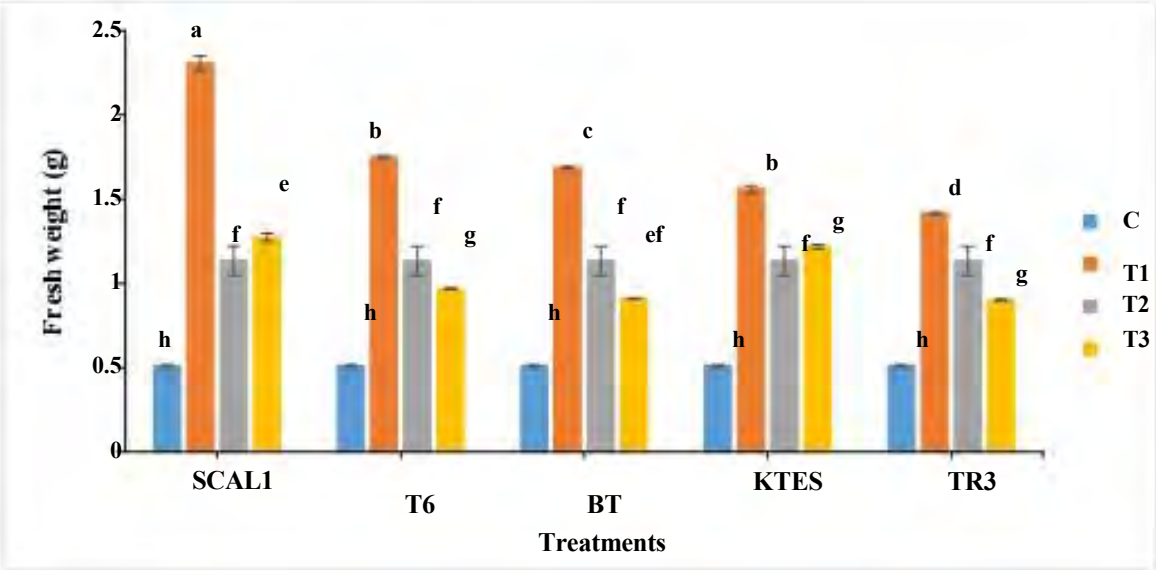

Figure 4.3. Effects of thermo-tolerant strains *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on fresh weight of tomato plant variety (Riogrande) under non-heat and heat condition. C: control, T1- Inoculated plants under normal condition, T2- Un-inoculated under heat condition and T3- inoculated plants under heat condition at $p>0.05$.

4.4.4. Dry weight

Inoculation of heat tolerant plant growth promoting bacteria (PGPB) enhanced dry weight under heat stress and normal condition. The applied PGPB, increased weight of tomato plant under heat stress and normal condition. Inoculation of ***Bacillus safensis*** **(SCAL 1)** revealed maximum significant increase **(32.3%)** in dry weight was observed under normal (T1) and heat stress condition (T3) **(48.8%)** as compared to respective control (C and T2). The dry weight of un-inoculated tomato plant was decreased (42.7%) under heat stress condition as compared to the control condition, as shown in Figure 4.4.

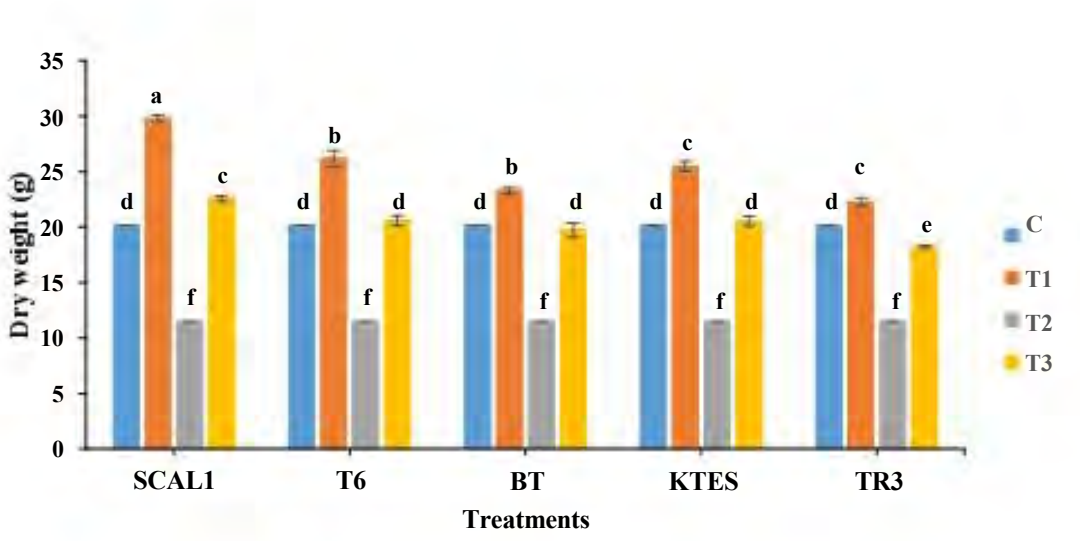

Figure 4.4. Effects of thermo-tolerant strains *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) dry weight of tomato plant variety (Riogrande) under non-heat and heat condition. C: control, T1- Inoculated plants under normal condition, T2- Un-inoculated under heat condition and T3- inoculated plants under heat condition at $p>0.05$.

4.4.5. Number of flowers

Inoculation of heat tolerant plant growth promoting bacteria (PGPB) enhanced flower numbers under heat stress and normal condition. The applied PGPB, increased number of flowers of tomato plant under heat stress and normal condition. Inoculation of ***Bacillus safensis*** **(SCAL1)** exhibited maximum significant increase **(34.3%)** in numbers of flower under normal (T1) and heat stress (T3) condition **(47.05%)** as compared to respective control (C and T2). The flower number of un-inoculated tomato plant was decreased (22.4%) under heat stress condition as shown in Figure 4.5.

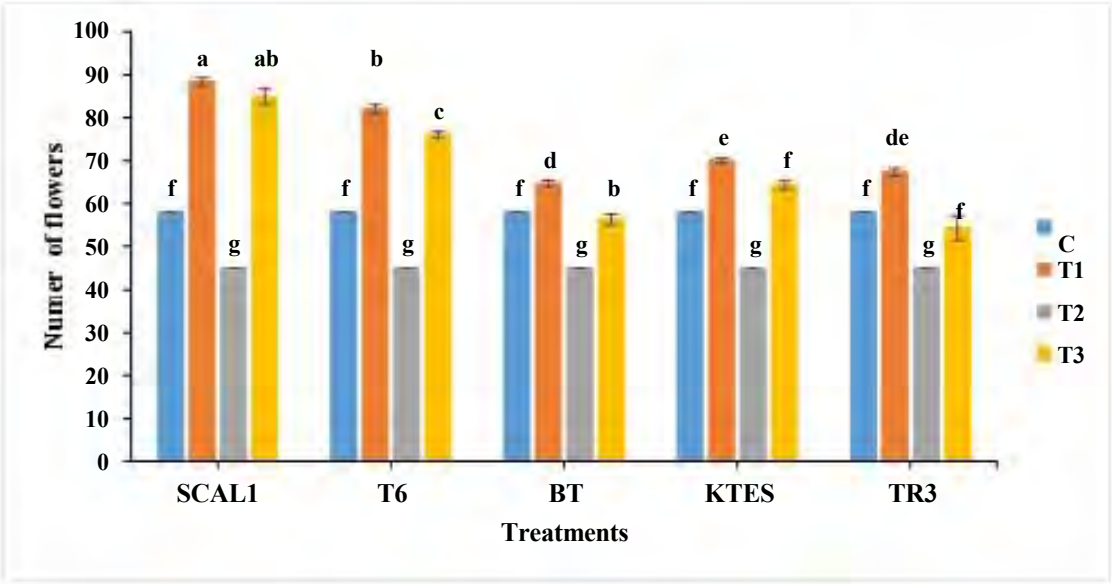

Figure. 4.5. Effects of thermo-tolerant strains *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on number of flowers of tomato plant variety (Riogrande) under non-heat and heat condition. C: control, T1- Inoculated plants under normal condition, T2- Un-inoculated under heat condition and T3- inoculated plants under heat condition at $p>0.05$.

4.4.6. Number of fruits

Inoculation of heat tolerant plant growth promoting bacteria (PGPB) enhanced fruits numbers under heat stress and normal condition. Inoculation of ***Bacillus safensis* (SCAL1),** revealed maximum significant increase **(33.9%)** in numbers of fruits under normal and heat stress **(31.1%)** condition as compared to respective control (C and T2). The numbers of the fruit of un-inoculated tomato plant were decreased (4.59%) under heat stress condition as compared to the normal control condition, as shown in Figure 4.6.

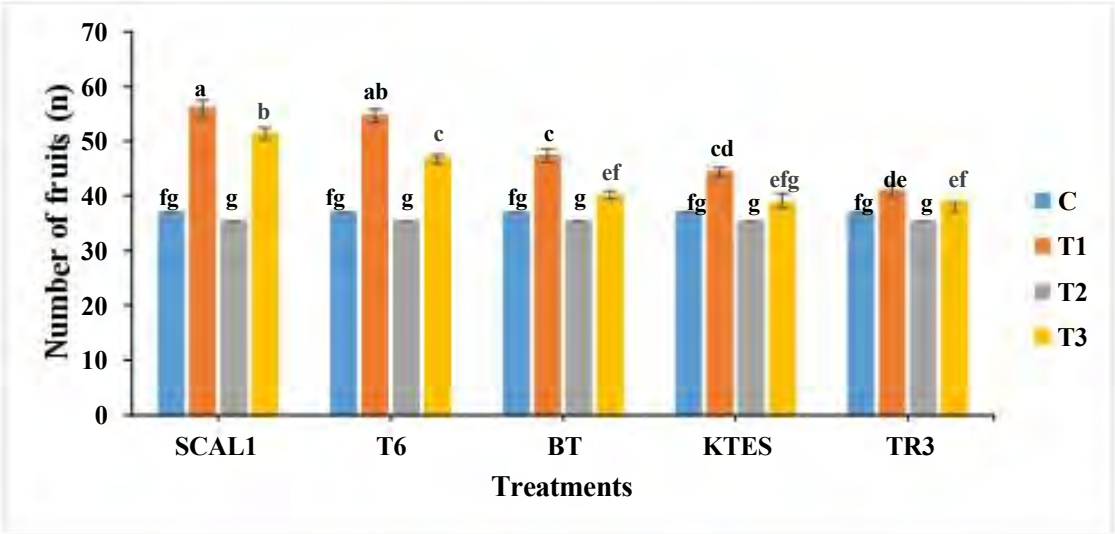

Figure 4.6. Effects of thermo-tolerant strains *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on number of fruits of tomato plant variety (Riogrande) under non-heat and heat condition. C: control, T1- Inoculated plants under normal condition, T2- Un-inoculated under heat condition and T3- inoculated plants under heat condition at *p>0.05.*

4.4.7. Chlorophyll *a*

Inoculation of heat tolerant plant growth promoting bacteria (PGPB) enhanced Chl *a* under heat stress and normal condition. The applied PGPB increased chl *a* content of tomato plant under heat stress and normal condition. Inoculation of ***Bacillus safensis*** **(SCAL1),** revealed a maximum significant increase in chl *a* under normal **(60.5%)** and heat stress **(78.9%)** condition as compared to respective control (C and T2). The chlorophyll *a* content of un-inoculated tomato plant was decreased (62.9%) under heat stress condition as compared to control, as shown in Figure 4.7.

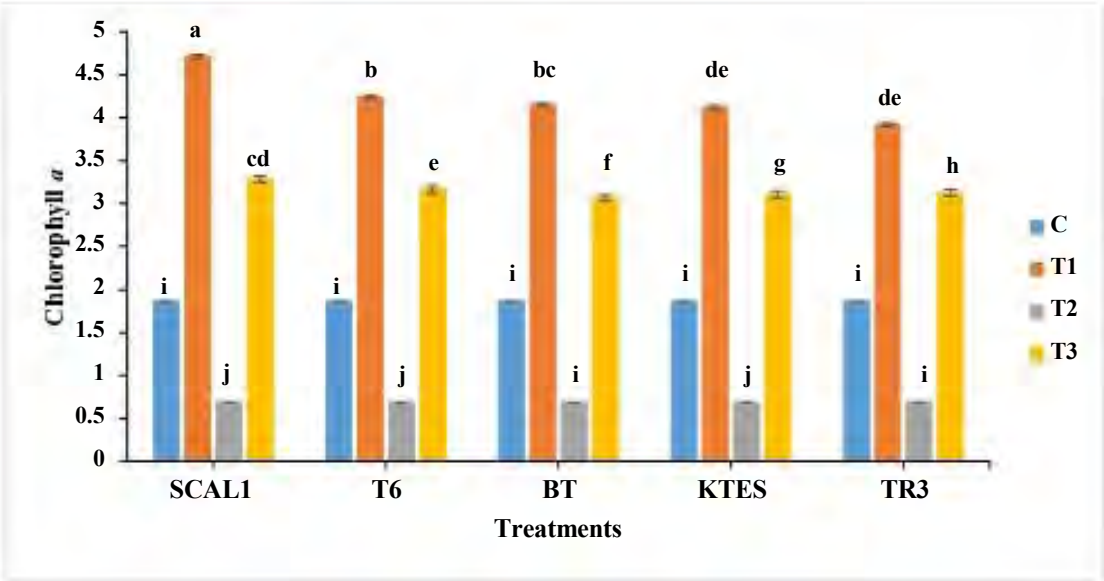
Chlorophyll a
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a
b
bc
de
de
cd
e
f
g
h
i
i
i
i
i
j
j
i
j
i
SCAL1
T6
BT
KTES
TR3
Treatments
C
T1
T2
T3

Figure 4.7. Effects of thermo-tolerant strains, *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on chlorophyll *a* of tomato plant variety (Riogrande) under non-heat and heat condition. C: control, T1- Inoculated plants under normal condition, T2- Un-inoculated under heat condition and T3- inoculated plants under heat conditions at $p>0.05$

4.4.8. Chlorophyll *b*

Inoculation of heat tolerant plant growth promoting bacteria (PGPB) enhanced Chl *b* under heat stress and normal condition. The applied PGPB increased chl *b* content of tomato plant under heat stress and normal condition. Inoculation of ***Bacillus safensis*** **(SCAL1)**, revealed a maximum significant increase in chl *b* **(55.9%)** under normal and heat stress condition **(75.8%)** as compared to respective control (C and T2). The chlorophyll *b* content of un-inoculated tomato plant was decreased (5.35%) under heat stress condition as compared to control (C) as shown in Figure 4.8.

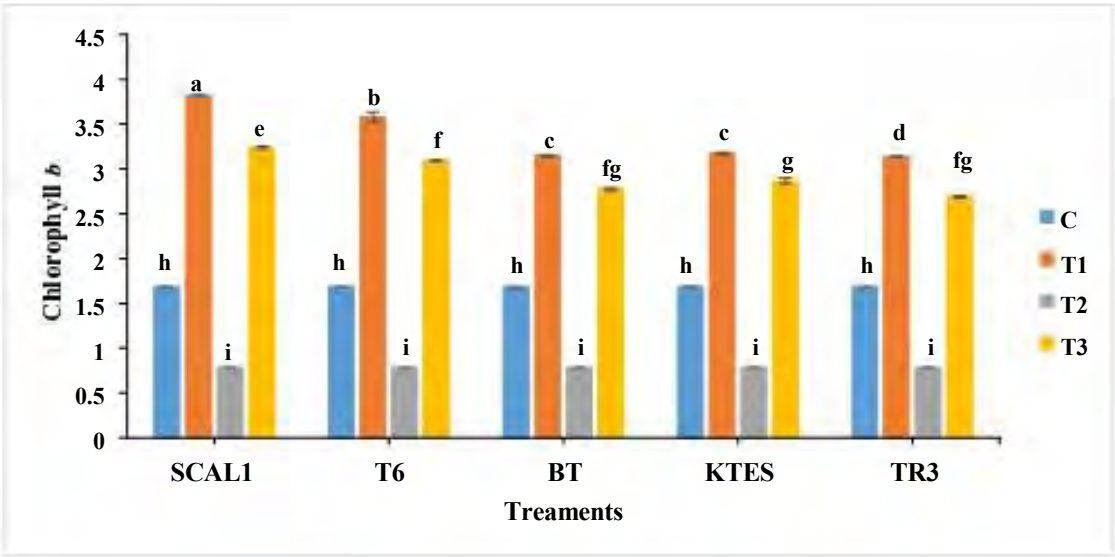

Figure 4.8. Effects of thermo-tolerant strains *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on chlorophyll b of tomato plant variety (Riogrande) under non-heat and heat condition. C: control, T1- Inoculated plants under normal condition, T2- Un-inoculated under heat condition and T3- inoculated plants under heat condition at $p>0.05$

4.4.9. Carotenoid content

Inoculation of heat tolerant plant growth promoting bacteria (PGPB) enhanced carotenoid under heat stress and normal condition. The applied PGPB increased carotenoid content of tomato plant under heat stress and normal condition. Inoculation of ***Bacillus safensis*** **(SCAL1)** bacteria, revealed a maximum significant increase in carotenoid content under normal **(63.5%)** and heat stress condition **(70.8%)** as compared to respective control. The carotenoid content of un-inoculated tomato plant was decreased (82.1%) under heat stress condition as compared to control, as shown in Figure 4.9.

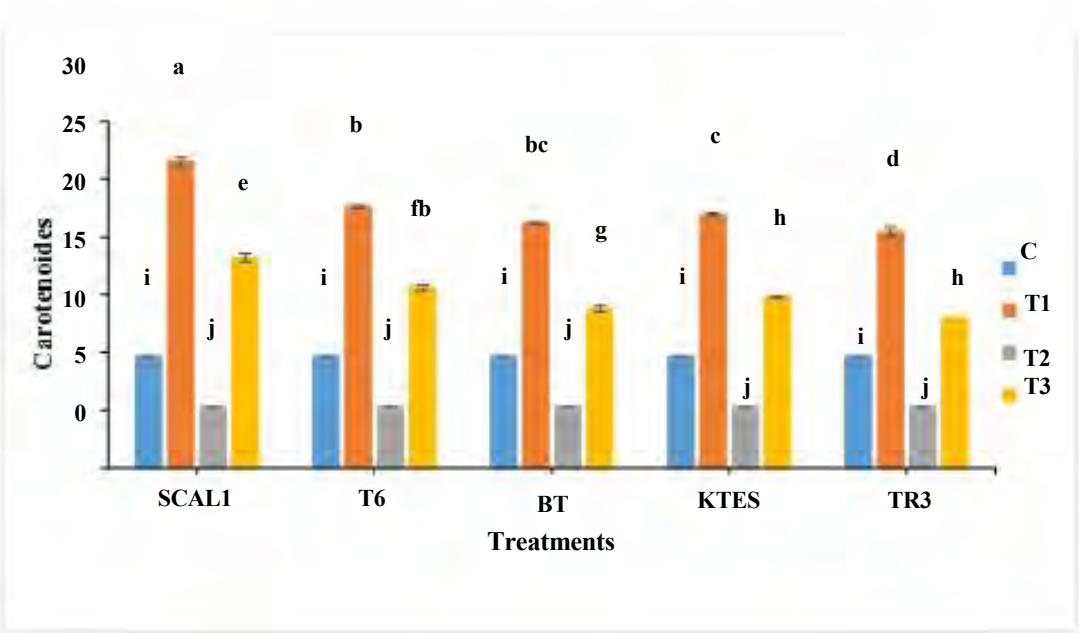

Figure 4.9. Effects of thermo-tolerant strains SCAL1 (*Bacillus safensis*), T6 (*Bacillus safensis*), BT (*Bacillus safensis*), KTES (*Bacillus cereus*) and TR3 (*Klebsiella variicola*) on carotenoids of tomato plant variety (Riogrande) under non-heat and heat condition. C: control, T1- Inoculated plants under normal condition, T2- Un-inoculated under heat condition and T3-inoculated plants under heat condition at $p>0.05$

4.4.10. Pearson correlation analysis of 2018

Pearson correlation bi-plot analysis revealed 89.14% of the total variation between the plotted data F1 and F2. F1 and F2 individually contributed about 77.99 % and 11.14 % variation respectively. Closely present variables in the same quadrant exhibited strong association with each. Furthermore, the correlation between parameters revealed by red dots on the other hand, blue dots demonstrated a correlation between the studied

treatments. Bacterial strains *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) exhibited a strong positive response towards various plant growth parameters under heat stress condition as revealed from principal component analysis. All studied parameters revealed the significantly positive correlation among each other except CNH (control non-heat), CH (control heat), B5H (Bacteria 5 under heat), B3H (Bacteria3 under heat) and B4H (Bacteria 4 under heat) that showed negative correlation as shown in the bi-plot analysis (Figure 4.10).

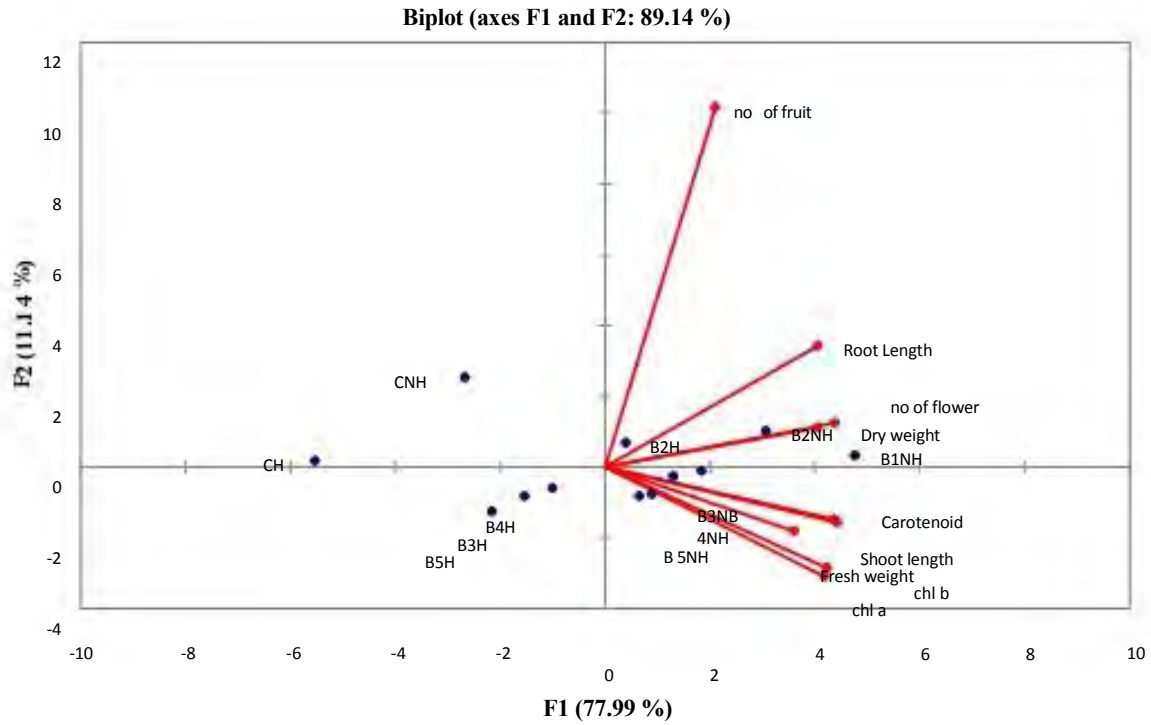

Figure. 4.10. Pearson analysis of field experiment of 2018

4.5. Results of field study conducted in National Agriculture Research centre (year 2019)

4.5.1. Shoot length

Inoculation of heat tolerant plant growth promoting bacteria (PGPB) enhanced shoot length under heat stress and normal condition. The applied, PGPB increased shoot length of tomato plant under normal and heat stress condition. Inoculation of ***Bacillus safensis*** **(SCAL1)** showed a maximum significant increase in shoot length **(57.3%)** under normal condition and heat stress **(59.2%)** as compared to

respective control (C and T2). The shoot length of un-inoculated tomato plant was decreased (27.8%) under heat stress condition as compared to control, as shown in Figure 4.11.

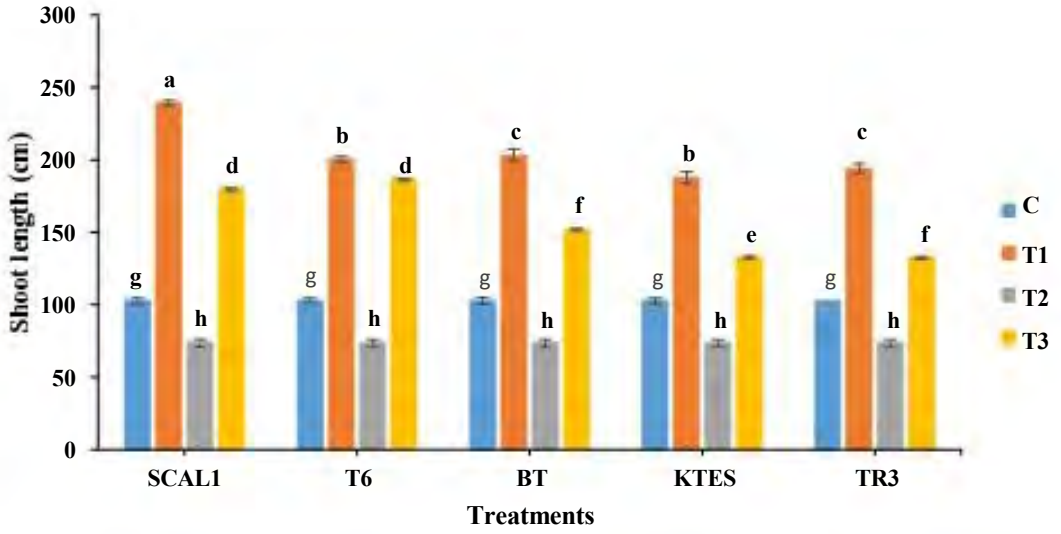

Figure 4.11. Effects of thermo-tolerant strains *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on shoot length of tomato plant variety (Riogrande) under non-heat and heat condition. C: control, T1-Inoculated plants under normal condition, T2- Un-inoculated under heat condition and T3- inoculated plants under heat condition at $p>0.05$

4.5.2. Root length

Inoculation of heat tolerant plant growth promoting bacteria (PGPB) enhanced root length under heat stress and normal condition. The applied PGPB increased root length of tomato plant under normal and heat stress condition. Inoculation of ***Bacillus safensis*** **(SCAL1)** revealed the maximum significant increase in root length **(41.3%)** under normal and heat stress (**60%**) condition as compared to respective control (C and T2). The root length of un-inoculated tomato plant was decreased (44.6%) under heat stress condition **(56.5%)** as compared to control (C) (Figure 4.12).

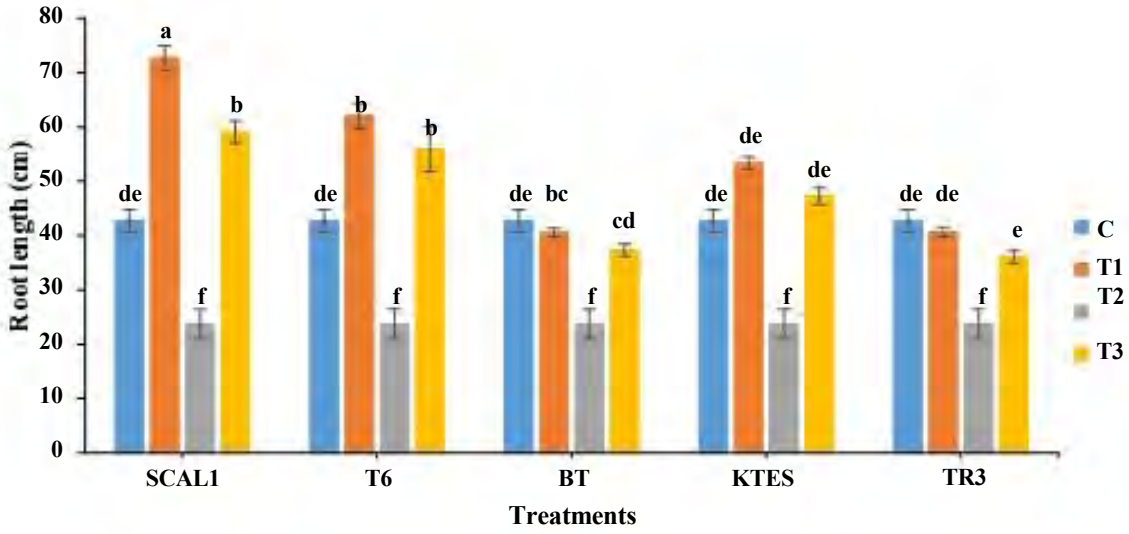

Figure 4.12. Effects of thermo-tolerant strains *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on root length of tomato plant variety (Riogrande) under non-heat and heat condition. C: control, T1- Inoculated plants under normal condition, T2- Un-inoculated under heat condition and T3- inoculated plants under heat condition at *p>0.05.*

3.5.3. Fresh weight

Inoculation of heat tolerant plant growth promoting bacteria (PGPB) enhanced fresh weight under heat stress and normal condition. Applied PGPB increased fresh weight of tomato plant under normal and heat stress condition. Inoculation of ***Bacillus safensis*** **(SCAL1)** represented a maximum significant increase in fresh weight **(77.3%)** under normal and heat stress condition **(16.03%)** as compared to respective control (C and T2) as shown in Figure 4.13.

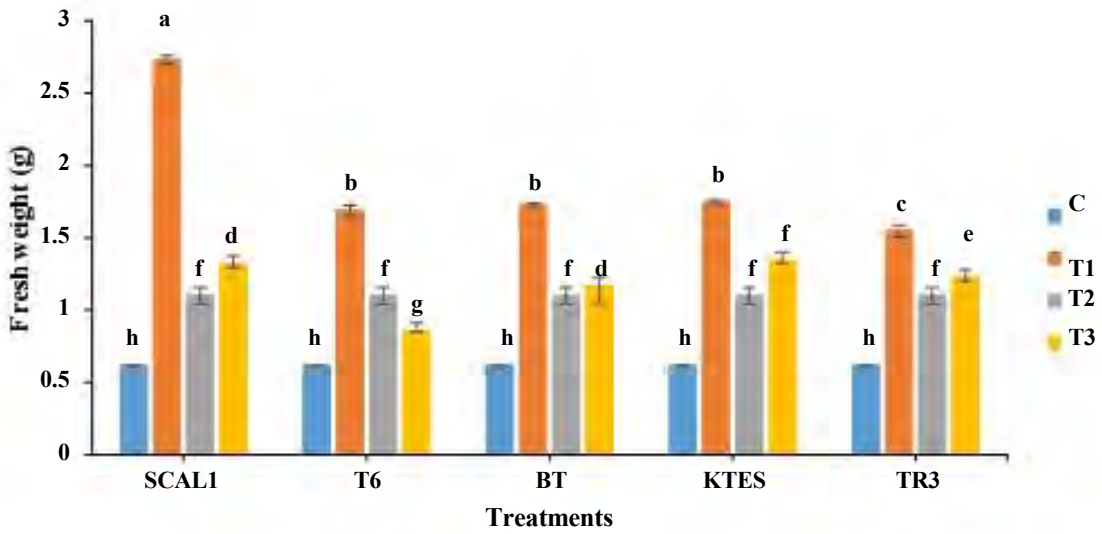

Figure 4.13. Effects of thermo-tolerant strains SCAL1 (*Bacillus safensis*), T6 (*Bacillus safensis*), BT (*Bacillus safensis*), KTES (*Bacillus cereus*) and TR3 (*Klebsiella variicola*) on fresh weight of tomato plant variety (Riogrande) under non-heat and heat condition. C: control, T1- Inoculated plants under normal condition, T2- Un-inoculated under heat condition and T3- inoculated plants under heat condition at $p>0.05$

4.5.4. Dry weight

Inoculation of heat tolerant plant growth promoting bacteria (PGPB) enhanced dry weight under heat stress and normal condition. Applied PGPB increased weight of tomato plant under normal and heat stress condition. Inoculation of ***Bacillus safensis* (SCAL1)** revealed a maximum significant increase in dry weight (**30.5**%) under normal and heat stress condition (**45.9**%) as compared to respective control (C and T2). The dry weight of un-inoculated tomato plant was decreased (46.7%) under heat stress condition as compared to control, (Figure 4.14).

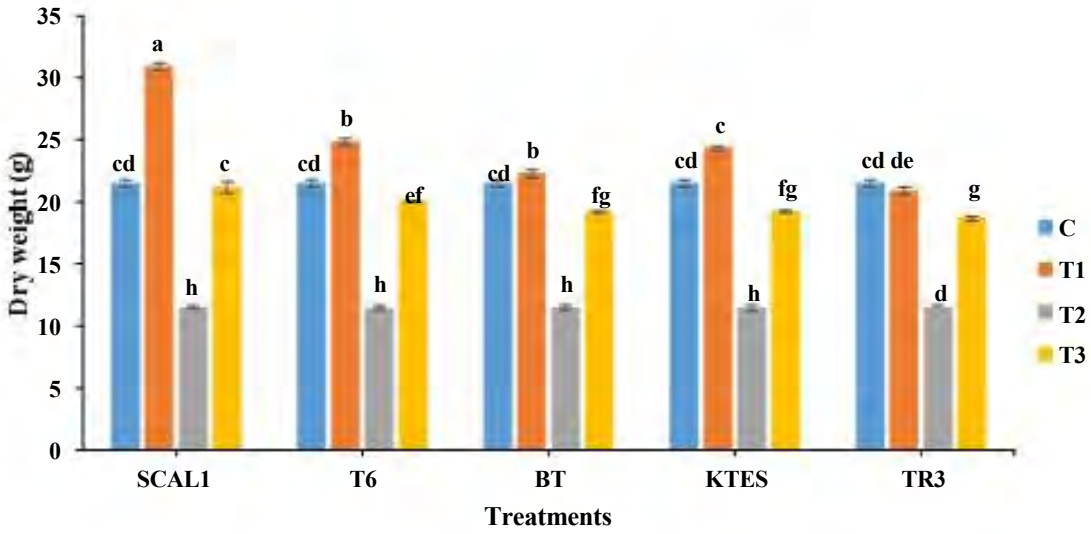

Figure 4.14. Effects of thermo-tolerant strains SCAL1 (*Bacillus safensis*), T6 (*Bacillus safensis*), BT (*Bacillus safensis*), KTES (*Bacillus cereus*) and TR3 (*Klebsiella variicola*) on dry weight of tomato plant variety (Riogrande) under non-heat and heat condition. C: control, T1- Inoculated plants under normal condition, T2- Un-inoculated under heat condition and T3- inoculated plants under heat condition at $p>0.05$

4.5.5. Number of flowers

Inoculation of heat tolerant plant growth promoting bacteria (PGPB) enhanced flower numbers under heat stress and normal condition. The applied PGPB increased the number of flowers of tomato plant under normal and heat stress condition. Inoculation of showed a maximum significant increase in the number of flowers **(36.1%)** under normal and heat stress condition **(44.1%)** as compared to respective control (C and T2). The flower number of un-inoculated tomato plant was decreased (18.8%) under heat stress condition as compared to control (Figure 4.15).

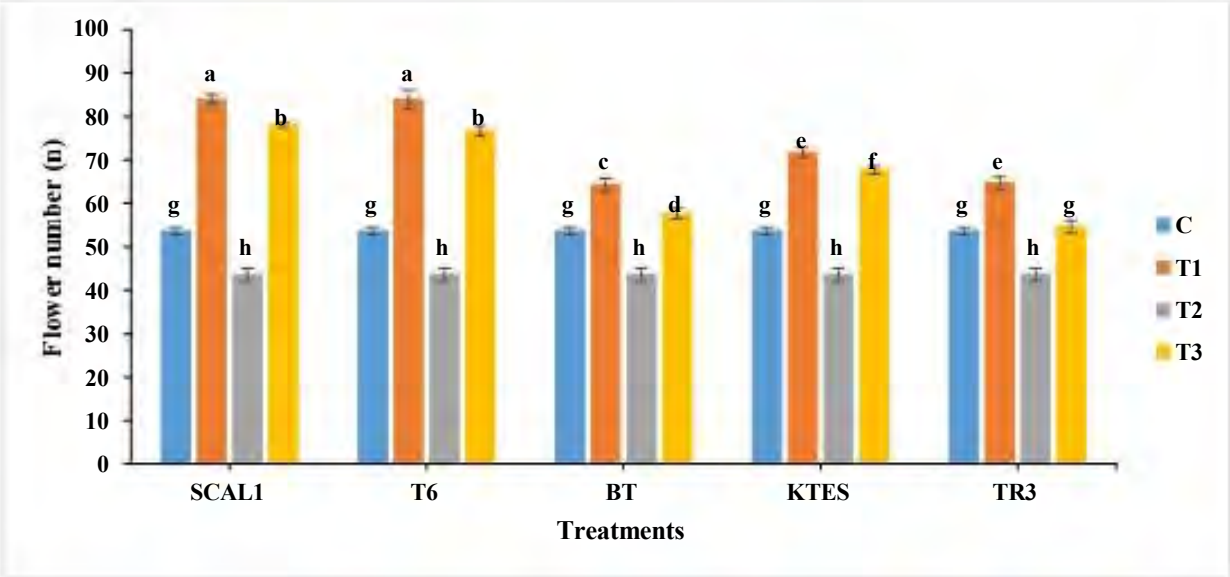

Figure 4.15. Effects of thermo-tolerant strains *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on flower no of tomato plant variety (Riogrande) under non-heat and heat condition. C: control, T1- Inoculated plants under normal condition, T2- Un-inoculated under heat condition and T3- inoculated plants under heat condition at $p>0.05$

4.5.6. Number of fruits

Inoculation of heat tolerant plant growth promoting bacteria (PGPB) enhanced fruit numbers under heat stress and normal condition. The applied PGPB increased the number of fruits of tomato plant under normal and heat stress condition. Inoculation of ***Bacillus safensis*** **(SCAL1)** revealed a maximum significant increase in numbers of fruits **(32.3%)** under normal condition under heat stress condition **(20.9%)** as compared to respective control (C and T2). The numbers of fruits of un-inoculated tomato plant were decreased under heat stress condition, (Figure 4.16, Appendix, plate: 37-46).

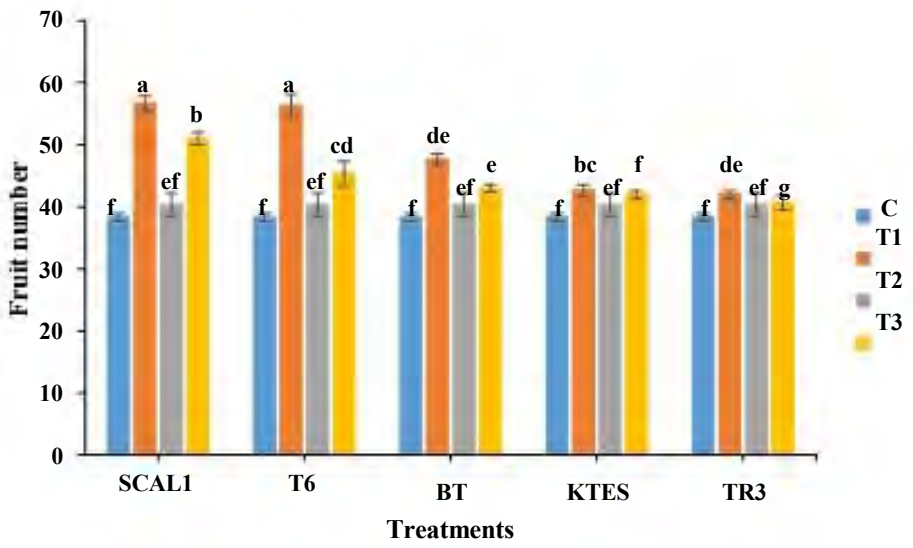

Figure 4.16. Effects of thermo-tolerant strains *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on fruit number of tomato plant variety (Riogrande) under non-heat and heat condition. C: control, T1- Inoculated plants under normal condition, T2- Un-inoculated under heat condition and T3- inoculated plants under heat condition at $p>0.05$.

4.5.7. Chlorophyll *a*

Inoculation of heat tolerant plant growth promoting bacteria (PGPB) enhanced Chl *a* under heat stress and normal condition. The applied PGPB increased chl *a* content of tomato plant under normal and heat stress condition. Inoculation of ***Bacillus safensis* (SCAL1)** revealed a maximum significant increase in chl *a* **(61.1%)** under normal and heat stress condition **(78.7 %)** as compared to respective control (C and T2). The chlorophyll *a* content of tomato plant was decreased (63.3) under heat stress condition, (Figure 4.17).

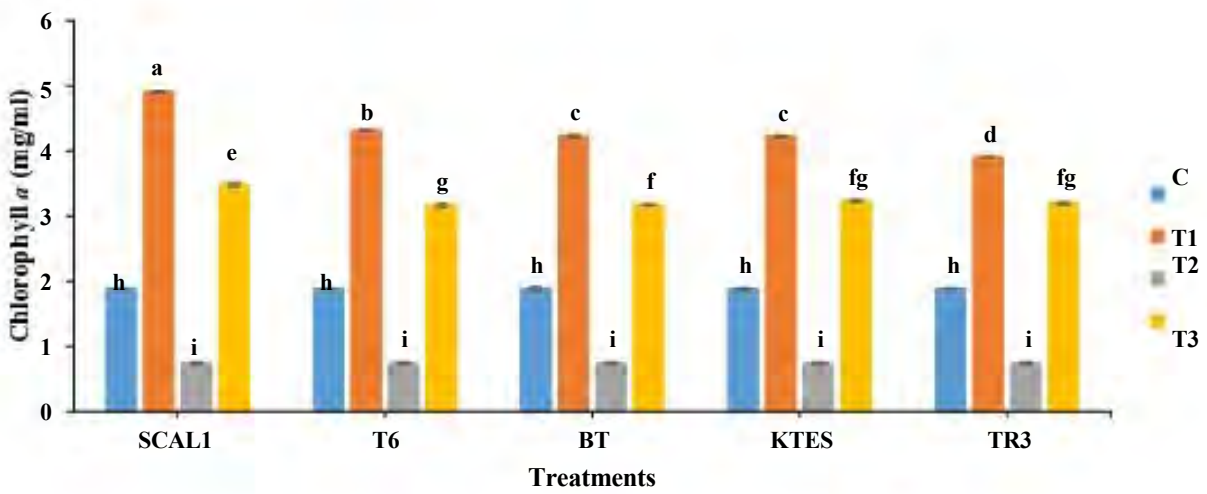

Figure 4.17. Effects of thermo-tolerant strains *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on chlorophyll a of tomato plant variety (Riogrande) under non-heat and heat condition. C: control, T1- Inoculated plants under normal condition, T2- Un-inoculated under heat condition and T3- inoculated plants under heat condition at $p>0.05$.

4.5.8. Chlorophyll *b*

Inoculation of heat tolerant plant growth promoting bacteria (PGPB) enhanced Chl *b* under heat stress and normal condition. Applied PGPB increased chl *b* content of tomato plant under normal and heat stress condition. Inoculation of ***Bacillus safensis* (SCAL1)**, revealed maximum significant increase in chl *b* **(55.7%)** under normal and heat stress condition **(75.3%)** as compared to respective control (C and T2). The chlorophyll *b* content of un-inoculated tomato plant was decreased (51.7) under heat stress condition as compared to control (Figure 4.18).

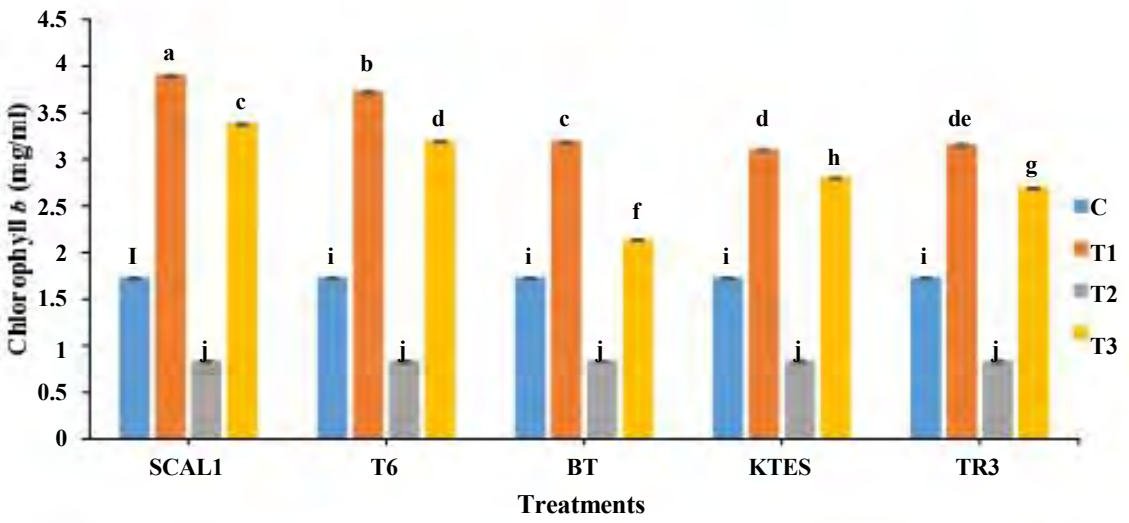

Figure 4.18. Effects of thermo-tolerant strains *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on chlorophyll *b* of tomato plant variety (Riogrande) under non-heat and heat condition. C: control, T1- Inoculated plants under normal condition, T2- un-inoculated under heat condition and T3- inoculated plants under heat condition at $p>0.05$.

4.5.9. Carotenoid content

Inoculation of heat tolerant plant growth promoting bacteria (PGPB) enhanced carotenoid under heat stress and normal condition. Applied PGPB increased carotenoid content of tomato plant under normal and heat stress condition. Inoculation of ***Bacillus safensis*** **(SCAL1)** showed maximum significant increase in carotenoid content **(65.4%)** under normal and heat stress condition **(78.9%)** as compared to respective control. The carotenoid content of un-inoculated tomato plant was decreased (62.6%) under heat stress condition as compared to control (Figure 4.19).

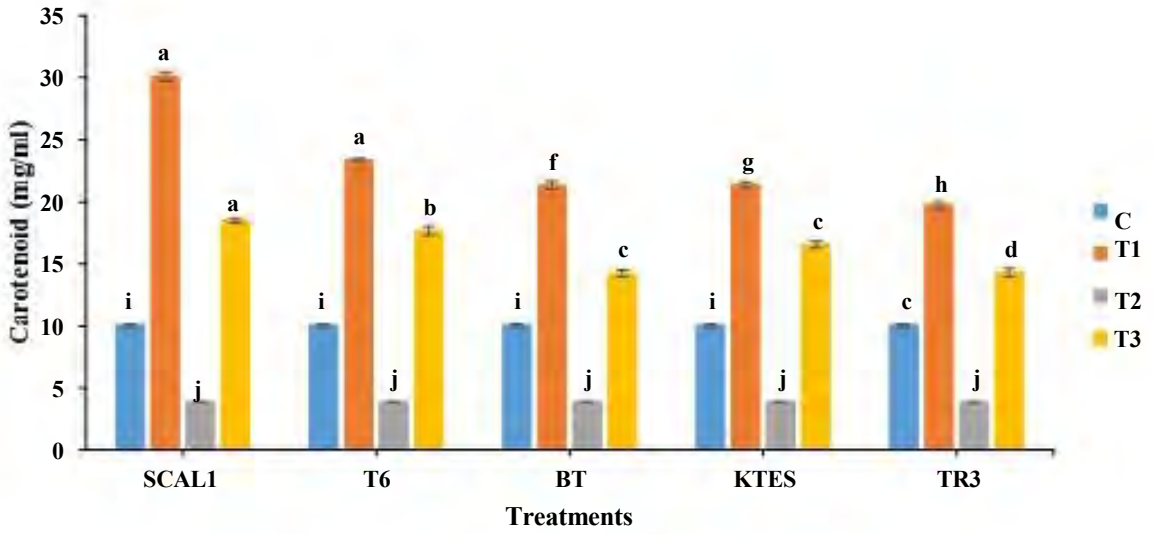

Figure. 4.19. Effects of thermo-tolerant strains *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) on carotenoids of tomato plant variety (Riogrande) under non-heat and heat condition. C: control, T1- Inoculated plants under normal condition, T2- Un-inoculated under heat condition and T3- inoculated plants under heat conditions at $p>0.05$.

4.5.10. Pearson correlation analysis of year 2019

Pearson correlation bi-plot analysis revealed 88.56% of the total variation between the plotted data F1 and F2. F1 and F2 individually contributed about 81.27 % and 7.29 % variation, respectively. Closely present variables in the same quadrant exhibited a strong association with each. Furthermore, the correlation between parameters shown by red dots while blue dots demonstrated a correlation between the studied treatments. Bacterial strains ***Bacillus safensis* (SCAL1)**, ***Bacillus safensis* (T6)**, ***Bacillus safensis* (BT)**, ***Bacillus cereus* (KTES)** and ***Klebsiella variicola* (TR3)** exhibited a strong positive response towards various plant growth parameters under heat stress condition as revealed from principal component analysis All studied parameters revealed the significantly positive correlation among each other CNH (control non-heat), CH (control heat), B5H (bacteria 5 under heat), B3H (bacteria3 under heat) and B4H (bacteria 4 under heat) that showed negative correlation as shown in the bi-plot analysis (Figure 4.20).

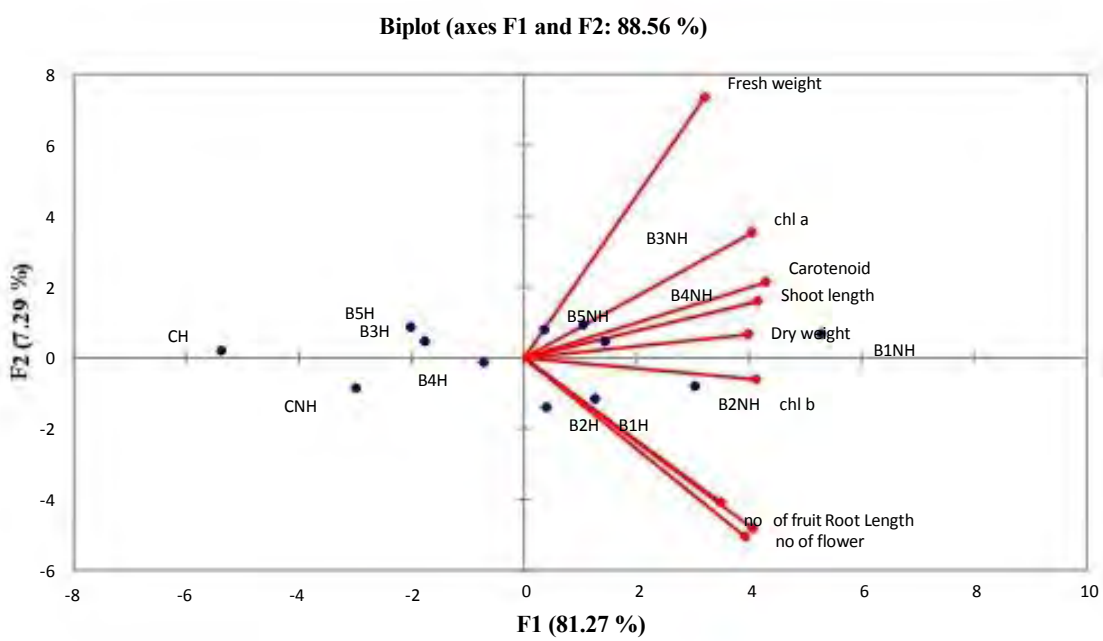

Figure 4.20. Pearson analysis of field experiment of 2019

4.6. Results of district Muzaffargarh experiment

The results of the field experiment conducted in district Muzaffargarh exhibited enhanced fresh weight **(29.01%)** as compared to respective control. The enhanced level of carotenoid **(65.01%)** was observed with the consortia treatment as compared to control. Increased number of flowers **(16.9%)** and fruits **(52.1%)** were also observed in consortia inoculated plants as compared to control (Figure 4.21, Apppendix, Plate-49-50).

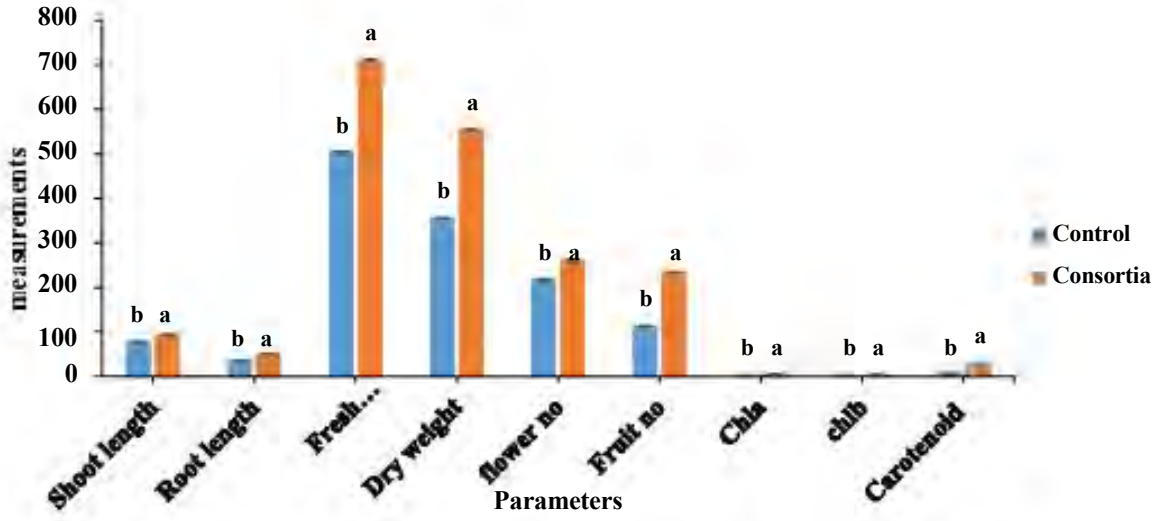

Figure 4.21. Effects of consortia of bacterial strains on the agronomic and biochemical parameters of tomato plants

4.7. Discussion

4.7.1. Field Experiments

A total of three field experiments were conducted at National Agriculture Research Centre, Islamabad, Pakistan (during 2018 and 2019) and districtMuzaffargarh, Punjab, Pakistan (during2019). The detailed discussion of the conducted field experiments is systematically described below.

4.7.1.1. Field experiment at National Agriculture Research Centre (NARC) during 2018

Inoculation of bacterial strains in the current field experiment enhanced all agronomic parameters and chlorophyll contents under heat stress and normal condition as compared to respective control and un-inoculated treatments. Fruit no per plant was enhanced significantly with inoculation of plant growth promoting bacteria (PGPB) under heat stress condition but heat stress caused poor fruit setting and led to less no of fruits in un-inoculated treatments. The temperature in the optimum scale is crucial for tomato fruit set. Studies showed that exposing plants to 3-h periods of temperatures above 104 °F on two successive days may cause fruit set failure (Adam *et al*., 2001). During fruit set the pollen development stage is very sensitive, which occurs about nine days before flowers open and any fluctuation in temperature during these days may lead to poor fruit set (Bertin, 2005). Plant growth promoting bacteria (PGPB**)** increases the vegetative and reproductive growth of plants by providing phosphorus, indole acetic acid and protect the plant from heat stress condition through ACC deaminase enzymes and it has a direct link with tomato yield in the form of enhanced flower and fruit yield (Aini *et al*., 2019). Studied bacterial strains also able to produce IAA, gibberellin and kinetin in significant amount. These plant hormones play important roles in plant growth and development, and can initiate the formation of flowers and fruits, as well as to increase fruit set ratio under heat stress condition (De Jong *et al*., 2009). ***Bacillus safensis* (SCAL1)** showed the maximum number of flower under heat stress (**40.7%)** condition as compared to respective control (T2-un-inoculated under heat stress). ***Bacillus safensis* (SCAL1)** produced the maximum number of fruits under heat condition at the increase of (**8.33%).** When comparing with Aini *et al*. (2019), we also found better trend, as they reported that inoculation of

plant growth promoting bacteria enhanced the flowers and fruit number in tomato plant.

Enhanced shoot and root length of the plant was observed with the inoculation of plant growth promoting bacteria under heat stress and normal condition in contrast with control and un-inoculated plants against heat stress. Our findings are supported with the findings of Ali *et al.,* (2011) as they reported that inoculation of heat tolerant plant growth promoting bacteria increased the shoot and root length.

Plant biomass of tomato was increased with inoculation of heat tolerant plant growth promoting bacteria (PGPB) under heat stress and normal condition as compared to respective control. Results of our experiments were strengthened with the findings of Mukhtar *et al.,* (2020), as they reported that heat tolerant plant growth promoting bacteria enhanced the fresh and dry weight under normal and stressed conditions in comparison with control.

Our results explained that photosynthetic pigments (chl *a*, chl *b* and carotenoid) were significantly increased with the inoculation of under heat stress and normal conditions as compared to respective control. Bacterial inoculation increased leaf surface area that increases the level of photosynthetic pigments and our results are supported with the findings of Afridi et al., (2019) as they documented that bacterial inoculation enhanced the chlorophyll contents under the heat stress condition as compared to control.

4.7.1.2. Field Experiment at National Agriculture Research Centre (NARC) during 2019

Flowering and fruit set has been influenced by high temperatures in tropical and temperate regions. The number of flowers can be reduced with exposure to heat stress as previously reported (Wahid *et al.,* 2007). The results of our current study showed that the number of flowers and fruits was increased with the inoculation of bacterial strains in tomato variety (Riogrande) under heat stress and normal conditions in comparison to variety without bacterial application. he Poor fruit setting has also been associated with low levels of carbohydrates and growth regulators which released in plant sink tissues due to elevated temperature (Kinet *et al.,* 1997). ***Bacillus safensis*** **(SCAL1)** showed the maximum number of flower **(36.1%)** under normal and heat stress **(44.1%)** condition as compared to respective control (C and T2).

Bacillus safensis **(SCAL1)** produced the maximum number of fruits among all bacterial strains under normal **(32.3%)** and under heat (**20.9 %)** condition. Current findings are found in better trend with the results of Aini *et al.,* (2019), as they reported that inoculation of plant growth promoting bacteria enhance the number of flowers and fruit production in the tomato plant.

Heat stress significantly alters physiological and biochemical processes of tomato plants. Reduction in tomato growth under heat stress has also been documented by (Kamara *et al.,* 2003). Inoculation of heat tolerant ACC- deaminase producing plant growth promoting bacteria (PGPB); *Bacillus safensis* (SCAL1, T6 and BT), *Bacillus cereus* (KTES) and *Klebsiella varricola* (TR3) and to tomato variety (Riogrande) significantly reduced the adverse effects of heat stress. Particularly, PGPB application was more effective to plants in stress and normal condition in terms of root and shoot length, fresh and dry weight and chlorophyll contents. Zahir *et al.,*(2008) also documented an increased root length in PGPB inoculated plants that led to efficient nutrient uptake from soil. In another study, tomato plants inoculated with *Klebsiella oxytoca* (10MKR7), *Enterobacter sakazakii* (8MR5) and *Pseudomonas* sp. (4MKS8) exhibited improvement in various agronomic variables including root elongation (Bhattacharyya & Jha, 2012). It has also been reported that PGPB more efficiently confer plant growth stimulation under heat stress than in a normal environment (Rubin *et al.,* 2017).

Chlorophyll content is an indicator of stability under stress conditions. The decline in chlorophyll content (*a*, *b*) under high temperature has been studied by Efeoğlu *et al.* (2009). Reduction in chlorophyll content is an indication of photooxidation (Ajithkumar *et al.,* 2012) as previously reported (Beinsan *et al.,* 2003) Bacterial inoculation considerably improved chlorophyll content (*a*, *b*) in tomato plants under heat stress. Kang *et al.,* (2014) further strengthen our results as they also observed enhanced chlorophyll content in plants with bacterial inoculation.

Carotenoids are non-enzymatic scavengers of reactive oxygen species present in substantial amounts in plants (Jung *et al.,* 2000). High carotenoids content attributed to heat stress tolerance, as they are responsible for the breakdown of singlet oxygen (Chandrasekar *et al.,* 2000). Since carotenoids are found in association with reaction centers (Efeoğlu *et al.,* 2009) and loss of photosynthetic reaction center was

observed in acute abiotic stress, the decline in carotenoids content was expected. Tomato plant inoculated with *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* (BT), *Bacillus cereus* (KTES) and *Klebsiella varricola* (TR3) exhibited positive response in terms of greater carotenoides contents under different water regimes.

4.7.1.3. Field Experiment at District Muzaffargarh (2019)

The number of flowers and fruits significantly enhanced with bacterial inoculation of consortia in tomato plants as compared to control condition. The data showed the maximum number of flower **(16.9%)** under normal and heat stress (**52.1%)** condition as compared to control Current findings are in line with the results of Aini *et al.* (2019), as they reported that inoculation of plant growth promoting bacteria enhance the number of flowers and fruit production in the tomato plant. Increased number of flowers and fruits under heat stress with bacterial inoculation might be due to better chlorophyll content in bacterial treated tomato plants as stated by Chun *et al.,* (2005). Various studies have documented the significantly enhanced health and productivity of different plant species with the application of plant growth promoting bacteria under normal and stressed conditions (Ahemad & Kibret, 2014). Ali *et al.,* (2011) applied *Pseudomonas* spp., *Rhizobium s*pp. and *Agrobacterium* spp. that promoted the growth of young plants and increased the yields under heat stress conditions.

Enhanced root and shoot length of *Solanum lycopersicum* was appeared under heat stress with bacterial inoculation as compared to un-inoculated stressed control. García *et al.,* 2017 reported enhanced root length in *S. lycopersicum* under water stress treated with plant growth promoting bacteria. Jha, Gontia *et al.,* (2012) and Barnawal, Bharti *et al.* (2014) explained that the growth of plant is enhanced by ACC-deaminase producing bacteria under abiotic stress. Application of *Pseudomonas putida* (UW4) to plant under abiotic stress produced ACC deaminase enzyme that improved various important agronomic parameters (Cheng *et al.,* 2007; Singh, 2015). The number of leaves, root length and shoot length increased under abiotic stress due to ACC- deaminase activity (Zhang *et al.,* 2014).

The plant growth promoting bacteria have several influences on plant hormones, improving hormones level in the shoot, enhance growth of the plant and its physiological processes under stress condition (Dodd *et al.,*. 2010). Several

biochemical and physiological damages occurred to plants, when exposed to diverse level of elevated temperature (Mittler 2006).

The existence of ACC- deaminase activity facilitated the chlorophyll content recovery in consortia treated plants and overcome the fatal effects of heat stress through low ethylene production (Nadeem *et al.,* 2017). High level of chlorophyll contents is due to enhanced leaf surface area in bacterial treated plants incontrast to un-inoculated plants against heat stress condition (Mukhtar *et al.,* 2020).

4.8. Conclusion

The outcomes of the current study recommended that seed inoculated with plant growth promoting bacteria (PGPB) have significant potential in promoting tomato plant growth under heat stress in field conditions. Taking into account the negative effects of global warming, the bacteria tested in our study may be promising alternatives for heat tolerance in tomato crop in sustainable agricultural systems. Plant growth promoting bacteria enhanced the flowers and fruit number under heat condition incontrast to un-inoculated plants in the experiment which was conducted at National Agriculture Research Centre, Islamabad, Pakistan during 2018 and 2019. The maximum number of flower (36.1%) and fruits (44.4%) were obtained with bacterial inoculation against heat stress. Applied bacterial consortia not only enhance the agronomic parameters but also enhanced the yield parameters of tomato plants in the field of district Muzffargarh, Punjab, Pakistan.

Conclusion

Overall conclusion

Seventy bacterial strains were isolated, characterized and screened for heat stress tolerance and plant growth promoting activities. Five out of 70 strains were selected on the basis of heat tolerance potential. Plant growth promoting bacterial strains have abilities that linked to plant growth promotion such as IAA, solubilization of phosphate, ammonia production, HCN, siderophores, protease, amylase, pectinase and catalase. These bacterial strains were characterized for ACC-deaminase, exopolysaccharide production and quantification of growth regulators. These selected strains were identified through 16S rRNA gene sequencing as with their closely related species, strain code along with gene bank accession number in brackets i.e. *Bacillus safensis* (SCAL1) (PRJNA286914), *Bacillus safensis* (T6) (MK910213), and *Bacillus safensis* (BT) (MK910212), *Bacillus cereus* (KTES) (MK784894) and *Klebsiella variicola* (TR3) (MK410214). The study provides data of potential plant growth promoting bacterial isolates *Bacillus safensis* (SCAL1), *Bacillus safensis* (T6), *Bacillus safensis* BT), *Bacillus cereus* (KTES) and *Klebsiella variicola* (TR3) for further evaluation under green house and field conditions.

On the basis of 1st study results we proceeded to greenhouse analysis of screened thermotolerant strains. This study investigated the five thermo-tolerant bacterial strains on tomato growth, physiology, antioxidants under greenhouse conditions. The findings of this study also revealed the mitigation of the adverse effects of heat stress. We have observed plants inoculated with strains performed well possibly through homeostasis of physiological machinery. Best performing strain ***Bacillus safensis* (SCAL1)** showed the maximum number of flower under normal and heat stress condition (**55.6% and 75.72%** respectively) and fruits under normal and heat stress **(69.4% and 77.54%** respectively) in variety Sweetie and this strain could be a strong candidate for field trial. The strains performed well in greenhouse condition encouraging enough for further trial in the field condition.

After evidence revealed in greenhouse analysis, the strains were checked for their efficiency under field condition. 1st year in 2018 study was conducted in **NARC, Islamabad, Pakistan**. The maximum number of flower (40.7%) and fruits (31.1%) were obtained with inoculation of plant growth promoting bacteria ***Bacillus safensis* (SCAL1)** under heat stress.

Second year study in 2019 was conducted in the same location of **NARC, Islamabad, Pakistan** and another **climatically important** location of **District Muzffargarh, Punjab, Pakistan.** Results were given respectively as in field of NARC, 2019. Maximum number of flower (44.1%) and fruits (20.9%) were obtained with inoculation of ***Bacillus safensis*** **(SCAL1)** under heat stress.The results were given respectively as in field of District Muzffargarh, 2019. Maximum number of flower (16.9%) and fruits (52.1%) were obtained with consortia against heat stress.

Current findings revealed that inoculated bacterial strains played a significant improvement in the formation of flower and setting of fruit (as important yield parameters for farmer community) which was evident in increased percentage in all field studies. When compared the results were significant enough to be reported as to the best of our knowledge. We tried our best with the purpose to identify potential strains which could be used in mitigation of heat stress under field condition in order to help in increasing challenges due to climate change.

Future perspectives

- The identified promising thermo-tolerant plant growth promoting bacterial strains (*Bacillus safensis* **SCAL1** (PRJNA286914), *Bacillus safensis* **T6** (MK910213), and *Bacillus safensis* **BT** (MK910212), *Bacillus cereus* **KTES** (MK784894) and *Klebsiella variicola* **TR3** (MK410214) will be submitted for patent in different culture collection centres.
- It is proposed that heat tolerant bacterial strains could be amplified for the expression of important heat tolerant genes i.e. dnak and heat shock factor (HSF).
- Expression of heat tolerant genes in plants after inoculation of thermo-tolerant bacterial strains should be checked with RT-PCR.
- These bacterial strains could be evaluated for multi-stress tolerance potential i.e. cold, drought, salt and heavy metals etc.

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Appendix

Plate: 01. Green house experiment in McGill University, Canada

Plate: 02. Greenhouse experiment

Plate: 03. Flowering Stage

Plate: 04. SCAL inoculated plants

Plate 05: Tomato plant with T6 inoculation

Plate 06. Bacterial inoculated tomato plants

Plate: 07. Fruit of inoculated plants under heat condition

Plate: 08. Fruit of inoculated plants under normal condition

Plate. 9: Fruits of Sweetie variety under normal condition with bacteria

Plate 10: Fruits of inoculated plants under normal condition

Plate 11: Fruit ripening of inoculated plant

Plate 12: Comparison of inoculated plant with control under heat

Plate 13: Comparison of inoculated plant with control under heat

Plate 14: Comparison of inoculated plant with control under heat

Plate 15: Comparison of inoculated plant with control under heat

Plate 16: Comparison of inoculated plant with control under heat

Plate 17: Comparison of inoculated plant with control under heat

Plate 18: Plants after heat exposure

Field Experiment conducted at Land Resources Research Institute at NARC, Islamabad during 2018- 2019

Plate 19: Nursery raising of tomato variety Riogrande under control condition

Plate 20: Tomato seedling at the time of transplantation under open field

Plate 21: Growth of tomato plants grown under field condition

Plate 22: Growth of tomato plants grown under field condition

Plate 23: Tomato plants grown under control condition

Plate 24: Inoculated (SCAL1) tomato plants grown under Non-heat stress condition at flowering phase.

Plate 25: Inoculated (KTES) tomato plants grown under Non-heat stress condition at flowering phase.

Plate 26: Inoculated (TR3) tomato plants grown under Non-heat stress condition at flowering phase.

Plate 27: Inoculated (BT) tomato plants grown under Non-heat stress condition at vegetative phase.

Plate 28: Inoculated (T6) tomato plants grown under Non-heat stress condition

Plate 29 Tomato plants grown under heat stress condition

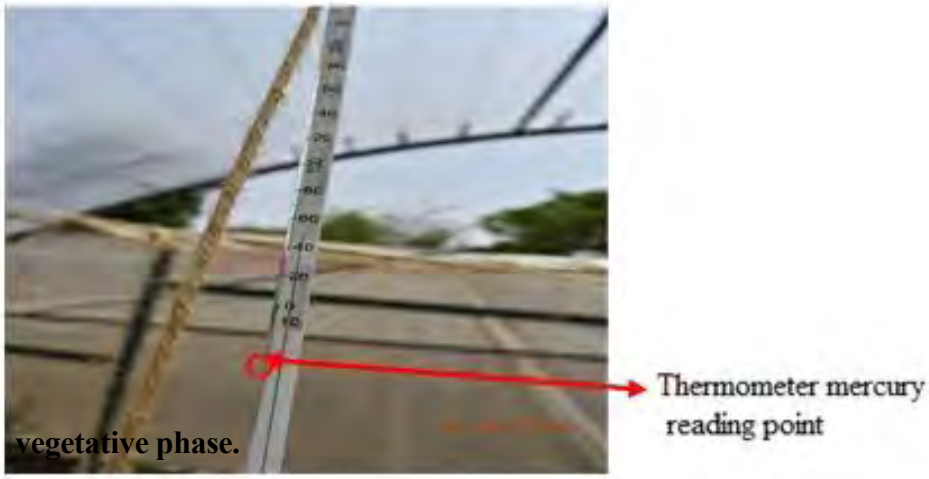
Plate 30: Tomato plants grown under heat stress condition

Plate 31: Inoculated (T6) tomato plants grown under heat stress condition at vegetative phase.

Plate 32: Inoculated (SCAL 1) tomato plants grown under heat stress condition at vegetative phase.

Plate 33: Inoculated (TR3) tomato plants grown under heat stress condition at vegetative phase.

Plate 34: Inoculated (KTES) tomato plants grown under heat stress condition at vegetative phase



vegetative phase.

Plate 35: Thermometer showing temperature measurements under field condition

Plate 36: Inoculated (BT) tomato plants grown under heat stress condition at flowering phase.

Plate 37: Tomato plants grown under control condition at fruiting stage

Plate 38: Inoculated (BT) tomato plants grown under Non-heat stress condition at fruiting phase.

Plate 39: Inoculated (KTES) tomato plants grown under Non-heat stress condition at fruiting phase.

Plate 40: Inoculated (TR3) tomato plants grown under Non-heat stress condition

Plate 41: Inoculated (SCAL1) tomato plants grown under heat stress condition at fruiting phase.

Plate 42: Tomato plants grown under heat stress condition at fruiting stage

Plate 43: Inoculated (T6) tomato plants grown under heat stress condition at fruiting phase.

Plate 44: Inoculated (KTES) tomato plants grown under heat stress condition at fruiting phase.

Plate 45: Inoculated (T6) tomato plants grown under heat stress condition at fruiting phase.

Plate 46: Inoculated (TR3) tomato plants grown under heat stress condition at fruiting phase.

Stock pictures of field experiment conducted at District Muzaffargarh (2019)

Plate 47: Application of the selected consortium to tomato nursery at the time of transplantation on farmer field located in District Muzaffargarh

Plate 48: Transplantation of inoculated tomato nursery in the farmer field at District Muzaffargarh

Plate: 49. Control field

Plate: 50. Consortia field

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Mitigation of Heat Stress in *Solanum lycopersicum* L. by ACC-deaminase and Exopolysaccharide Producing *Bacillus cereus*: Effects on Biochemical Profiling

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Abstract: Soil microorganisms might be assessed for their capabilities of plant growth promotion in order to identify heat tolerant strategies for crop production. The planned study was conducted to determine the potential of heat tolerant plant growth promoting rhizobacteria (PGPR) in mitigating heat stress effects in tomato. *Bacillus cereus* was evaluated for plant growth promoting activities and assessed for 1-aminocyclopropane-1-carboxylate (ACC-deaminase) (0.76–C0.9 μM/mg protein/h), and exopolysaccharide (0.66–C0.91 mg/mL) under normal and heat stressed conditions. Plant growth regulators were evaluated through High Performance Liquid Chromatography. Bacterial inoculation effects on important physiological and biochemical parameters were evaluated under normal and heat stressed conditions in growth chamber. The morphological-physiological traits significantly revealed drastic effects on both of un-inoculated tomato varieties under heat stress conditions. Bacterial augmentation significantly promoted shoot, root length, leaf surface area, fresh and dry weight. Heat stress enhanced extracellular polymeric substances (EPS) production and cleavage of ACC into a-ketobutyrate and ammonia due to ACC-deaminase producing bacteria that significantly reduced the adverse effects of heat on tomato growth. In conclusion, the applied plant growth promoting rhizobacteria (PGPR) bacterial strain proved as potential candidate for improving tomato crop growing under heat stressed conditions. However, it is highly suggested to validate the current results by conducting field trials.

Keywords: ACC-deaminase; heat tolerance; rhizobacteria; PGPR; plant growth regulators; tomato

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AUTHOR CORRECTION

Correction for Mukhtar et al., "Draft Genome Sequence of *Bacillus safensis* SCAL1, an Endophytic Heat-Tolerant Plant Growth-Promoting Bacterium"

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Page 1: The article title should read as given above.

Pages 1 and 2: The bacterial strain should be identified throughout as *Bacillus safensis* rather than *Bacillus pumilus*. In August 2018, NCBI staff performed an average nucleotide identity (ANI) analysis and determined that the organism name should be changed.

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