

Induction of heat tolerance in tomato cultivar with heat tolerant bacteria under field condition

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Abstract

Two planned studies (Isolation, characterization, and field studies) were piloted to assess the potential of heat-tolerant plant growth promoting bacteria (PGPB) to mitigate heat stress in the tomato plant. . The production of indole acetic acid (IAA), phosphate, solubilization and ammonia, as well as siderophores, hydrogen cyanide (HCN), extracellular enzyme activity (protease, amylase, pectinase, and catalase), ACC-deaminase, and exopolysaccharide synthesis, were all positively confirmed in the isolate. The selected strain was screened at high temperature of 60 °C. The promising potential heat-tolerant isolate was further identified through 16S rRNA gene sequencing technique and *Acds* gene was also successfully amplified. Morphological characterization revealed that strain was Gram positive. *B. safensis* (T6) strain showed higher quantity of Gibberellic acid (8.73 µg/ml) and Kinetin (34.8 µg/ml) under heat stress condition. The results of morpho-physiological parameters revealed significant effects of heat on un-inoculated and inoculated tomato plants under high temperature stress. Multi-year/multi-location field trials were done to extend studies of heat mitigation effects after encouraging results by bacterial strains. Plant growth promoting bacteria (PGPB) improved agronomic and yield metrics of tomato plants under heat stress in all field studies. First year 2018 & 2019 field trials at National Agriculture Research Centre, Islamabad, Pakistan showed that *Bacillus safensis* (T6) produced the maximum number of flower's (40.7 %) and fruits (24.8%) in 2018 and number of flowers (43 1%) and fruits (11.03%) in 2019 while the consortia treatments produced maximum flowers (16.9%) and fruits (52.1 %), respectively. The current study comprehensively exhibited the role of PGPB in the mitigation of heat stress.

Keywords: Microorganisms, PGPB, growth regulators, thermo-tolerance

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Introduction

Heat stress is a type of environmental stress defined by the temperature range, severity, and duration of the heat (Grover et al., 2011). Abiotic stress, such as high temperatures, can result in economic yield losses when they exceed a certain threshold (Ruelland and Zachowski, 2010). According to numerous studies and crop modelling methodologies, global temperature is anticipated to rise by the end of the twenty-first century (2081–2100). Even though temperature increase may be beneficial in some areas, crop production declines are probable unless adaptive strategies are used (Bita and Gerats, 2013). Heat stress has a significant impact on plant growth and development due to morphological and physiological changes. The first affected stage during plant growth is the germination stage. Abnormal growth of seedling, vigor, stunted radicle and plumule growth during initial stages are the responses of the plant under heat stress conditions. Sometime under unusual and abrupt temperature rise, the size and area of leaves may reduce, and it may lead to fall of the young leaves. This influences the photosynthetic efficiency of the plant (Greer and Weedon, 2011). High-temperature stress conditions are also indicated by sunburns and blistering of leaves, branches, and stems. Leaf abscission, senescence, stunted roots, shoot growth, fruit destruction, and decreased pigmentation are the most common concerns during heat stress. Heat stress affects almost all tissues of the plant at all stages; however, the reproductive tissues and organs are the most sensitive. Even heat spell of a short duration may result in damage of new floral buds and cause fruit abortion as well. Heat stress adversely affects fertilization process, germination of pollen, tube growth of pollen, viability of ovule, stigma and style positions of the flower, no flower production, poor pollination, the growth of the endosperm and poor fruit set (Grover et al., 2011).

The tomato (*Solanum lycopersicum*) is a member of the Solanaceae family that originated in South America and was introduced to Europe (Bauchet and Causse, 2012). Tomatoes are high in antioxidants such as flavonoids and phenolics, as well as vitamins, minerals, dietary fibers, and carotenoids, which are important nutraceutical molecules. 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 (Noonari et al., 2018). Baluchistan was the largest tomato producing province with production of 200 thousand tones planted an area of 27 thousand hectares, followed by Sindh, Khyber Pakhtunkhwa, and Punjab. Pakistan produces 4.2 million tonnes of tomato annually with a total cultivated area of more than 65 thousand hectares. More than 70% losses in harvesting of cultivated tomato crops have been reported because of hot summer season of many agricultural regions (Fahad et al., 2017).

Today's modern technologies such as genetic, biotechnological, and transgenic technologies have been developed to cope with heat stress issue. The thermo-resistant plants change the heat shock protein level in their body to regulate heat stress issue through heat shock proteins (HSPs). Advanced genetic editing and transformation along with techniques of engineering soluble metabolites like antioxidants and integral expression of HSPs have proved as key tools in conferring heat tolerance in tomato crops. Almost all the currently used heat tolerance solutions are both expensive and site-specific. Furthermore, conferring microbial relationships with plants to minimize abiotic challenges such as high temperature is being used in agriculture as a viable and less expensive strategy. Such microbes are called as plant growth-promoting bacteria (PGPB) that not only mitigate environmental stresses, but also enhance growth and yield of plants (Mukhtar et al., 2020b).

Plant growth is aided by symbiotic rhizo-bacterial strains through a variety of methods, including phosphorus and potassium solubilization, nitrogen fixation, and nodulation, among others. Plant growth promoting strains from a variety of taxa have been found so far, with *Bacillus* and *Pseudomonas* being the most investigated (Ahmad et al., 2018). Besides these stresses, PGPBs have also been tested for their ability to mitigate temperature stress in some other crops (Nadeem et al., 2014). The present investigation was undertaken to assess the ability of thermo-tolerant bacteria to enhance yield of tomato crop including different physiological parameters under both green house as well as field conditions.

Material and Methods

Soil, plants sampling and isolation of rhizobacteria

Soil and plant samples were collected in triplicates 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 4°C for further analyses. The dilution plate method was used for isolation of bacteria from samples (Amna et al., 2019).

Plant growth promoting potential of heat tolerant strains

Indole acetic acid (IAA)

Falcon tubes containing Luria Bertani (LB) broth that was amended with tryptophan (precursor of IAA) were used for the growth of bacterial cultures. Five drops of Kovac's reagent were also added to each tube for confirmation of IAA production (Hussain et al., 2019).

Phosphate solubilization

Phosphate solubilization test was performed following the procedure of Gupta et al. (2012). Sterilized Pikovaskaya's media was poured into the Petri plates. Inoculated plates were placed in an incubator for 7 days at 28°C.

Ammonia production

Peptone water (peptone 10g and NaCl 5g/L, pH 7±0.2) was used to test bacterial isolates for ammonia production (Dinesh et al., 2015). Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 48–72 h at 36±2°C. After that, Nessler's reagent (0.5 ml) was mixed in each tube. Development of brown to yellow color showed positive test for ammonia production.

Hydrogen cyanide (HCN) production

Bacterial isolates were screened out for HCN production by streaking bacteria on plate containing LB agar supplemented with 4.4 g/L glycine. The filter papers were cut in round shape. The dipped filter paper was placed in upper lid of plate. Para film was used to seal the plates to avoid gas discharge and incubated for 5 days at 30°C. Change in color of filter paper from yellow to orange, brown was considered as a positive indication of HCN production (Amna et al., 2019).

Siderophore production

The proposed protocol of Loudon et al. (2011) was implemented for the assay of siderophore production. A loop full of selected isolates was inoculated on

selective media (without iron) amended with CAS-substrate and kept incubated at 30°C for 7 days. The appearance of orange colored halo zone around the colony was an indication of siderophore production.

Extracellular enzyme activities of the isolates

Protease Production

Skim milk agar medium was used to evaluate the protease activity. Media was autoclaved and plates were prepared. Bacterial strains were spot inoculated on skimmed milk media plates and placed in an incubator at 30°C for 2-3 days. Formation of halo zone around bacterial colony was considered as a positive indication of protease production (Ahmed et al., 2016).

Pectinase Production

All 70 isolates were screened for their pectinase producing capability by spot inoculation on agar plates. After inoculation, plates were sealed with parafilm and incubated at 30°C for 2 days. After completion of incubation period plates were flooded with iodine solution, and the formation of halo zone was taken as a positive indication of pectinase production (Tiru et al., 2013).

Amylase production

Bacterial Isolates were tested for amylase production following the method of Ashwini and Gaurav (2011). A loop full of the bacterial colony was spot inoculated in agar plate. Incubation of plates was done for 2 days at 28°C. 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 showed positive result of amylase production

Catalase production

For testing catalase (CAT) enzyme activity, a single bacterial colony from 24hrs old bacterial culture was placed on a clean glass slide and a drop of 30% (H₂O₂) hydrogen peroxide was added upon it. Production of gas bubbles indicated that CAT enzyme is present in the bacteria (Naseem and Bano, 2014).

ACC- Deaminase Activity

The method of Pandey and Gupta (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 (Dworkin and Foster) salt media per liter



supplemented with and without ACC. DF salt media with ammonium sulfate was taken as positive control. The growth on ACC supplemented plates were compared to positive and negative controls after three days of incubation at 28 ± 2 °C and isolates using ACC as a source of nitrogen were selected.

Screening of bacteria for heat tolerance potential

The isolates were screened for their heat tolerance ability against different temperature ranges (32-60°C) on LB solid agar media (Ali et al., 2009).

Growth curve under heat stress

Selected isolates were cultured on LB broth under temperature ranges of 32 - 60°C 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 600nm wavelength after every 24 h up to seven days consecutively. The bacterial isolates having optical density value >0.2 at 600 nm at maximum temperature of 60°C were considered as thermo-tolerant and selected for further studies (Khan et al., 2020).

Morphological Characterization and colony morphology

Fresh colony of each isolate was streaked on LB agar plates and kept in incubator at 28°C for 24 h. The purified isolates were cultured on solid media for the observation of morphological characterization such as colony surface, margins, color, texture as well as shape and elevation, colony color, colony margins, colony surface texture, colony shape (Mehmood et al., 2021)

Gram staining and cell morphology

The purified isolates were further analyzed by Gram staining technique.

Quantitative assay of ACC-deaminase activity

Bacterial isolates were characterized for quantitative estimation of ACC-deaminase activity after qualitative screening. Selected isolate was grown in 5 ml of Tryptic Soy Broth (TSB) under normal and heat stressed condition and centrifuged after 24 h to obtain cell pellets. The pellets were washed with 0.1M Tris-HCl (pH 7.5). These cells were supplemented with DF minimal medium that was supplemented with 3mM ACC. Cells put into incubator shaker for 72 h. In each sample concentration of α -ketobutyrate was analyzed

to measure ACC-deaminase activity (Khan and Khan, 2014).

Characterization through QTS-24 kit

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

Qualitative and quantitative assay of exopolysaccharide (EPS) production

Qualitative and quantitative assessment of exopolysaccharide was done under normal and high temperature conditions by following Mukhtar et al. (2020a). The strain T6 was also checked for exopolysaccharide (EPS) production by culturing on ATCC medium no. 14 and incubated at 32°C for 3 to 4d. Slimy growth of strain was indication of EPS activity. Furthermore, EPS produced by strain PM31 was also quantified

DNA extraction

DNA of all selected bacterial strains was extracted by Phenol-chloroform. (Mukhtar et al., 2020b).

Polymerase Chain Reaction for 16S rRNA genes

Extracted DNA was amplified with 16S rRNA genes given below:

27F: 5-AGAGTTTGATC AC TGGCTCAG-3,

1492R: 5-CGG CTTACCTTGTTACGACTT-3.

These aligned sequences were used for the construction of phylogenetic tree through mega 6.0 software (Mukhtar et al., 2020b).

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'.

Agarose gel Electrophoresis

The PCR products were observed 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 (Mukhtar et al., 2020b).



Evaluation of plant growth regulators under normal and high temperature

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) was inoculated with 24hrs old bacterial cultures and incubated at 30°C on a shaker at 100 rpm for 7 days until OD of cultures became equivalent to 1 at 600nm.

Quantification of growth regulators

Quantification of growth regulators was carried out on HPLC (Agilent 1100) 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 (100µl) 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 280nm wavelength and GA3 and Kinetin at 254nm respectively (Kapoor et al., 2016)

Field experiment

Inoculum preparation

Luria Bertani media was used to make fresh bacterial cultures. Centrifugation of broth was done at 3000 rpm for 10 min. Pellet was collected and dispersed in autoclaved double distilled water (dH₂O), and optical density was set to 10⁹ colony forming unit (CFU) at 660nm. Tomato seeds (Riogrande Variety) was soaked in bacterial strain T6 for 2-4 hours before sowing in pots (Amna et al., 2019).

Experimental design for field condition

A total of three field experiments were conducted at National Agriculture Research Centre (NARC), Islamabad (during 2018 and 2019) and district Muzaffargarh (during 2019) systematically. Studied strain was analyzed in two consecutive years along with two different locations. Field experiments comprised of four treatments i.e. C, control (un-inoculated, 30 °C); T1, plants with bacterial inoculation; T2, plants under heat stress without

inoculation of bacteria; T3, plants under heat stress with inoculation of bacteria. Temperature of control was 30 °C. Heat stress was maintained using a transparent plastic sheet. Remaining all practices including irrigation was done according to production technology of tomato (Chand et al., 2021). Experiment was conducted with three replicates. Field experiment was conducted with completely randomized block design. The tomato plants were harvested after 96 days of sowing.

Agronomical, photosynthetic, and biochemical analyses of plants

Shoot and root length

Length of freshly harvested shoots and roots was measured using measuring tape (Hussain et al., 2019).

Fresh and dry weight of tomato plant

Fresh weight of harvested plants was measured using digital balance. Dry weight was measured after drying the plants in oven at 70°C for 2 days in paper bags. Weight of fully dried plants was measured using digital balance (Hussain et al., 2019).

Number of flowers and fruits

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

Chlorophyll a, b and carotenoid contents

Chlorophyll contents were estimated using 0.1 g leaf material from each treatment. Small pieces of fresh leaves were incubated in 4 mL DMSO₄ (Dimethylsulfoxide) (Muneer et al., 2020a; Muneer et al., 2020b). Absorbance was recorded using spectrophotometer (752 N UV-VIS, Beijing, China). Chlorophyll *a*, chlorophyll *b* and carotenoid contents were calculated by following formulas.

$$\text{Chl } a \text{ (mg/g)} = [1.07 (\text{OD } 663) - 0.09 (\text{OD } 645)]$$

$$\text{Chl } b \text{ (mg/g)} = [1.77 (\text{OD } 645) - 0.280 (\text{OD } 663)]$$

$$\text{Carotenoid (mg/g)} = (\text{O.D.}480 \text{ nm}) - 0.144(\text{O.D.}663 \text{ nm}) - 0.6308(\text{O.D.}645 \text{ nm})$$

Statistical analysis

Analysis of variance was performed using Statistix software (Version 8.1) for both varieties based on bacterial (control or inoculated) and temperature treatments (non-heat and under heat stress). Completely randomized block design was used for current field experiment. Adjustments for multiple comparisons were made using the LSD test, keeping significant level at $p \geq 0.05$. The application of bi-plots



correlation analysis was performed on mean values of all variables using XL-STAT 2015. Shapiro wilk test (Wang and Riffel, 2011) was conducted for checking the normality level field data in current experiment.

Results

Sample collection and isolation

Seventy isolates were obtained from samples. These seventy isolates were purified, preserved and characterized. Most promising isolate was obtained that survived against high temperature and played role in plant growth promotion and extracellular enzyme production.

Table-1: Morphological and biochemical characterization of *Bacillus Safensis* strain T6

Traits	Properties		
Morphological	Temperature range	30-60 °C	
	Colony color	off white	
	Shape	rod	
	Elevation	convex	
	Form	circular	
	Opacity	transparent	
	Margin	lobate	
	Gram staining	Gram Positive	
	Biochemical	ACC deaminase	+
		Phosphorous solubilization	+
IAA		+	
EPS production		+	
HCN		+	
Ammonia		+	
Catalase		+	
Amylase		+	
Pectinase and protease		+	
Tryptophan deaminase		+	
Urea		+	
Indole		+	
Glucose		+	
Sodium citrate		+	
Melibiose		+	
Ornithine decarboxylase		+	
H ₂ S		+	
Maltose		+	
Sucrose		+	
Sorbitol		+	
Inositol		+	
Raffinose		+	
Sodium malonate		-	
Lysine decarboxylase		-	
Gelatin hydrolysis		-	
Ortho nitro phenyl β-D-galactopyranoside		-	
Arginine dihydrolase		-	
Mannitol		-	

Indole acetic acid (IAA) production

Cherry red rings were formed by studied strain (T6) due to IAA production on the top of the test tube (Table.1).

Phosphate solubilization, ammonia and HCN production

Studied strain showed positive result for P-solubilization (Table 1). Ammonia production was confirmed upon formation of brown to yellow color in all 70 strains (Table.1) Bacterial isolate (T6) out of seventy, showed the positive result for HCN production. (Table. 1).

Extracellular enzyme tests

The strain T6 showed a positive response towards extracellular enzymes protease, pectinase, amylase and catalase producing ability (Table. 1).

Morphological observation through compound microscope and naked eye, plant growth promoting activities and biochemical characterization of bacterial strain through QTS-24 kits.

Screening of isolates against heat stress

Only 1 bacterial isolate out of 70 survived till 60°C (Table. 2, Figure.1).

Table-2: Number of heat tolerant bacterial isolates

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

Number of isolates against different temperature



Figure-1: Growth of bacterial isolates on LB solid media against different temperature

Growth curve analysis of selected bacterial isolates

Growth curve analysis of selected bacterial isolates against different temperature ranges for 7 days is shown in Figure 2. The growth curve analysis of bacterial isolate T6 revealed peak growth at 2nd day and started decline at optimum temperature of selected bacterial isolate. Peak growth at 2nd and 3rd day was noted at 50°C and 60°C, respectively, as shown in Figure 2.

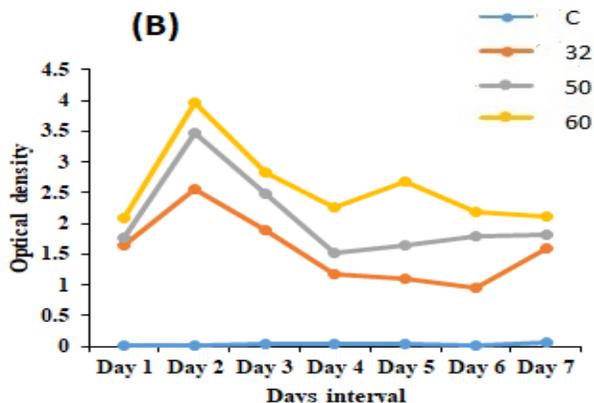


Figure-2: Growth curve analysis of bacterial isolate T6, under different temperature level against control

Morphological characterization

Bacterial isolates showed their characteristics through cell morphology, color, shape and Gram staining reaction (Table 1).

Quantitative assay of ACC-deaminase and exopolysaccharide (EPS) production

Bacterial Isolate T6 produced maximum quantity of enzyme activity (0.95 μM/mg protein/h) under the stress condition. EPS production in isolate T6 was 0.9 and 0.71mg/ml under heat stress and non-heat stress conditions as shown in table.3

Table-3: Quantitative assessment of ACC-deaminase and exopolysaccharides (EPS) in culture of B. safensis T6 with and without heat stress

Strain	Acc-deaminase Activity (μM/mg protein/h)		EPS production (mg/mL)	
	Non-heat	Heat condition	Non-heat	Heat condition
<i>B. safensis</i>	0.83 ± 0.012	0.95 ± 0.011	0.71 ± 0.02	0.9 ± 0.01

Acds gene amplification

The PCR mediated amplification of *acds* gene of

studied strain was performed using a universal set of primers (Figure 3). 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 amplified product was captured using agarose gel electrophoresis.

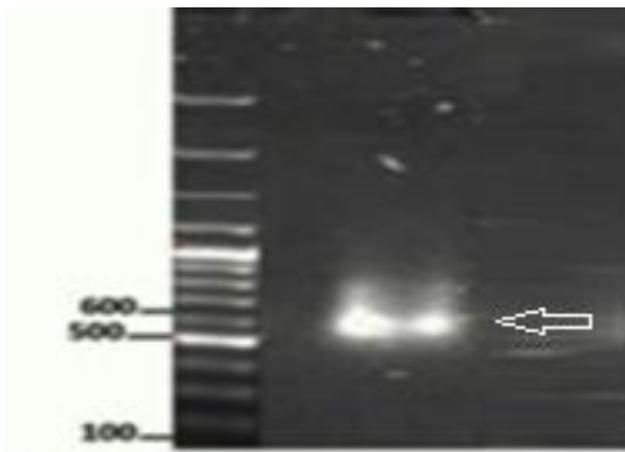


Figure-3: Gel imaging picture of amplification and presence of *acdc* gene in the selected bacterial Isolate (T6,)

Characterization of selected bacterial strain through QTS-24 kit

The selected strain that exhibited positive results for different tests was characterized through microbial identification kit QTS-24 (Table1). Whereas T6 showed positive for all tests done with microbial identification kit.

Identification of selected bacterial strain

Molecular identification of studied isolate (T6) was carried through 16S rRNA gene sequencing. Studied isolate was identified as *Bacillus safensis* (Table.4)

Table-4: Identification of the bacterial isolate by 16S rRNA gene technique.

Sr. No.	Isolates Code	Scientific name	Accession number
1.	T6	<i>Bacillus safensis</i>	MK910213

Agronomic parameters

Analysis of growth parameters

Plant biomass parameters and number of flowers, fruits and leaf surface area were significantly increased with inoculation of *B. safensis* under heat stress (T3) in two field trials at National Agriculture



Research Centre Islamabad, Pakistan during 2018-2019 and in Muzaffargarh, Punjab, Pakistan during 2019. Heat stress reduced plants shoot and root length in Riogrande (tomato variety) and reduced growth, in general, was observed in direct relationship with the increasing level of temperature. Heat stress along with bacterium inoculation demonstrated maximum shoot and root length in Riogrande. Heat stress without bacterium inoculation revealed minimum fresh and dry weight in Riogrande. Heat stress with bacterium inoculation revealed maximum fresh and dry weight in Riogrande. Heat stress without bacterium inoculation showed minimum number of flowers and fruits in Riogrande. Bacterium inoculation revealed maximum

number of flowers and fruits in Riogrande under heat stress (Table.5, 6 and 7).

Effects of *B. safensis* on photosynthetic pigments

Photosynthetic pigments such as Chlorophyll *a*, *b* and carotenoids of Riogrande (tomato variety) was observed in increased concentration in bacterium inoculated treatment under normal conditions (T1) as compared with control treatment (C). Similarly, photosynthetic pigments increased in heat stress with bacterium inoculation treatment (T3) in comparison to heat stress without bacterium inoculation treatment (T2) (Table 5, 6 and 7).

Table-5: Results of field study conducted in National Agriculture Research center (year 2018)

Treatments	Shoot length (cm)	Root length (cm)	Fresh weight (kg)	Dry weight (kg)	No of flowers	No of fruits (n)	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Carotenoid content (mg/g)
Control	96.6± 1.4c	43±2.6dc	0.50± 0.01d	20.1±0.3b	58±0.5c	37±1.15c	1.8±0.01c	1.6±0.02c	9.6±0.03b
T1	197.3±2.3a	64±2.0a	1.74± 0.01a	26.1±0.7a	82±101a	54.6±1.2a	4.2±0.01a	3.5±0.05a	22.6±1.7a
T2	71.3±2.0d	24±1.7d	0.96±0.08c	11.5±0.5c	45±1.1d	35.3±1.4cd	0.9±0.02d	0.7±0.03d	5.3±0.01c
T3	177.6±1.8b	57±2.2b	1.13± 0.03b	20.5±0.4b	76±1.0b	47±1.15b	3.1±0.01b	3±0.005b	15.6±0.3d

Control represents un-inoculated; T1: *Bacillus safensis*; T2: heat stress; T3: *B. safensis*+heat stress

Table-6: Results of field study conducted in National Agriculture Research center (year 2019)

Treatments	Shoot length (cm)	Root length (cm)	Fresh weight (kg)	Dry weight (kg)	No of flowers (n)	No of fruits (n)	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Carotenoid (mg/g)
Control	102.6±2.1c	42.6±2.0dc	0.61±0.02d	21.4±0.28b	53.6±0.88c	38.3±0.6d	1.88±0.04c	1.72±0.01c	10±0.13c
T1	200.3±2.0a	62±2.3a	1.69±0.03a	24.8±0.27a	84.2±2.08a	56.3±1.76a	4.31±0.00a	3.72±0.0a	23.3±0.14a
T2	73.3±2.3d	23.6±2.7d	1.1±0.05b	11.4±0.25d	43.6±1.45d	40.3±1.85c	0.74±0.02d	0.83±0.01d	3.88±0.02d
T3	186.3±2.2b	55.9 ± 4.1b	0.85±0.05c	20±0.05bc	76.6±1.20b	45.3±2.02b	3.1±0.02b	3.19±0.01b	17.6±0.37b

Control represents un-inoculated; T1: *Bacillus safensis*; T2: heat stress; T3: *B. safensis*+heat stress

Table-7: Results of field study conducted in Muzaffargarh (year 2019)

Treatment s	Shoot length (cm)	Root length (cm)	Fresh weight (kg)	Dry weight (kg)	No of flowers	No of fruits	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Carotenoids (mg/g)
Control	96.6± 1.4c	43±2.6c	0.50± 0.01d	20.1±0.3b	58±0.5c	37±1.1fc	1.8±0.01c	1.6±0.02c	9.6±0.03c
T1	197.3±2.3a	64±2.1a	1.74± 0.01a	26.1±0.7a	82±1.1a	54.6±1.2a	4.2±0.01a	3.5±0.05a	22.6±1.73a
T2	71.3±2.0d	24±1.7d	0.96±0.01c	11.5±0.2c	45±1.1d	35.3±1.4d	0.9±0.02d	0.7±0.03d	5.3±0.01d
T3	177.6±1.8b	57±2.2b	1.13± 0.03b	20.5±0.4b	76±1.1b	47±1.15b	3.1±0.01b	3±0.005b	15.6±0.36b

Control represents un-inoculated; T1: *Bacillus safensis*; T2: heat stress; T3: *B. safensis*+heat stress



Pearson correlation analysis of *B. safensis* (T6) in field condition during 2018 and 2019

Treatment graph showed that less variation among the blue dots means that treatments are significantly correlated with each other (Figure. 4. A-c). Pearson correlation bi-plot analysis revealed 97.16 % of the total variation between the plotted data F1 and F2. F1 and F2 individually contributed about 88.2 % and 8.96 % variation, respectively during 2018. Pearson correlation bi-plot analysis revealed 95.5 % of the total variation between the plotted data F1 and F2. F1 and F2 individually contributed about 83.9 % and 11.6 % variation, respectively during 2019. All growth parameters of tomato plants were positively correlated with inoculation of strain T6 under heat stress conditions. Bacterial strain *B. 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 significant positive correlation among growth parameters (Figure.4. b-d).

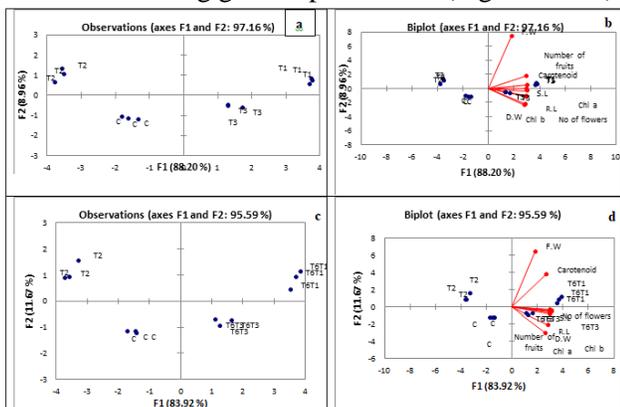


Figure-4: Pearson correlation of interaction of bacteria with plants in field condition. a-c (treatment test) and b-d (Biplot analysis).

Normality test

Field 2018	Shapiro-Wilk		
	Statistic	df	Sig.
Shoot length	0.901	4	0.437
Root Length	0.9	4	0.429
Fresh weight	0.955	4	0.746
Dry weight	0.987	4	0.94
Noor flower	0.981	4	0.91
Noof fruit	0.977	4	0.883
Chl A	0.944	4	0.681
Chlo B	0.986	4	0.938
Carotenoid	0.98	4	0.902

Field 2019	Shapiro-Wilk		
	Statistic	df	Sig.
Shoot length	.954	4	.738
Root Length	.972	4	.853
Fresh weight	.924	4	.558
Dry weight	.940	4	.657
No of flower	.981	4	.910
No of fruit	.977	4	.883
Chlorophyll contents	.961	4	.786

Discussion

Different environmental stresses, particularly heat stress, have a substantial impact on agricultural production, which is becoming increasingly worrying with the passage of time. To overcome heat stress, sophisticated environmentally friendly and cost-effective solutions must be used (Batool et al., 2019; Mukhtar et al., 2020a; Mehmood et al., 2021). The Plant growth promoting bacteria (PGPB) is well reported to improve plant growth as well as and discussed tolerant to different abiotic stresses (Amna et al., 2019; Ali et al., 2021). The inoculation of PGPB resulted in increased agronomic variables such as roots, shoot length, leaf surface area, number of flowers and fruits, fresh and dry weight, and chlorophyll a, b, and carotenoid content in the current experiment. PGPB is a microbial flora found asymptotically in all plant sections that improves nutrient uptake and protects host plants from the negative effects of heat stress (Ali et al., 2018; Mukhtar et al., 2020b; Zainab et al., 2020).

In the recent investigation, enhanced shoot, and root length under normal (T1) and heat stress (T3) compared to control (C) and un-inoculated heat treatment (T2) were observed. Also, Riogrande revealed higher shoot and root length. Our findings are supported by Mehmood et al. (2021) as they obtained enhanced shoot and root length and chlorophyll content with application of bacterial strain *Bacillus subtilis* (MPB 2.1). IAA production aids plant growth as well as the ability to cope with various environmental conditions. Furthermore, under salinity and heat stress, *Klebsiella* sp. (SBP-8) considerably increased plant development. Furthermore, the current findings are consistent with Liu et al. (2013) and Din et al. (2020) who found that cytokinin-producing *Bacillus* spp. are resistant to abiotic stress.

In the present findings, plant biomass (fresh and dry



weight) was enhanced under normal (T1) and heat stress (T3) PGPB inoculation as compared with un-inoculated heat stress (T2) and control (C) treatments. Present results are strongly supported findings of Khan and Khan (2014) as they revealed that Bacterial inoculation enhanced plant growth attributes of wild-type and Got-3 tomato cultivars. Similar trends were documented in the study of Ali et al. (2011). Inoculation of plant growth promoting bacteria reported to decrease the toxic heat stress effects. Similar trend was observed that revealed increased plant growth with inoculation of PGPB under heat stress conditions.

Increased plant biomass of Riogrand variety was observed under heat stress. Similarly, Ali et al. (2009) reported increased plant growth and biomass production under heat stress. Furthermore, shoot, root lengths, fresh and dry weight significantly increased with *Pseudomonas* sp inoculation under heat stress (Chandra et al., 2018).

With PGPB inoculation, increased plant growth and biomass production may be owing to improved plant growth regulators that improve nutrient and water absorption capacity (Ali et al., 2011). In Riogrande, heat stress treatments exhibited increased chlorophyll b levels as compared to control treatments. One reason of increased chlorophyll content may be due to the enhanced photosynthetic activity in PGPB inoculated plants under normal conditions that was decreased in un-inoculated plants in stress conditions.

Fahad and Bano (2012), reported that heat stress significantly declined plant photosynthesis which might be possibly due to alteration in photosynthetic mechanisms balance. Present results are in line with Ali et al. (2018) that showed enhanced chlorophyll content in shoots with inoculation *P. putida*. Increased plant growth and chlorophyll contents were observed in plants inoculated with *Brevibacterium* sp. (FAB3) (Asari et al., 2017, Ahmadi et al., 2015).

A total of three field experiments were conducted at National Agriculture Research Centre, Islamabad, Pakistan (during 2018 and 2019) and district Muzaffargarh, Punjab, Pakistan (during 2019). Inoculation of bacterial strains in current field experiment enhanced all agronomic parameters and chlorophyll contents under normal and heat stress condition as compared to respective control and un-inoculated treatments. Fruit number 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 number of fruits in un-inoculated treatments. Temperature in the optimum scale is crucial for tomato fruit set. Fruit failure occurred in plants exposed to temperature above 104 °F for two days over 2-hour duration.

During fruit set the pollen development stage is very sensitive, that starts approximately nine days before flowering stage. During these days any variation in temperature results in poor fruit development and fruit set. PGPB increases the vegetative and reproductive growth of plants by providing phosphorus, indole acetic acid and protect plant from heat stress condition through ACC deaminase enzymes and it has direct link with tomato yield in the form of enhanced flower and fruit yield (Aini et al., 2019; Maqbool et al., 2021). Studied bacterial strain was also able to produce IAA, gibberellin, and kinetin in significant amount. Under heat stress conditions plants hormones play a vital role in plant growth as well as development, thus start fruit and flower development and finally fruit set (de Jong et al., 2009).

Bacillus safensis (T6) showed the maximum number of flowers under heat stress (40.7%) condition as compared to respective control (T2- un-inoculated under heat stress). The *Bacillus safensis* (T6) 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. Previous study (Wahid et al., 2007) showed that number of flowers decreased upon exposure to heat stress. In comparison to non-inoculated tomato plants, bacterial inoculation enhanced the number of flowers and fruits under heat stress and normal conditions. In addition, increasing temperature and low carbohydrate concentrations, as well as growth regulators, resulted in poor fruit setting. (Mukhtar et al., 2020a). The *Bacillus safensis* (T6) 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* (T6) produced the maximum number of fruits among all bacterial strains under normal (32.3%) and under heat stress (20.9 %) condition similar to the findings of Aini et al. (2019).

Enhanced root and shoot length of plant was showed with the inoculation of plant growth promoting bacteria under normal and heat stress condition in comparison with control and un-inoculated plants under heat stress. Plant biomass of tomato was increased with inoculation of heat tolerant PGPB under normal and heat stress condition as compared to respective control. Results of our experiments were



strengthened with the findings of Mukhtar et al. (2020a). Current results explained that photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoid) were significantly increased with inoculation of *Bacillus safensis* (T6) under heat stress as well as normal conditions in comparison to respective control. Bacterial inoculation increased leaf surface area along with level of photosynthetic pigments and our results are supported with the findings of Afridi et al. (2019). Heat stress significantly alters the physiological and biochemical processes of tomato plants. Reduction in tomato growth under heat stress has also been documented (Kamara et al., 2003). Inoculation of heat tolerant ACC- deaminase producing PGPB; *Bacillus safensis* (T6) 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 chlorophyll, carotenoids contents, shoots, root length, fresh and dry weight. Previous study (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. Chlorophyll content is an indicator of stability under stress conditions. Decline in chlorophyll content (a, b) under high temperature has been studied by Efeoğlu et al. (2009). Reduction in chlorophyll content is indication of photo-oxidation as previously reported (Beinsan et al., 2003)

Bacterial inoculation considerably improved chlorophyll content (a, b) in tomato plants under heat stress. Carotenoids are non-enzymatic scavengers of reactive oxygen species present in substantial amount in plants (Jung et al., 2000). High carotenoids content attributed to heat stress tolerance, as they are responsible for breakdown of singlet oxygen. 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 *B. safensis* (T6) exhibited positive response in terms of greater carotenoids contents under different water regimes. The plant growth promoting bacteria have several influences on plant hormones, improving hormonal level in shoot, enhance growth of plant and its physiological processes under stress condition (Dodd

et al., 2010). The occurrence of ACC- deaminase activity helped in the chlorophyll content recovery in consortia treated plants and overcome the fatal effects of heat stress through low ethylene production (Nadeem and Imran, 2017). In stress conditions, high level of chlorophyll contents was due to enhanced leaf surface area in bacterial treated plants as compared to un-inoculated plants.

Conclusion

Seventy bacterial strains were characterized and tested for heat stress tolerance and plant growth promotion. One strain out of seventy had significant heat stress tolerance and was chosen. These bacteria were tested for ACC-deaminase, exopolysaccharide synthesis, and growth regulator quantification. The strain was tested for efficiency in the field after evidence was revealed in greenhouse study. Maximum number of flower (40.7%) and fruits (31.1%); flowers and fruits (44.1 and 20.9%) flowers (16.9%) and fruits (52.1%) were obtained with inoculation of plant growth promoting bacteria *Bacillus safensis* (T6) under heat stress in the year of 2018, 2019 and Muzfargarh as compared with heat stress un-inoculated. According to recent findings, inoculated bacterial strains significantly improved flower formation and fruit setting (both important yield parameters for farmers), as evidenced by increased percentages in all field studies. When compared the results were significant enough to be reported to the best of our knowledge. To the best of our knowledge in Pakistan this is the first study in which heat stress tolerant bacterial strains was used for mitigation of heat stress under field condition.

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