Thermotolerant PGPR consortium B3P modulates physio-biochemical and molecular machinery for enhanced heat tolerance in maize during early vegetative growth

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Abstract

Global maize productivity has decreased due to sudden temperature fluctuations and heat waves. The current study demonstrates the potential of beneficial bacteria for evaluating plant heat tolerance during early growth. Three *Bacillus* spp. AH-08, AH-67, SH-16, and one *Pseudomonas* spp. SH-29 showed the ability to grow and exhibited multiple plant-beneficial traits up to 45 ± 2°C. In *Bacillus* sp. SH-16 two small heat shock proteins (HSP) of 15 and 30kDa and in SH-16 and AH-67 two large HSP of 65 and 100kDa were upregulated at 45 and 50°C. Plant-inoculation with the consortium B3P was carried out on six maize varieties pre-grown at 25 ± 2 ºC and then applied heat shock at 10-day for 3h at 38ºC, and then 48h at 42ºC. The B3P treatment showed significant improvement in the plant growth parameters and level of catalase, peroxidase, chlorophyll, and carotenoids. The expression of *HSP1* and *HSP18* in Malka and YH-5427 while *HSP70* and *HSP101* were higher in FH-1046 and Gohar as compared to control. The results indicate that PGPR exert multiphasic responses to improve plant growth and heat-tolerance during seedling growth. Further studies will be focused on the field evaluation of this consortium under high heat to evaluate the impact on crop yield.

INTRODUCTION

In natural and agricultural habitats, plants are exposed to continually varying or sudden fluctuations in temperature. Plant growth is severely affected at high temperatures due to stress-induced cell damage, generation and accumulation of reactive oxygen species, protein misfolding (Mathur et al. 2019; Sapkota and Pokhrel 2010; Tariq and Iqbal 2010), and disruption of the photosynthetic machinery (Kajla et al. 2015). Maize (*Zea mays*) is among the major grains highly affected due to increased temperature, where each degree rise in temperature may reduce its production by up to 13% (Izaurralde et al. 2011). The reproductive stage is more sensitive as the anthesis and grain filling in cereals are severely affected by heat (Schauberger et al. 2017) but a temp. of 42 ºC could inhibit both shoot and root growth during early seedling development (Iloh et al. 2014). Maize is suffering from serious heat stress events as a result of climate change which will be more intense, frequent, and longer lasting in the 2nd half of this century causing even larger yield reductions in the future (El-Sappah and Rather 2022). In addition, the climate variability is large enough to offset the impact of the increase in production due to technological improvements in both advanced (China, Brazil, Russia, USA) as well as developing countries (South Asia and Southern Africa) increasing the risks and uncertainties on global food security.

Plants exhibit both basal and acquired thermotolerance which is implemented by the production of molecular chaperones such as heat shock proteins (HSPs), antioxidants, accumulation, and adjustment of compatible solutes (Wahid et al. 2007). Based on these mechanisms, traditional and contemporary molecular breeding, and transgenic strategies have been exploited to increase plant thermotolerance but with limited success mainly because of the complexity, and limitation of genetic resources, phenotypic flexibility, and assimilate partitioning under heat stress (Wahid 2007).

Beneficial microbes are a major constituent of the rhizosphere microbiome that exert a growth-stimulating impact on plants. These plant growth-promoting rhizobacteria (PGPR) directly assist plants by supplying vital nutrients (e.g., nitrogen, phosphorous, iron or zinc, etc.), improved water and nutrient intake by stimulating root proliferation due to the production of phytohormones (e.g., indole acetic acid, cytokinin, and gibberellin). Likewise, they indirectly assist plants by inducing biotic and abiotic stress tolerance due to their antagonistic ability, exopolysaccharide production, and ACC-Deaminase activity (Ahmad et al. 2022; García et al. 2017). The heat-resilient microbes exhibit these plant-beneficial functions at elevated temperatures enabling plants to withstand heat stress (Elshafie et al. 2017; Kaushal and Wani 2016). At present, microbial consortia based on beneficial bacteria are available in the market and applied widely in farmer fields (Imran et al. 2022). By contrast, climate-resilient especially heat-resilient formulations are very little known.

The present study aimed to design a heat-resilient microbial inoculum and to test microbe-mediated heat stress tolerance primarily in maize and then other crops. We hypothesized that multi-stress tolerant PGPR will act smartly to protect against climate stress. The bacteria were isolated from the desert and tested *in vitro* and *in vivo* to stimulate early vegetative growth of maize under high temperatures. The current study indicates that a bacterial consortium B3P based on heat-resilient bacteria have the potential to stimulate early vegetative growth of maize and can be tested further as heat-resilient microbial inoculum in maize and other crops under field condition.

MATERIAL AND METHODS

Site description, sampling, bacterial isolation, and screening for heat tolerance

The rhizosphere soil and plant roots samples were collected from the Cholistan desert and surrounding areas of Ahmadpur and Bahawalpur (Supplementary Fig. 1). The average soil and environmental temperature of the Cholistan desert are high with low rainfall (100 mm), high soil pH (7.5–8.6), and high electric conductivity (EC) (Zia et al. 2021).

Bacteria were isolated by enrichment technique from roots. One gram root (rinsed in distilled water) was macerated in 1 mL saline and 100 mL of macerate was inoculated separately to test tubes containing 5mL LB broth and Nitrogen free malate (NFM) media (Okon and Kapulnik 1986), respectively, in four replicates per root sample. One set of replicate tubes was incubated at 28°C while 2nd set was incubated at 38°C, 3rd set at 45°C and 4th set at 50°C ± 2. From soil samples, 1 g each was serially diluted in saline as described (Somasegaran and Hoben 1994), and 10^{-4} – 10^{-6} dilution was spread on 4-replicate LB agar plates and incubated at different temperatures as was done for roots. The tubes and plates were incubated until the appearance of visible growth in the tubes and colonies on the plates. The cultures from tubes were streaked onto LB/NMF plates for obtaining the pure colonies. Different morphotypes from each root/soil sample were selected and purified by repeated sub-culturing at respective media and temperatures. The bacteria were validated for tolerance by growing them in LB broth with continuous shaking for 3 to 4 days at 28°C, 38°C, 41°C, 43°C, 45°C, 48°C, and 50°C ± 2. The temperature tolerance/survival rate was analyzed by taking optical density at 600nm on a spectrophotometer.

Identification of bacteria and functional characterization

The growth characteristics and colony morphologies were determined as per standard microbial procedure along with the cell size, shape, and mobility. Gram staining was performed as described (Vincent and Humphrey 1970). The catalase activity was observed by putting one drop of 5% H_2O_2 on a glass slide containing a bacterial colony and observing bubble formation within 10–15 seconds which indicated catalase-positive for catalase enzyme. A potassium hydroxide (KOH) test was used to identify the bacteria following (Halebian et al. 1981). A drop of KOH (3%) was dropped on a glass slide and with the help of a 2 mm loop, the bacterial colony was agitated on the slide in a circular motion, the bacterial colony became mucoid and viscous within 20 seconds and stuck to the loop and move upward which was an indication of gram-negative bacteria. The DNA was extracted from bacterial isolates by using CTAB methods (Gomes et al. 2000). The genomic DNA was used to amplify the 1500bp 16S rRNA gene for taxonomic identification by using universal primers P1/P6. The sequencing was done commercially from Macrogen and analysis was done by using Mega 10X and the trimmed sequence was submitted to NCBI. The different carbon source utilization and enzymatic reaction were performed through the QTS-24 kit (DESTO, Karachi) according to the manufacturer protocol as described (Zia et al. 2021)

The production of indole-3-acetic acid was checked using colorimetric methods with some in LB broth containing tryptophan (0.1%) (Ehmann 1977) for 3 to 5 days incubated at 28 ± 2°C, 38 ± 2°C, 45 ± 2°C, and 50 ± 2°C. After incubation, the culture was centrifuged at 10,000 rpm and the supernatant was mixed with Salkowski reagent (1:1) as described by (Gordon and Weber 1951). The 100ppm IAA standard was used as positive control while water was as a negative control. The plate was incubated for 30 minutes at 28 ± 2°C. The results were observed by the development of color from pink to purple and purple to purplish-pink which was an indicator of IAA presence.

The nutrient solubilization was checked at different temperatures. Zinc and Phosphorus solubilization were checked by inoculation of a single bacterial colony on LGI medium containing zinc oxide (Bunt and Rovira 1955), and Pikoviskaya's media containing tri-calcium phosphate (Pikovskaya 1948) and calcite media contained calcium carbonate ($CaCo_3$) as an insoluble calcium source, respectively (Nautiyal et al. 2000). The plates were incubated at 28 ± 2°C, 38 ± 2°C, 45 ± 2°C, and 50 ± 2°C for 4 to 6 days. The development of the halo zone was considered positive for respective nutrient solubilization and the solubilization index was calculated (Pathak et al. 2017).

For salt tolerance, the bacteria were grown on LB media supplemented with 2.5%, 5%, 7.5%, and 10% NaCl and were incubated at 28 ± 2°C, 38 ± 2°C, 45 ± 2°C, and 50 ± 2°C. The ability of bacteria to fix atmospheric nitrogen was screened on a semi-solid Nitrogen free malate medium (NFM) as described (Baldani et al. 2014). The bacterial strains were grown on L.B media for 24 hours at 30°C. After incubation, 20 µL culture was transferred into an Eppendorf tube containing 1mL NFM medium and was incubated at 28 ± 2°C, 38 ± 2°C, 45 ± 2°C, and 50 ± 2°C for 5 to 10 days. The change of color from green to blue and the development of a pellicle-like structure were considered an indication of the fixation of the nitrogen potential of the bacterium (Baldani et al. 2014).

Biofilm formation ability was tested by the crystal violet method at different temperatures (28–45°C) (Pratt and Kolter 1999). The bacteria were grown in 3mL L.B broth for 4 days until biofilm developed. After the formation of the biofilm, the culture was decanted carefully. The attached biofilm was washed with 4 ml distilled water and stained with 0.1% crystal violet and incubated at room temperature for 15 minutes. After incubation, the crystal violet solution was drained out and the biofilm was washed with 4ml of 95% ethanol. The biofilm dissolved in distilled water. The final optical density was checked through a spectrophotometer at OD_{590nm}. Biofilm quantification was done by using the Stepanovic method (Stepanović et al. 2000). Three standard deviations above the mean OD_{590nm} of negative control were defined as the cut-off value (ODC).

The bacteria were cultured in a flask containing L.B broth for 2–4 days with continuous shaking at 38 ± 2ºC. One mL of bacterial culture was spread onto solid ASS agar (Antibiotic sensitivity sulphonamide agar; Merck, Germany) plates [40 g/L, pH 7.4] with the help of a sterilized cotton swab until the culture is completely absorbed into the agar surface. The intrinsic antibiotic resistance pattern was determined by the disc diffusion method as described by (Sarker et al. 2014) using ready-to-use antibiotic discs (Bioanalyse®, Turkey). Antibiotic discs used were ampicillin AM (10 µg), chloramphenicol C (30 µg), aztreonam ATM (30 µg), gentamycin CN (10 µg), rifampicin RA (5 µg), cefixime CFM (5 µg), amikacin AK (30 µg), ciprofloxacin CIP (5 µg), tetracycline TE (30 µg), nalidixic acid NA (30 µg), ofloxacin OFX (5 µg) and erythromycin E (15 µg). Antibiogram was observed after 24–48 h of incubation at 38 ± 2ºC and analyzed using the standard chart (Sarker et al. 2014).

Determination of bacterial total cell protein

Total bacterial soluble protein was determined by using Laemmli buffer (He 2011). The bacterial culture was incubated at 28,45, and 50°C for 15 days. The bacterial culture was centrifuged and dissolved in 200 µL Laemmli buffer and 1ml autoclaved distilled water and then incubated at 95°C for five minutes. The suspension was cooled down to ice and centrifuged at 12000 rpm for 10 minutes. The supernatant containing cell protein was mixed with SDS loading dye and loaded on SDS PAGE gel with a 100kDa ladder to analyze the total cell proteins.

Plant inoculation experiments

Based on the temperature tolerance and PGP trait at high temperatures, 4 bacteria were selected for plant inoculation. Potential best strains from the collection of heat-resilient PGPR were selected for inoculation response on maize (Malka-16, Sahiwal Gold, Gohar-16, FH-1046, YH-5427, FH-1745) under high temperature at the controlled condition.

Development of inoculum-B3P

Bacteria were individually grown in 100 mL L.B broth for two days with continuous shaking at 45°C, then the pre-culture was further inoculated to one liter L.B broth. After two days of growth, the cell pellet was obtained by centrifugation and suspended in 0.85% sterilized saline, centrifuged, and again

resuspended in 100mL dH_2O. The inoculum was mixed in 1:1 and the final OD was adjusted as 10^8 mL^{-1}. The inoculum was mixed @ 30% v/w in the sterilized carrier material (i.e., Filter mud).

Effect of B3P treatment on plant growth under induced heat stress

A total of six maize varieties including two moderately heat-tolerant (YH-5427, FH-1046) and four heat-sensitive (Malka, Sahiwal Gold, Gohar, FH-1745) obtained from Ayyub Agriculture Research Institute (AARI) was used in these experiments. These varieties were selected based on their performance in the AARI field under natural heat stress during previous years (Supplementary Fig. 2). Seeds were washed in 2% (v/v) sodium hypochlorite with shaking for 2 minutes, then it was washed with autoclaved distilled water 3–4 times. After surface sterilization seeds were coated/pelleted with filter mud containing the inoculum as described by Imran et al. (2015).

Pots were filled with 100 g autoclaved peat moss and properly labeled. Coated seeds were sown in the pot (3 seeds/pot). The seeds were germinated at 25–28 ± 2°C at 50–60% humidity. After nine days of seed germination, the pots were placed at 38 ± 2°C for 3 hours for induction of heat stress (HS-1) to seedlings. After 3h incubation, the pots were replaced at 25–28 ± 2°C at 50–60% humidity for the recovery period of two days. After two days, the pots were incubated at 44 ± 2°C for 48 hours for 2nd stress (HS-2). And then shifted back to 25–28 ± 2°C. On day 14 of seed germination, the flag leaves were plucked, placed on white paper, and photographed with a digital camera. Photographs of leaves were further processed in the ImageJ software to measure the leaf area as described by (Abràmoff et al. 2004). The leaves were collected for enzyme and gene expression analysis. Plants were uprooted from peat moss and washed with distilled water. Roots were processed for colonization studies and fresh and dry weight analysis. The effectiveness of bacterial inoculation (B3P) was determined via the following formula:

$$\mathrm{TreatmentEffectiveness} = 100\mathrm{x}\left(\left(\mathrm{B3P} - \mathrm{TreatedPlant} - \mathrm{Controlplant}\right) / \left(\mathrm{non} - \mathrm{treatedplant}\right)\right)$$

Root colonization and plant enzymes analysis

The root colonization was checked under a confocal laser scanning microscope (CLSM) using 20 µL methyl acridine orange dye (0.1 mg/mL) for 10 minutes in the dark by following the protocol of (Harrison et al. 2006). The samples were observed under a confocal laser scanning microscope (CLSM) (Olympus FV 1000, Japan) using an acridine orange specific filter and imaged at 100X magnification. The bacterial population was quantified by intensity estimation using image J software.

For analysis of plant enzyme expression under heat stress, 0.1 g fresh leaf of B3P-treated and non-treated plants were homogenized in 2 mL of sodium phosphate buffer. Three biological replications were processed for enzyme analysis. Leaf samples were taken 24 and 48 hours after HS-2. The leaf samples were crushed in a mortar pestle and centrifuged at 12000 rpm for 15 minutes at 4°C and stored at -20°C till enzyme analysis (Moore and Stein 1954).

The total chlorophyll contents were measured as described (Wellburn 1994). The fresh leaves were homogenized in 80% acetone and centrifuged at 10,000 rpm for 15 minutes, the supernatant was directly transferred into the cuvette, and absorbance was taken at λ663nm for chlorophyll a, λ645nm for chlorophyll b, and λ480nm for carotenoids.

$$\mathit{Chlorophyll\ a}\left({}^{mg}/_{mL}\right) = 12.7A_{663} - 2.69\ A_{645}\mathit{Chlorophyll\ b}\left({}^{mg}/_{mL}\right)$$

$$= 22.9\ A_{645} - 4.68A_{663}$$

$$\mathit{Chlorophyll\ a+b}\left({}^{mg}/_{mL}\right) = \mathit{Chlorophyll\ a} + \mathit{Chlotophyll\ b}$$

For the peroxidase (POD) enzyme, 0.1 mL supernatant of fresh leaves was taken and mixed with the reaction mixture containing 2.7 mL PBS (pH 7.0), 0.1 mL Hydrogen peroxide (H_2O_2), and 0.1 mL guaiacol. The solution was mixed gently, and absorbance was measured at λ_{470nm} for 2 minutes at the interval of 20 seconds (Zia et al. 2021). The solution of PBS, H_2O_2, and guaiacol was used blank. Enzyme activity was determined by the equation below.

$$POD = Totalvolumeofextractionbuffer/totalweightofleaftissue \times 1/volumeofsampleused$$

Catalase (CAT) enzyme activity was determined by using the protocol of (Aebi 1984). The substrate was prepared by mixing 0.15 mL of H_2O_2 with 25 mL of 0.1 M sodium phosphate buffer. Wavelength was set at 240 nm, 1 mL of the substrate, 1.9 mL of distilled water, and 0.1 mL of sample was mixed, and absorbance was recorded at 0 seconds and 1 minute after thoroughly mixing the solution. The mixture of H_2O_2 and sodium phosphate buffer was used as blank. CAT activity was determined by the equation below.

$$CAT = \frac{changeinabsorbance}{min} \times \frac{100}{43.6}g \times weightofsamplepermLofreaction$$

Total amino acids were measured by following the protocol of (Moore and Stein 1954). The 0.5 mL supernatant was added into test tubes containing the 0.5 ml 2% Ninhydrin and 0.5 mL 10% pyridine. Test tubes were incubated at 100°C for 30 minutes and absorbance was taken at λ_{570nm}. The concentration of amino acid was measured through a standard curve of Ninhydrin.

The activity of the Malonaldehyde (MDA) enzyme was calculated by following the protocol of (Gaweł et al. 2004). The 0.25g fresh leaf was ground into 7.5% trichloroacetic acid TCA and centrifuged for 12 minutes at 10,000 rpm. The 0.1 ml supernatant was mixed with 1.5 ml thiobarbituric acid TBA (0.9g TBA in 150 ml of 10% TCA) and mixed gently. The reaction mixture was incubated at 100°C for 30 minutes. Absorbance was measured at λ_{600nm}, λ_{532nm}, and λ_{450nm} on a spectrophotometer.

$$MDA\left(\mu mol/g\right) = 6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{645}$$

Total soluble protein was quantified by using the Bradford method (Bradford 1976). The fresh leaf sample (0.25 g) was homogenized in 5mL chilled sodium phosphate buffer and centrifuged at 12000 rpm for 10 minutes. The 0.3 mL supernatant was mixed with 3 mL of Bradford reagent and mixed gently. The reaction mixture was incubated at room temperature for 10 minutes and absorbance was measured at λ_{595nm} by using a spectrophotometer. Protein concentration was measured through a standard curve by using bovine serum albumin (BSA).

RNA extraction and gene expression analysis

Total RNA was extracted from maize leaves using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Three biological replicates from each treated/non-treated sample were used for extraction. The quality and quantity of RNA were assessed by electrophoresis on 2% agarose gels and by a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). The cDNA was synthesized using the Revert Aid First Strand cDNA Synthesis Kit following the manufacturer's protocol. The SYBR™ Green PCR Master Mix and CFX Maestro Software for Bio-Rad CFX Real-Time PCR Systems were used to measure the gene expression of heat shock proteins. Maize-specific primers for heat shock proteins were designed (Table 2) and synthesized commercially. *Actin* was the housekeeping gene (HSG) used as an endogenous reference. The *HSP1, HSP18, HSP70*, and *HSP101* were the gene of interest (GOI). The qPCR condition was initial denaturation at 94°C for 2 minutes, denaturation at 94°C for 20 seconds, annealing at 54 for 30 seconds, first extension for 30 seconds at 72°C, 35 cycles, final extension for 5 minutes at 72°C, Hold at 8°C. The gene expression analysis was measured by using the following formula (Naqvi et al. 2017)

$$Geneexpressionanalysis = (\%ageefficiencyofGOI)^{\Delta CTofGOI} / (\%ageefficiencyofHSG)^{\Delta CTofHSG}$$

Statistical analysis

The correlation analysis and plant inoculation data were statistically analyzed by one-way and/or two-way ANOVA using the software Statistix 10. The comparison among treatment means was done at a 5% probability level using Tukey's HSD or Fischer's LSD.

RESULTS

Isolation of heat-tolerant bacteria

A large collection of different bacteria/morphotypes (n = 135) were obtained from different soil and root samples during this study on both LB and NFM at different temperatures. Of a total of 135, four bacteria were selected that contained PGP traits as well as high-temperature tolerance. These four bacterial isolated *AH-08, AH-67, SH-16*, and *SH-29* were able to grow up to 50°C and showed plant growth-promoting traits up to 45°C. The morphological, biochemical, and physiological data of these strains and functional characterization i.e., IAA production, nitrogen fixation, phosphorus, zinc, and calcium solubilization are mentioned in Table 1 and Fig. 1.

Table 1
Detailed morphological, biochemical and physiological characteristics of bacterial isolates obtained from desert

Characteristics	*B. subtilis* AH-08	*B. cereus* AH-67	*B. badius* SH-16	*P. koreensis* SH-29	Characteristics	*B. subtilis* AH-08	*B. cereus* AH-67	*B. badius* SH-16	*P. koreensis* SH-29
Isolation from/ medium	Soil/ L. B	Soil/ L. B	Soil/L. B	Soil/L. B	KOH test	-	-	-	-
Colony color / motility	Half white/ highly motile	Cottony White/ highly motile	Light Yellow/ motile	White/ motile	Catalase	+	+	+	+
Colony morphology/size	Wrinkled/ medium	Filamentous/ large	Round/ Large	Wrinkled/ medium	Biofilm	Strong	Intermediate	Week	intermediate
Cell shape	Rod	Rod	Rod	Rod	Temperature tolerance	50°C	50°C	50°C	50°C
16S rRNA accession	MZ779051	MZ779053	ON340744	MZ779057	Salt tolerance	7.5%	2.5%	7.5%	7.5%
Gram staining	positive	Positive	Positive	Negative	Siderophore Production	+	-	-	+
Phosphorus Solubilization	+	+	+	+	Zinc Solubilization	-	+	+	-
Calcium Solubilization	+	+	+	-	IAA Production	++	++	+	+
Antibiotic Resistance									
Gentamycin (10mg)	S	S	S	S	Streptomycin (10mg)	R	R	R	R
Rifampicin (5mg)	R	R	R	R	Ciprofloxacin (5mg)	S	S	S	S
Nalidixic acid (30mg)	MS	MS	MS	MS	Kanamycin (30mg)	R	R	R	R
Ceftriaxone (30mg)	S	S	S	S	Neomycin (10mg)	S	S	S	S
Amikacin (30mg)	R	R	R	R					
Biochemical Characterization									
ONPG	+	+	+	+	SODIUM CITRATE	+	-	-	+
SODIUM MALONATE	-	-	-	+	LYSINE DECORBOXYLASE	+	+	+	+
ARGININE DIHYDROLASE	-	-	-	-	ORNITHINE DECORBOXYLASE	-	-	-	-
H2S PRODUCTION	-	-	-	-	UREA HYDROLYSIS	+	-	+	+
TRYPTOPHANE DEAMINASE	-	-	-	+	INDOLE	-	-	-	-
ACETION	+	+	+	+	GELATIN HYDROLYSIS	+	+	+	+
ACID FROM GLUCOSE	+	+	+	+	NO3/N2 PRODUCTION	+	+	+	-/+
ACID FROM MALTOSE	+	+	+	+	ACID FROM SUCROSE	+	+	+	+
ACID FROM MANNITOL	+	-	+	+	ACID FROM ARABINOSE	+	+	+	+
ACID FROM RHAMNOSE	+	+	+	+	ACID FROM SORBITOL	+	-	+	+
ACID FROM INOSITOL	-	-	+	+	ACID FROM ADONITOL	-	-	-	-
ACID FROM MELIBIOSE	+	+	-	+	ACID FROM RAFFINOSE	-	-	+	-

Table 2: Sequence of primer pairs and their respective product size designed in this study for qPCR analysis of heat induced genes in maize

Gene	Primers Sequence (5 → 3)		Product Size (bp)
HSP1	Forward:	*ATTGCGACCACACCTCACAA*	190
	Reverse:	*GAAGATGTACCAGGGCGAGG*	
HSP18	Forward:	*CGATCCGACATCCGAGAGAT*	340
	Reverse:	*GGTACTTGGCGTCGTCCTC*	
HSP70	Forward:	*CAACGACACACGACAAGCAG*	284
	Reverse:	*TTTGCTAGAGCTTGCCCACA*	
HSP101	Forward:	*CTGAGACGGGGATGAAGTCG*	224
	Reverse:	*TCACGGGCTTATCTACACGC*	
Actin	Forward:	*ATGGCTGACGGTGAGGACATCCAGCC*	205
	Reverse:	*AGGTGAGGATACCCCTCTTGGATTGG*	

Morphological and Functional characters of AH-08

Morphological characterization revealed that *AH-08* is a Gram-positive, rod-shaped bacterium with an off-white gummy, wrinkled, medium-size colony having undulate edges and flat elevation. The bacterium was motile, catalase-positive, and KOH-negative, and identified as *Bacillus subtilis sub sp. spizizenii* (Accession No. MZ779051) based on 16S *rRNA* gene sequence analysis.

The strains *B. subtilis AH-08* showed a P-solubilization zone of 5.00 mm at 6 days post-inoculation (dpi) with solubilized phosphorus 331.07µg/L after 14dpi, 5.03mm for calcium after 6dpi up to 45°C. The bacterium produced 50ppm IAA compared with the control, tolerate 7.5% NaCl and fix nitrogen at 50°C. The strain showed a good root colonization ability.

Morphological and Functional characters of AH-67

The bacterium *AH-67* is a Gram-positive, rod-shaped strain with a large white filamentous colony with entire edges and flat elevation. This is catalase-positive, KOH-negative, and identified as *Bacillus cereus* based on 16S *rRNA* gene sequence analysis (Accession No. MZ779053).

The *AH-67* bacterium solubilized phosphorus 4.00mm after 6dpi with solubilized phosphorus 203.0 µg/L after 14dpi, 4.5mm for calcium, and 2.21mm for zinc after 6dpi up to 45°C. The bacterium produced 100ppm IAA, and tolerate 2.5% NaCl at 38°C at 50°C. The bacterium, not fixed nitrogen at all. The strain showed a good root colonization ability.

Morphological and Functional characters of SH-16

The strain *SH-16 was* a rod-shaped, Gram-positive, pale-yellow bacterium with a round colony, filamentous edges, and flat elevation. It is KOH-negative and catalase-positive and identified as *Bacillus badius* (Accession No. ON340744) based on 16S *rRNA* gene sequencing analysis.

The solubilization indices of *SH-16* strain 3.00mm for phosphorus with 331.0 µg/L after 6 and 14dpi respectively, 4.5 mm for calcium at 6 dpi and fix nitrogen, produced 75 ppm IAA up to 45°C, grow well at 38°C with 7.5% NaCl, form a biofilm with roots. The strain showed a good root colonization ability.

Morphological and Functional characters of SH-29

The bacterium *SH-29* is Gram-negative, rod-shaped, with a white, wrinkled, medium-sized colony having lobate edges and flat elevation. The bacterium was KOH-negative and catalase-positive and identified as *Pseudomonas koreensis* (Accession No. MZ779057) based on 16S *rRNA* gene sequencing analysis.

The *SH-29* bacterium solubilized phosphorus 1.83mm with 140.8 µg/L after 6 and 14dpi, and 1.5mm zinc after 6dpi, producing 75 ppm IAA, and fix nitrogen up to 45°C, tolerates 7.5% NaCl at 50°C. The bacterium formed a weak biofilm on glass tubes as well as on plant roots. The strain showed a good root colonization ability.

Determination of total cell proteins under heat stress

The analysis of whole cell proteins under heat stress indicates that in bacterial strains AH-08, AH-67, and SH-16 many proteins were unregulated at higher temperatures (Fig. 5). The heat shock protein upregulated includes 100, 90, 75, 70, 40, 45, and 18 kDa.

Inoculation effect on early growth of maize under induced heat stress

The data from the pot experiment collected for different plant-growth traits showed significant differences with B3P-treated under heat stress compared to non-treated plants both in heat-tolerant and heat-sensitive maize varieties.

Shoot and root length

Data shows that the shoot length of B3P-treated plants was generally higher in all maize varieties compared to their respective non-inoculated plants under heat stress (Fig. 3A). Plant height was maximum under stress in the B3P-treated Malka variety which is a heat-sensitive variety. A maximum percent increase in shoot length was observed in FH-1046 (63%) followed by Malka (25.66%). Data regarding root length also showed a strong positive impact of B3P treatment on all maize varieties under heat stress (Fig. 3B). A maximum percent increase in root length was observed in treated plants of varieties Gohar and FH-1046 (89%) followed by Sahiwal Gold (51%).

Shoot and root weight

Plant fresh weight (both shoot and root; Fig. 3C, D) was generally higher in all B3P-treated varieties with the highest fresh weight in Malka showing a 73% increase over respective non-inoculated control. While maximum percentage increase (treatment effectiveness) was observed in variety FH-1046 which exhibited an 84% increase in shoot fresh weight after inoculation. For root fresh weight, B3P treatment was most effective in the Sahiwal Gold variety showing an increase of 88% over control. Both shoot and root dry weights (Fig. 3E, F) followed almost the similar trend as mentioned for fresh weight. The maximum percentage increase (over control) in shoot dry weight was 92% in Sahiwal Gold while in root dry weight was 109% in YH-1745.

Leaf and root area

The data show that B3P inoculation significantly increased leaf length, leaf area, and root area in all varieties (Fig. 4). Leaf area and length were maximum in the Gohar variety showing an increase of 95% and 62% over respective non-inoculated control (Fig. 4A). The leaf area of B3P-treated varieties of Sahiwal Gold showed a non-significant response compared to the non-treated control (Fig. 4A). The minimum increase in flag leaf length was observed in Sahiwal Gold after inoculation with B3P under heat stress (Fig. 4B). Leaf chlorophyll contents (measured through SPAD) and chlorophyll a/b were generally higher in B3P-treated leaves compared to non-treated leaves in all varieties (Fig. 4C, D). A significant increase in chlorophyll contents (especially chl b) was observed for B3P-treated Gohar and Sahiwal Gold varieties under stress conditions (Fig. 4D). The data further revealed that B3P-treated plants had significantly well-developed roots (Fig. 4E) compared to non-treated control plants. A maximum increase (100%) was observed in Gohar followed by FH-1046 (42%). Maize variety Malka, however, exhibited a non-significant response of inoculation on root area parameters.

Analysis of heat shock gene expression

Quantitative PCR analysis was performed on RNA extracted from B3P-treated and non-treated plants after 48 hours of heat stress induction to see the expression of genes related to heat shock proteins. The qPCR data related to four genes (*HSP1, HSP18, HSP70*, *HSP101*) show a differential response of varieties between B3P-treated and non-treated plants. In the case of *HSP1*, all B3P-treated plants showed higher expression levels as compared to non-treated ones, but Gohar showed the highest expression of *HSP1* whereas, FH-10446 showed a maximum percentage increase over non-treated control (Fig. 5A). Similarly, B3P treatment increased the expression of *HSP18* in all varieties after heat stress compared with non-treated plants, where maximum expression was observed in variety Malka followed by YH-5427 (Fig. 5B). A similar expression response was observed for *HSP70*, where mostly B3P-treated plants showed increased expression compared to non-treated plants (Fig. 5C). Maximum expression was observed in Malka, followed by YH-5427 and FH-1046. On contrary, the expression of *HSP101*, was maximum in B3P-treated FH-1046 followed by Gohar and Sahiwal Gold while other varieties showed a relatively lower expression (Fig. 5D).

Antioxidant enzyme activities, total proteins, and amino acids under heat stress

Plant enzyme activities were analyzed 24 and 48h after HS-2. In general, the activities of antioxidant enzymes CAT and POD increased while MDA increased after B3P-inoculation under stress. Malonaldehyde activities at 24HPI (24 hours post induction of HS-2) and 48HPI were significantly reduced in B3P-treated plants as compared to non-treated plants. MDA activity was reduced by up to 42% in the YH-5427 variety after B3P treatment followed by a significant reduction in varieties Gohar, Malka, and YH-1745. The highest MDA activity was observed in non-treated plants of variety YH-175 (Fig. 6A).

Peroxidase activity was lower in all varieties during early hours after stress i.e., 24HPI. As time passed, POD activities increased significantly (Fig. 6B) in most of the plants (both treated and non-treated). A maximum increase (635%) in POD activity was observed in B3P-treated plants of variety FH1046, followed by YH-5427 (96%). POD activity in varieties Malk and Gohar also increased over respective non-inoculated controls, but the increase was statistically non-significant.

Catalase activity (CAT) started during the early hours of stress in all varieties except FH-1046 (Fig. 6C). The activity increased significantly afterward (both treated and non-treated). Maximum CAT activity was observed in B3P-treated variety YH-1745 with a percent increase of 635%, followed by FH-1046 (96%). CAT activity in varieties Malk and Gohar also increased over respective non-inoculated controls, but the increase was statistically non-significant.

Total cell proteins at 24HPI were initially higher in all B3P-treated plants, whereas YH-1745, Sahiwal Gold, and Malka varieties showed higher total cell proteins in plants (Fig. 6D). Protein concentration increased gradually with time after heat stress and significantly higher total cell proteins were observed in all B3P-treated varieties compared to respective non-treated control plants. Comparatively, protein contents were higher for Gohar, Sahiwal Gold, YH-1745, FH-1046, and YH-5247.

Total amino acids did not change significantly with or without B3P treatment after the heat stress (Fig. 6E). During initial hours (24HPI), the amino acids were maximum in YH-1745 which remained so after 48HPI.

Regression, interaction, and correlation analysis

The analysis of correlation (Supplementary Fig. 3) revealed a positive linear relationship between different growth parameters, gene expression, and enzyme activities (r = 0.1–0.98**). A higher correlation coefficient ratio (r-value) was observed for SL: SFW (r = 0.90), and RFW: RDW (r = 0.98). Correlation analysis further revealed that enzyme and gene expression has also a significant positive correlation (r > 0.5) with growth parameters e.g., SL with CAT, HSP18, HSP70 (r = 0.5–0.6), RL with CAT, HSP18, HSP70 (r = 0.52–0.69), SFW/SDW with CAT, HSP18, HSP70 (r = 0.57–0.7), LA with HSP18, HSP70 (r = 0.67, 0.70), POD with HSP1 (r = 0.75), MDA: RA (r = 0.52), HSP1 with POD, CAT, HSP101 (r = 0.64–0.86).

Linear regression effectively modeled the positive relationship of morphological parameters with gene expression and enzyme activity, accounting for 70% of the total variance. A positive linear regression was observed for dependent variables i.e., CAT (R^2 = 0.874), POD (R^2 = 0.822), MDA (R^2 = 0.990), total proteins (R^2 = 1.00), expression of HSP1 (R^2 = 0.835), expression of HSP18 (R^2 = 0.762), expression of HSP70 (R^2 = 0.868), expression of HSP101 (R^2 = 0.880) with SFW/SDW, RFW/RDW, FLL, LA, RA, and chlorophyll contents as independent variables. The PCA (Fig. 7) captured more than 89% of the variance and demonstrated the key varietal differences in the treatment response. The effect of bacterial inoculation under heat stress was more pronounced in different varieties as treated plants loaded positively on PC1 than non-treated plants.

DISCUSSION

The current research describes the PGPR traits of bacteria native to the Desert; their heat resilience and investigates their likely role in inducing heat tolerance in maize during the early vegetative growth stage. Maize is more sensitive to heat stress than wheat and rice (Zhang et al. 2019; Zhao et al. 2017) where for each 1ºC increase in global temperature a yield penalty of 10% has been simulated (Dong et al. 2021). Each decade is becoming hotter than the last while an increase of 1.14°C in global mean temperature has been recorded above 1850–1900 industrial average until 2022. Climate-smart agricultural strategies advocate high-yielding climate-resilient crop varieties with lesser emissions and losses. Plant-associated microbes are native to the stressed environment help plants to endure stress by means of a wide range of enzymes and metabolites along with their innate plant-growth-promoting potential (Niu et al. 2008). The present study lays the foundation to exploit the heat-tolerant PGPR for stimulation of plant growth under heat stress with minimum impact on the yield.

Heat-tolerant PGPR were isolated from the Cholistan desert and surrounding areas (latitude: 28˚46 N - 29˚16 N, longitude: 69˚52 E - 71˚29 E, altitude: 112 m). The climate of this desert is sub-tropical, arid, semi-arid, and scorching harsh, with low monsoon rainfall which increases the soil and environment temperature. The summer temperature ranges from 46 to 51°C during the months of drought while the winter temperature varies from 0 to 1°C. The soil is non-saline, low in organic matter with alkaline pH (Zia et al. 2021). A total of 130 distinct morphotypes (bacteria) were obtained from these samples while only 30 were able to grow at 50 ± 2°C and contained PGP traits. The strains were mostly *Bacillus* and *Pseudomonas* spp. These bacteria also showed differential responses to tolerate different levels of NaCl salt (2.5, 5, 7.5%) at a range of different temperatures ranging from 28 ± 2°C to 50 ± 2°C. Rhizospheric bacteria from high-temperature soil exhibiting PGP traits and desert PGPR (mostly *Bacillus* spp.) have been reported for PEG tolerance of up to 13%, NaCl tolerance of up to 15%, and temperature tolerance up to 70°C (Shekhawat et al. 2021).

The isolates have shown catalase activity which shows their ability to tolerate ROS which is produced in response to stress conditions. Extracellular catalase (microbes on the roots) in the vicinity of the roots may indirectly benefit the plants under stress by detoxifying ROS species produced in root cells. Similar work has been reported in *Arabidopsis* knockout of the catalase-regulated genes and compared the mutant with control plants (Mhamdi et al. 2010). Root colonization and biofilm formation are mainly considered in plant-microbes interaction. Confocal laser scanning microscopy results showed that AH-08 had a good root colonization ability as compared to AH-67 and SH-16. This root colonization helps to interact with the host as described in earlier studies for *Bacillus subtilis* sp. under stress conditions (Beauregard et al. 2013). In heat stress, carbon, and sugar contents are mainly affected in plants that cause cellular damage. These bacterial strains contain the ability to utilize the maximum number of carbon sources, hence, can survive on different forms of soil carbon under heat-stress conditions as reported earlier under heat and drought conditions (Eida et al. 2018).

Several plant hormones are involved in signaling metabolic regulation during heat stress. Bacteria also produce phytohormones such as auxins, cytokinins, gibberellins, and abscisic acid in the vicinity of plant roots that directly regulate plant growth similar to the plant's hormones. The AH-08, SH-16, and SH-29 produced IAA up to 45°C while AH-67 produced IAA at 50°C. Similar results were also reported that bacteria produce IAA at 28 ± 2°C and 50 ± 2°C (Shekhawat et al. 2021). It is reported that IAA-producing rhizospheric bacteria play an important role in plant growth, especially under heat stress, but their activity reduces at high temperatures. A study reports a 50% reduction in IAA production by *Pseudomonas* spp. and 16% by *Enterobacter* spp. at high temperatures while IAA production by *Acetobacter* spp. did not show any change at high temperatures (Kachhap et al. 2015) but the such reduction was not observed in the strains used in the present study.

Nitrogen (N), Phosphorous (P), Calcium (Ca), and Zinc (Zn) are the essential macro and micronutrients required for plant growth and play important role in improving soil fertility. The bacteria are able to fix nitrogen and solubilize P, Zn, or Ca at higher temperatures i.e., 45 and 50 ± 2°C increase nutrient availability and stimulate nutrient uptake under stressed conditions. It has been reported earlier that PGPR solubilize phosphorus (Kachhap et al. 2015) and fixed nitrogen (Hungria and Franco 1993) at higher temperatures i.e., 45°C.

Bacteria express heat shock proteins to counteract and maintain metabolic activity during heat stress. The present study demonstrates differential protein expression in heat-tolerant PGPR at different temperatures. The PAGE gel shows that few proteins not expressed at 28 ± 2°C appeared at 45 ± 2°C and 50 ± 2°C. These include 60–65 kDa and 10 to 30 kDa proteins that show the expression of small heat shock proteins at 45 ± 2°C and 50 ± 2°C. Similar 65kDa proteins have been reported earlier in *Bacillus* spp. grown at 50 ± 2°C (Richter and Hecker, 1986). Mostly *Bacillus subtilis* and *Pseudomonas* spp. exposed to high temperatures usually produce small heat shock proteins (Reischl et al. 2001). It is also reported that PGPR inoculation modulates the induction of the heat shock protein or heat shock factors in a plant that protect the integrity and normal functioning of the cellular proteins. In this study, four heat shock

proteins *HSP1, HSP18, HSP70*, and *HSP101* were analyzed in maize plants under heat stress conditions after B3P inoculation. Results showed that PGPR enhanced the expression of *HSP1* and *HSP18* in Malka and YH-5427, while the expression of *HSP70* and *HSP101* was higher in FH-1046 and Gohar, suggesting that heat resilient PGPR induced gene expression in host plants which help plants under stress conditions. HSPs play a key role in anti-stress processes in plants. A previous study reported that PGPR enhanced the level of *CAPx*, *rbcL*, and *rbcS* in wheat plants under water deficit conditions (Zia et al. 2021).

The present study showed significant improvement in the morphological and biochemical parameters of maize under stress. The plant height, root & shoot fresh & dry weight, root, and leaf area, root length, and plant biomass of treated plants under both normal and high-temperature condition was higher generally compared to non-treated plants. Further, B3P-treated plants showed a higher level of CAT, POD, and high levels of chlorophyll a, b, proteins, and amino acids while the concentration of MDA was low in B3P-treated plants as compared to the control. The higher expression of ROS-scavenging systems i.e., CAT (Catalase), POD (peroxidase), and SOD (Superoxidase dismutase) are known to reduce the impact of ROS on plants under stress (Chen et al. 2008). Co-inoculation of lettuce with the PGPR strain *Pseudomonas* sp. and an arbuscular mycorrhiza significantly enhanced the peroxidase (POD), and catalase (CAT) in the leaves under moderate and severe abiotic stress (Kohler et al. 2008). The stress decreases the total chlorophyll, cell protein, and amino acid and increases the MDA concentration that is mainly released after cell rupturing. Thermotolerant *Pseudomonas putida* strain *AKMP7* inoculated to wheat plants, increased the plant biomass and dry weight, enhanced root and shoot length, increased the number of tillers and spikelet, and enhanced grain formation. The inoculated bacteria prevent the plant from cellular injury, enhanced the antioxidant enzymatic activities (SOD, APX, CAT), and improve cellular metabolism e.g., increase the level of protein, enhanced proline contents, increase cellular sugar, amino acid, and starch, and increase the efficiency of chlorophyll under heat stress condition. *AKMP7* forms biofilm on plant roots which helps plants to combat adverse heat stress conditions (Ali et al. 2011). *Bacillus* spp. and *Pseudomonas* spp. from roots of desert cacti were reported to significantly enhance leaf surface area, stalk length, and fresh & dry biomass in *Z. mays* under heat stress (Kavamura et al. 2013). It has also been shown that heat-tolerant endophyte SA187 increases heat tolerance in *Arabidopsis* and wheat under control and field condition and enhance their morphological parameter (Shekhawat et al. 2021). The current study also shows that photosynthetic pigments are higher in B3P-treated plants as compared to control under stress conditions. Heat-resilient *Bacillus cereus* inoculated tomatoes have previously shown increased chlorophyll a and b, relative water contents in plants under heat stress as compared with control (Mukhtar et al. 2020).

Conclusions

This study provides an insight into heat-resilient bacteria with distinctive plant-growth-promoting traits. Increasing temperature is the main climate stress, the plants are facing today due to climate change and it is expected to increase further in the coming decades. The present study describes those bacteria that have multiple mechanisms to cope with the heat and induce plant-heat tolerance using a combination of these mechanisms e.g., expression of heat shock genes/proteins, and modulation of enzymatic activity of stress-related enzymes. The bacteria described in this study are mostly bacilli strains that are more tolerant and resistant to multiple stresses e.g., salt, water, etc. We anticipate that amelioration of heat-stress tolerance will improve plant growth not only during the early vegetative stage but plant growth and yield during later stages as well. The same may be validated in future experiments on the same or other crops.

Declarations

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Author contribution

MA: Data curation; Formal analysis, Investigation; Methodology, Writing - original draft, **MI:** Investigation; Supervision, **SN:** Validation; Visualization, **FM:** Investigation; Methodology, **YS:** Methodology, **MH:** Methodology, Data curation, **MA:** Investigation; Methodology, **RZN:** Methodology, Software, **MA:** Project administration, **AI:** Conceptualization; Funding acquisition; Investigation; Project administration; Resources; Software; Supervision; Writing - review & editing**.**

Data availability statement

The gene data related to the manuscript is available in the NCBI.

Declaration of Ethical standards

The study does not involve any human and animal objects so the approvals from appropriate ethics committee was not applicable.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. All persons gave their informed consent prior to their inclusion in the study.

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Figures

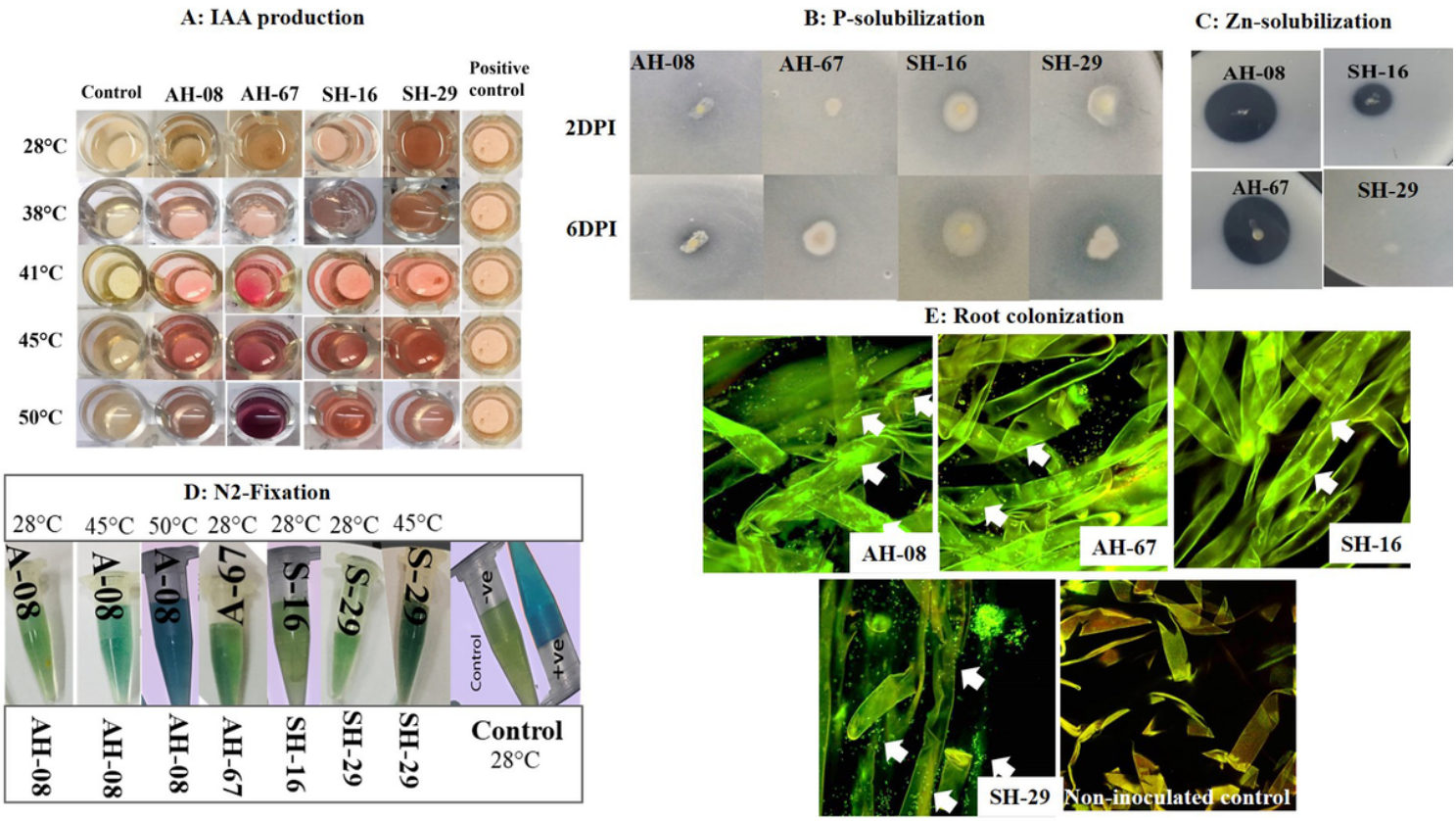

Figure 1

In vitro PGP-activity assays for production of IAA (A), P-solubilization (B), Zn-solubilization (C), Nitrogen fixation (D), and root colonization (E) by heat tolerant bacteria obtained from Cholistan desert.

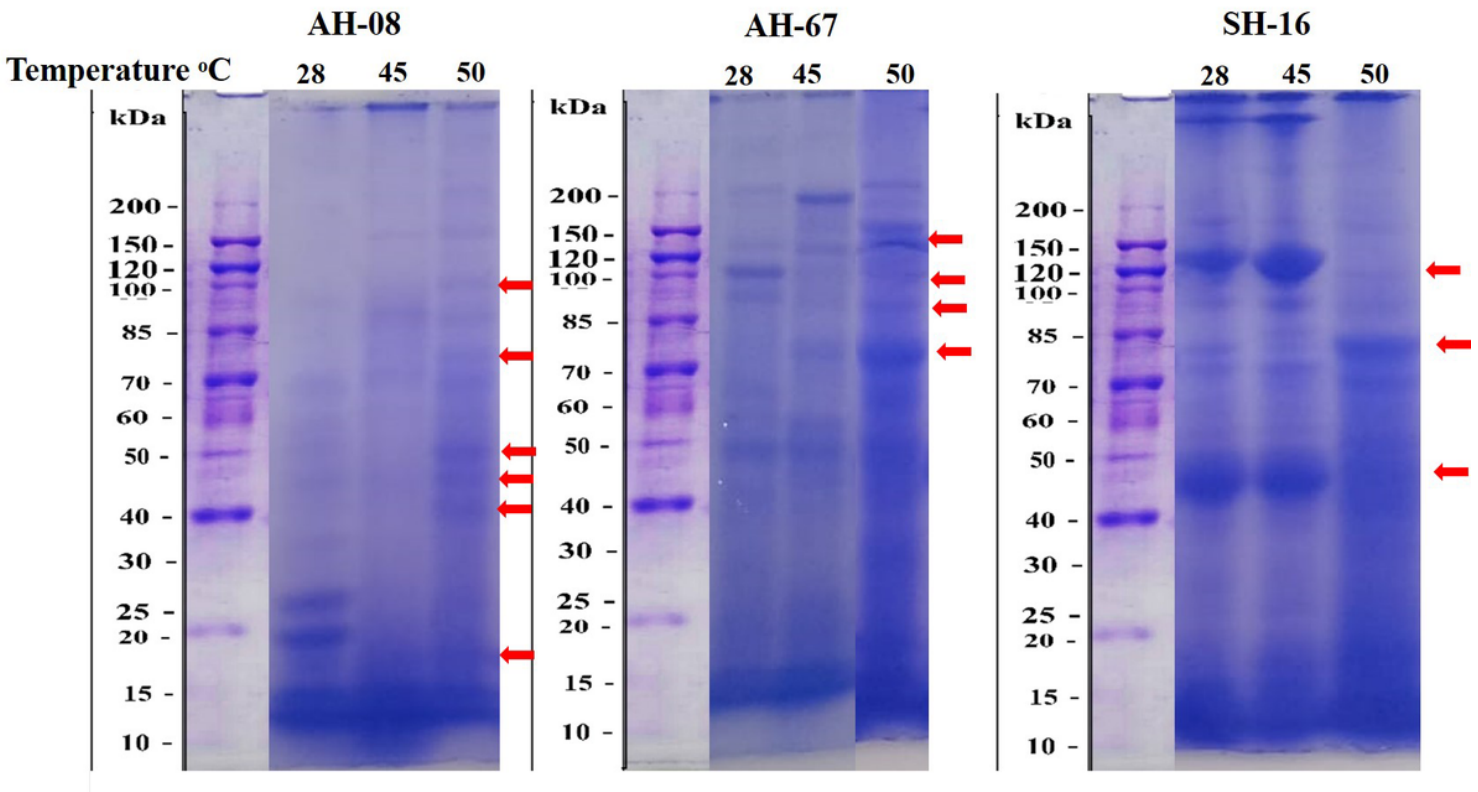

Figure 2

Whole-cell protein profiling of microbes grown under different temperature stress levels on SDS-PAGE

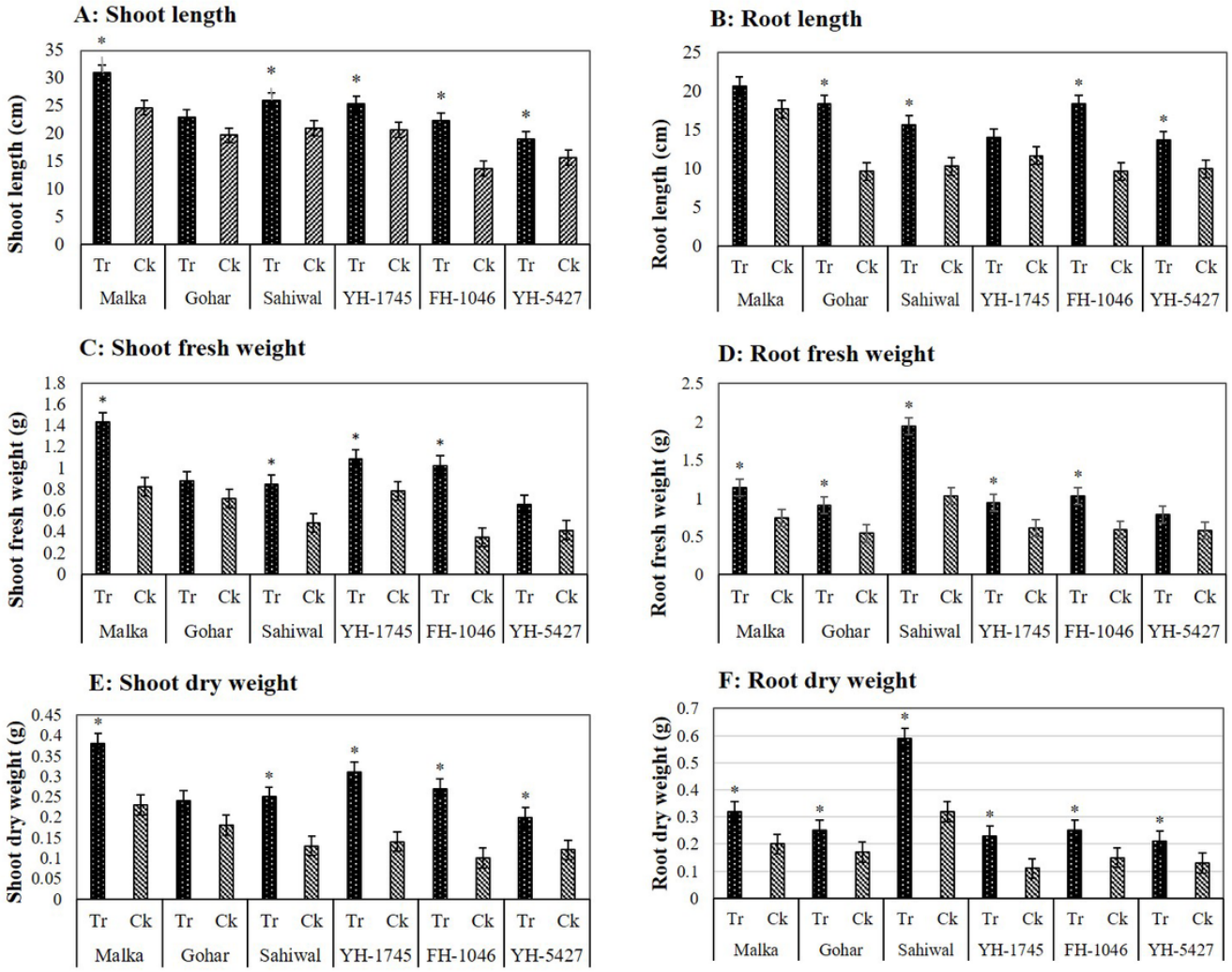

Figure 3

Graphical representation of above ground and below ground plant parameters showing the effect of bacterial inoculation on shoot length (A), root length (B), shoot fresh weight (C), root fresh weight (D), shoot dry weight (E), root dry weight (F) of different maize varieties grown under heat stress.

The data presented is mean of 3 independent replicates. The bars represent the standard deviation. The data was subjected to ANOVA. The on the bar indicates that treatment mean different significantly at P <0.05 according to HSD.

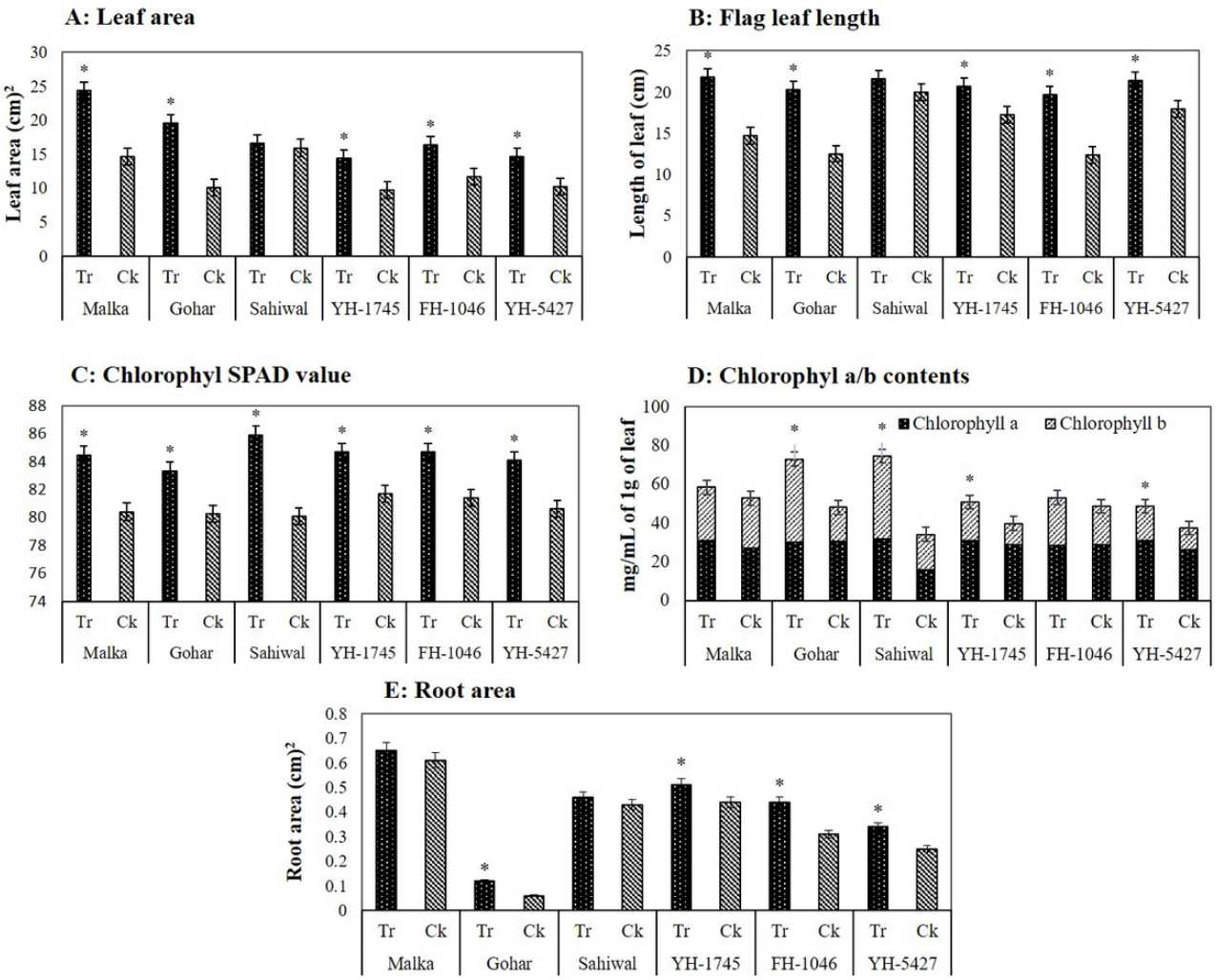

Figure 4

Graphical representation of plant parameters showing the effect of bacterial inoculation on leaf area (A), flag leaf length (B), chlorophyl SPAD value (C), chlorophyl a/b contents (D), root area (E) of different maize varieties grown under heat stress

The data presented is mean of 3 independent replicates. The bars represent the standard deviation. The data was subjected to ANOVA. The on the bar indicates that treatment mean different significantly at P <0.05 according to LSD.

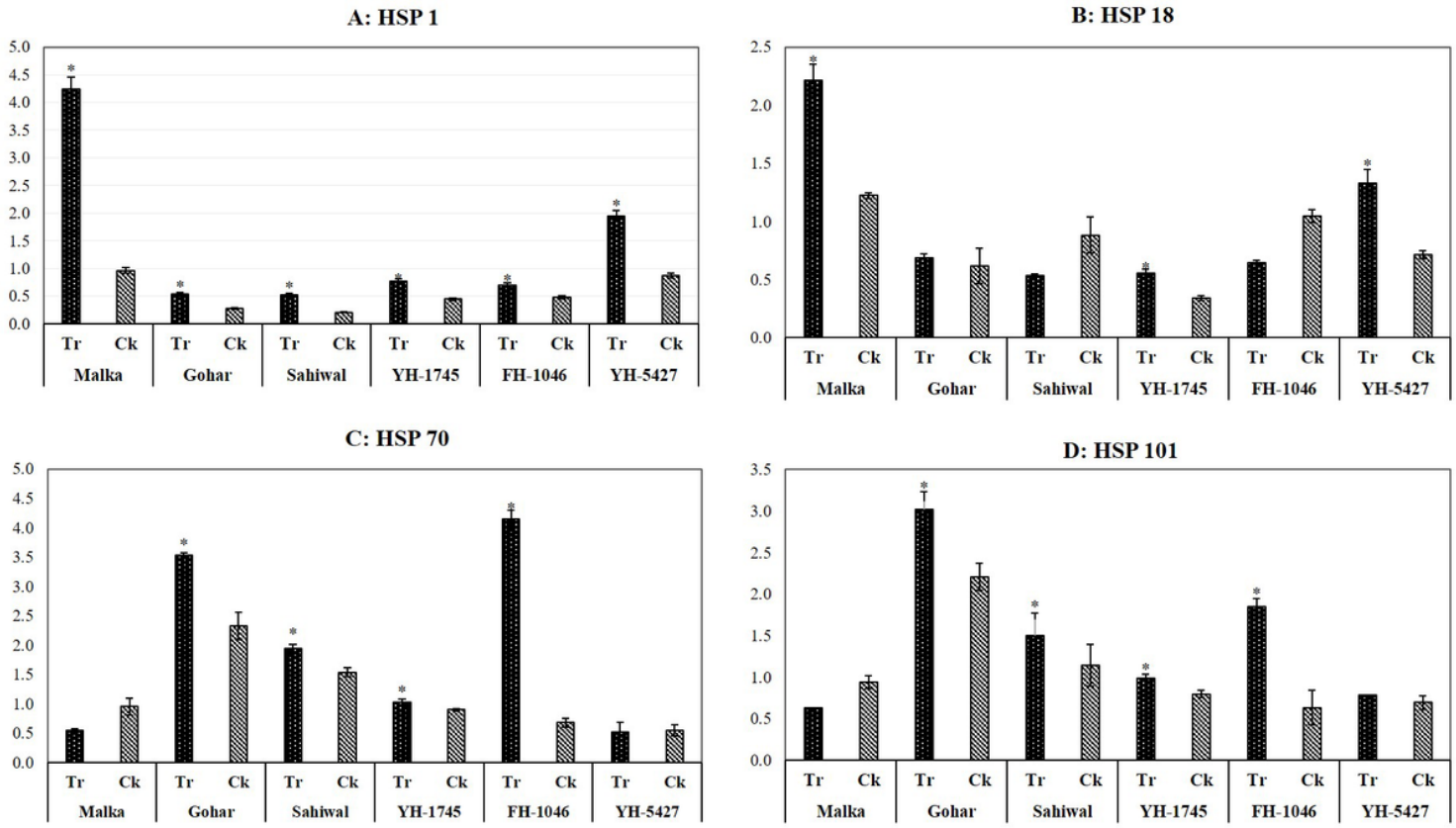

Figure 5

Effect of B3P inoculation on expression of HSP1 (A), HSP18 (B) HSP70 (C) and HSP101 (D) post induction of heat stress in maize varieties

The data presented is mean of 3 independent replicates. The data was normalized with the actin gene and then subjected to ANOVA. The on the bar indicates that treatment mean different significantly at P <0.05 according to LSD.

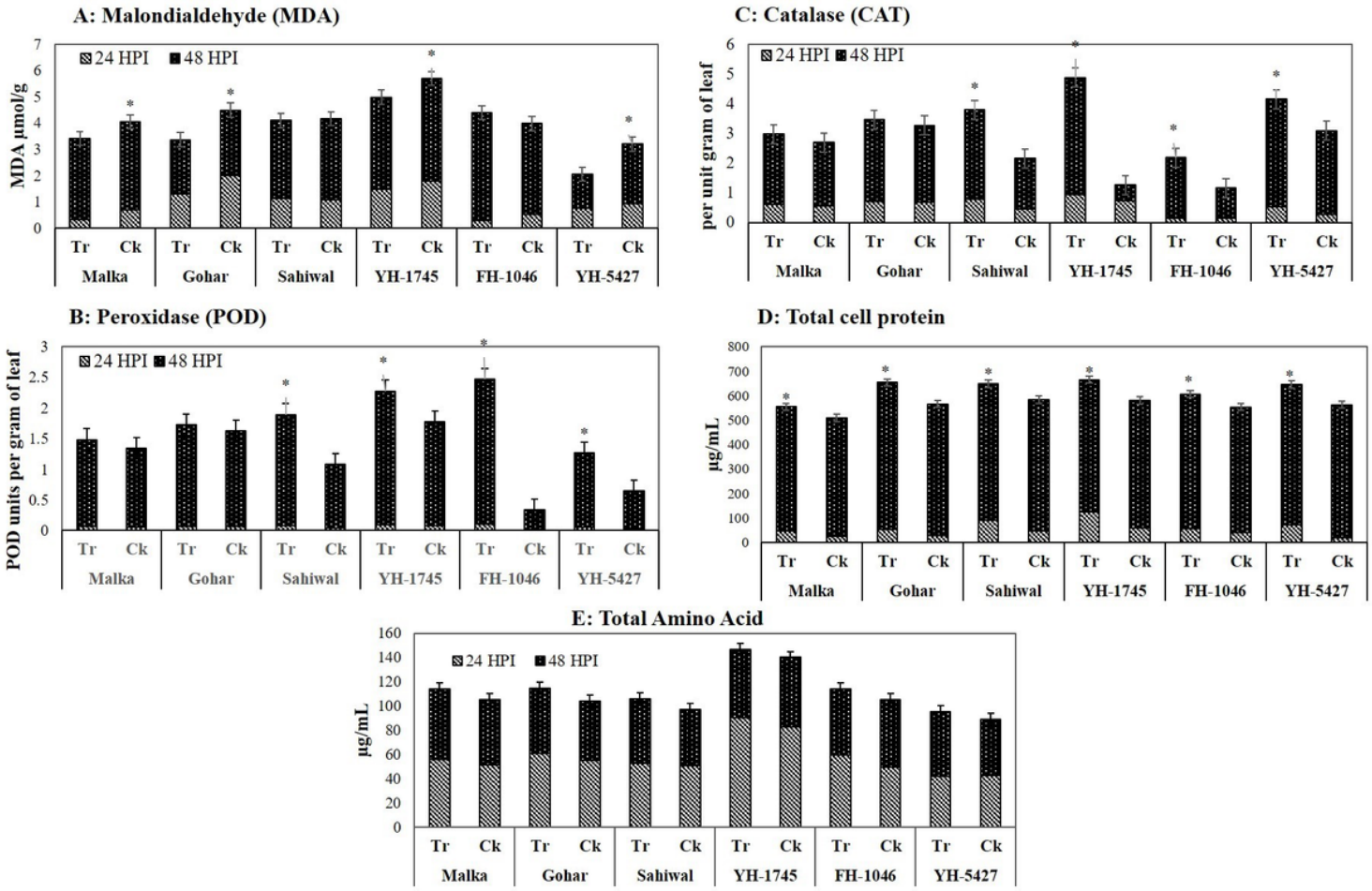

Figure 6

Effect of B3P inoculation on enzyme activities Malondialdehyde (A), peroxidase (B) catalase (C), total cell proteins (D) and amino acids (E) post induction of heat stress in maize varieties

The data presented is mean of 3 independent replicates. The on the bar indicates that treatment mean different significantly at P <0.05 according to LSD.

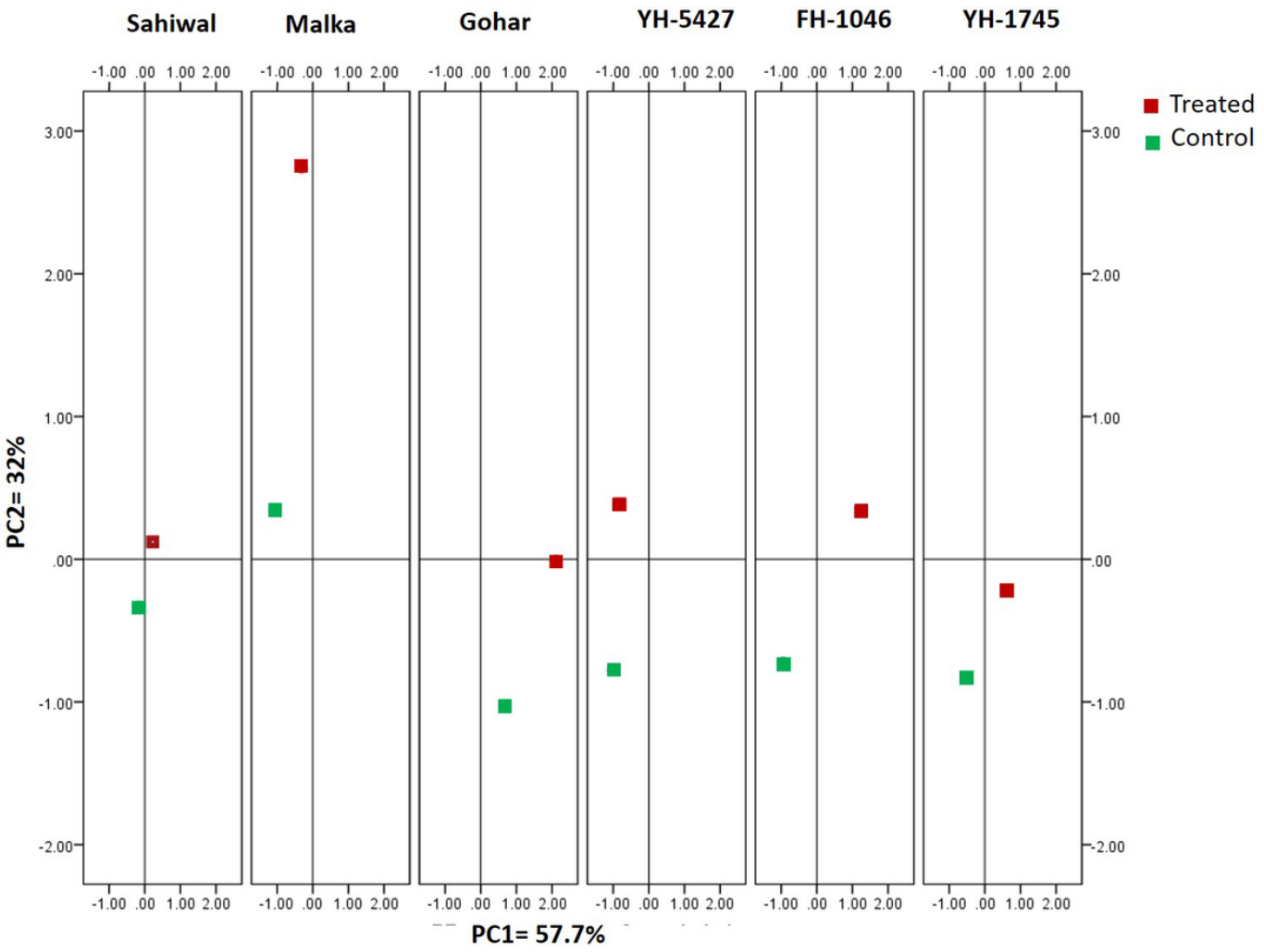

Figure 7

Principal component analysis (PCA) sowing the response of maize varieties after heat stress towards inoculation (Total variance explained= 89.7%).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigure1.jpg
- SupplementaryFigure2.jpg
- SupplementaryFigure3.jpg