An invasive weed-associated bacteria confers enhanced heat stress tolerance in wheat

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ABSTRACT

Global temperatures are expected to increase due to climate change, and heat stress is one of the major limiting factors affecting future agriculture. To identify plant-associated microorganisms which can promote heat stress tolerance in wheat, we have screened several bacteria isolated from etiolated seedlings of the invasive noxious weed *Parthenium hysterophorus*. One isolate designated as Ph-04 was found to confer enhanced heat stress tolerance in wheat. The 16S rRNA gene sequence analysis showed that Ph-04 isolate shared highest sequence identity with *Bacillus paramycoides* species of the *Bacillus cereus* group. Ph-04 treated wheat seeds exhibited enhanced germination, longer coleoptile, radicle and seminal root length than control seedlings when grown in the dark at optimum and high temperatures. Similarly, under autotrophic conditions, Ph-04 treated plants also exhibited enhanced heat stress tolerance with a significant increase in membrane integrity and significantly reduced levels of H2O2 under heat stress compared to control plants. This observed heat stress tolerance is associated with constitutively higher basal levels of proline, and activity of antioxidant enzymes, catalase (CAT) and ascorbate peroxidase (APX) in Ph-04 treated plants grown under unstressed conditions with further increase under heat stress conditions compared to controls. Plant recovery after heat stress also showed that the Ph-04 treated plants exhibited significantly less damage in terms of survival percentage and exhibited better morphological and physiological characteristics compared to control plants. The study proves that invasive weeds can harbour potentially beneficial microorganisms, which can be transferred to non-native crop (host) plants to improve climate resilience characteristics.

1. Introduction

The rate of food production must be increased by 25%–70% above current production levels to meet the demand of food production by 2050 for the world’s increasing population (Hunter et al., 2017). It has been projected that by the end of the 21st century, global mean temperature could increase by 1.8 °C–4.0 °C, and with little or no mitigation efforts, it is more likely to exceed 4.0 °C (IPCC, 2014). Such an increase in temperature would pose a greater risk to future global and regional food security (IPCC, 2014). Wheat (*Triticum aestivum* L.) is being grown on more than 200 million hectares worldwide. The global production is reaching more than 750 million tons per year, with India being the second-largest producer of wheat (FAOSTAT, 2019). It has been projected that the Indian region may experience double the number of heat events in the next fifty years and is projected to get warmer faster than the projected global average increase (IPCC, 2014). Further, high temperatures are expected to limit the national wheat production of India (Asseng et al., 2017). High temperatures due to heatwaves can create acute heat stress in plants and have a detrimental effect on plants. In cool-season annuals such as wheat, high temperature negatively affects the vegetative growth, development and reproduction (Porter & Gawith 1999; Driedonks et al., 2016). It was estimated that, with every 1 °C increase in global temperatures, the yield of wheat is projected to decline by 4.1–6.4%, and it is going to be similar for major wheat-producing countries China, India, USA and France (Liu et al., 2016). Further, the impact of climate change on future soil temperatures and its effect on seed germination and establishment has not received enough attention.

Higher soil temperatures require deeper sowing under low irrigation...
conditions. High soil temperature causes reduced seed germination and reduced coleoptile length in cereals, resulting in the lesser establishment of seedlings and subsequent reductions in yield (Rebetzke et al., 2016). Earlier occurrence of phenological stages was observed in wheat due to high temperature, and studies in Europe and worldwide suggested accelerated phenology due to climate change (Gouache et al., 2012). The impact of heat stress depends on the intensity, duration and the developmental stage exposed (Balla et al., 2019). Heat stress disrupts cellular homeostasis and adversely influences growth, physiology and development of plants (Sinha and Kumar 2022). Heat stress accelerates the leaf senescence, reduces chlorophyll content, and damages chloroplast integrity in wheat (Haque et al., 2014). High temperature increases the level of reactive oxygen species (ROS), causing oxidative stress and disturbing the redox homeostasis of the cell (Sinha and Kumar 2022). Oxidative stress causes membrane lipid peroxidation, increases electrolyte leakage and decreases membrane thermostability in wheat (Savicka and Skute 2010). Studies on thermo-tolerant and susceptible genotypes of wheat showed that heat stress tolerance is associated with increased ROS scavenging capacity (De Pinto et al., 2015). Further, ROS-scavenging activity exhibits a positive correlation with chlorophyll content and a negative correlation with membrane damage and heat susceptibility (Hameed et al., 2012).

Among the agronomic adaptation strategies, improving tolerance to heat stress in plants is considered to be the most efficient one (Gouache et al., 2012). Therefore, improving heat stress tolerance in wheat is an urgent necessity either by developing new wheat varieties through breeding or developing novel strategies for improving heat tolerance suitable for future climatic conditions. Although conventional breeding and genetic engineering are viable options, both are time-consuming and have limitations like delayed outcomes and objections by environmental protection groups, respectively. Concerns about horizontal gene transfer and its impact on biodiversity have also enhanced the skepticism towards transgenics. In addition to that, extensive usage of chemical fertilizers and pesticides has caused significant damage to the soil quality, water bodies and the environment. Hence, sustainable and environmentally friendly approaches are required. Using plant-associated microorganisms such as plant growth-promoting rhizobacteria (PGPR) and endophytes is an environmentally friendly and cost-effective way of improving the growth and abiotic stress tolerance of crop plants (Lata et al., 2018). Recently, endophytic bacteria and PGPR were shown to enhance heat stress tolerance in different plant species such as sorghum (Khan et al., 2020), tomato (Mukhtar et al., 2022), rice (Choi et al., 2022) and wheat (Abd El-Daim et al., 2014; Sarkar et al., 2018; Shekhawat et al., 2021). This enhanced heat stress tolerance is associated with increased exopolysaccharide production, synthesizing and modulating phytohormones, enhanced accumulation of osmoprotectants, increased activity of antioxidant enzymes and enhanced expression of heat shock protein (HSP) genes (Abd El-Daim et al., 2014; Sarkar et al., 2018; Khan et al., 2020; Mukhtar et al., 2022). Hence, the isolation and identification of effective plant-associated microorganisms that can confer consistent and reproducible growth-promoting effects or stress tolerance effects is an important approach for crop improvement.

Plant growth-promoting bacteria belonging to the genera Bacillus are considered suitable biofertilizers, as they can survive in diverse biotic and abiotic environments (Bokhari et al., 2019). Several Bacillus species have been identified as plant growth-promoting bacteria with their ability to promote stress tolerance or otherwise promote plant growth (Shahzad et al., 2019). The Bacillus cereus group is a subfamily of the genus Bacillus and comprises several closely related species (Liu et al., 2017). Though Bacillus cereus is popularly known for its pathogenicity in humans, lately there are many reports on plant associated Bacillus cereus strains and its closely related species from Bacillus cereus group having ability to promote growth and improve stress tolerance in plants (Zeng et al., 2018). Recently, Bacillus cereus strains were shown to confer tolerance to heat stress in soybean (Khan et al., 2020) and tomato (Mukhtar et al., 2020). Bacillus paramycoideus is one of the newly proposed species within the Bacillus cereus group (Liu et al., 2017). However, there are very few reports on the association of Bacillus paramycoideus with plants. For example, Saran et al. (2020) reported isolation of endophytic Bacillus paramycoideus from Helianthus pettolaris with plant growth-promoting traits and use in bioaugmentation. Pandey and Gupta (2020) reported the isolation of Bacillus paramycoideus isolate from coconut tree rhizosphere which improved growth and salinity tolerance in Phaseolus vulgaris.

Invasive weed plants can germinate, grow and reproduce even under sub-optimal conditions. Recent studies have shown that endophytes and other plant-associated microorganisms play a significant role in weed establishment and growth under suboptimal environmental conditions (Trognitz et al., 2016). Although the complete mechanism by which invasive weeds survive in diverse climatic conditions is not known, it can be speculated that apart from evolving their own adaptive mechanisms, these invasive weeds might maintain their native interaction with different microbiota, which help them to grow and survive under stress conditions. Parthenium hysterophorus L. is a global invasive weed growing in different habitats. There are very few reports of endophytes from P. hysterophorus (Romero et al., 2001). Only fungal endophytes from P. hysterophorus were shown to have a growth promotion potential (Priyadharsini & Muthukumar 2017).

Germination and subsequent heterotrophic growth in the dark (beneath soil surface) are crucial developmental stages before the seedling become autotrophic upon exposure to sunlight. This developmental stage is sensitive to environmental changes, which can dramatically affect their survival. We hypothesized that if a weed to be successful in becoming invasive in diverse environmental conditions, it needs to maintain a set of microbiota and transmit them vertically to the next generation. Such a microorganism can have potentially beneficial characteristics such as promoting seed germination and subsequent skoto-morphogenic growth under sub-optimal conditions. Therefore, our study aimed at isolating and identifying bacteria associated with P. hysterophorus, which can help mitigate the effects of heat stress in wheat. In this study, we show that one of the bacterial isolates from etiolated seedlings of P. hysterophorus, could promote tolerance to heat stress in wheat during the heterotrophic and autotrophic developmental stages of wheat.

2. Materials and methods

2.1. Plant materials

P. hysterophorus seeds were collected during the dry season in the wild near the IGNTU campus, Amarkantak, India. This region is located at an altitude of more than 900 m above sea level. Seeds were collected from three different locations (22° 48’ 15” N, 81° 44’ 47” E; 22° 47’ 47” N, 81° 47’ 04” E; 22° 45’ 54” N, 81° 44’ 48” E). P. hysterophorus seeds were surface sterilized and germinated on sterile moist filter paper at 28 °C in the dark to obtain etiolated seedlings. Wheat seeds (JW3211) (Kindly provided by Krishi Vigyan Kendra, IGNTU Amarkantak, India) were surface sterilized using sodium hypochlorite and 70% ethanol, followed by several washes with sterile Milli-Q water. Surface sterilized seeds were used in all experiments. All experiments were repeated three times with three technical replicates.

2.2. Isolation and screening for heat stress tolerance promoting bacteria from P. hysterophorus

Six-day old etiolated seedlings of P. hysterophorus growing in vitro were surface sterilized as described above and ground into an aqueous extract using a mortar and pestle under aseptic conditions. This seedling extract was serially diluted with sterile Milli-Q water and spread on different nutrient mediums such as Tryptone agar (HiMedia), Luria Bertani Agar (HiMedia), Glucose yeast extract agar (HiMedia), King’s B medium (HiMedia), and incubated at 28 °C for two days. The last water
wash of the seedlings during surface sterilization and the sterile Milli-Q water used in the experiment were also plated separately to confirm effective sterilization and the absence of any unintended contamination. Different bacterial colonies obtained were selected based on their morphology and pigmentation characteristics. Pure cultures of the selected isolates were obtained and preserved in glycerol at -80 °C for subsequent use. For identifying the isolates that can confer heat stress tolerance and/or growth promotion in wheat, wheat seeds were germinated at high temperatures (40 °C) after incubation in different bacterial suspensions. During screening for heat stress tolerance, suspension of each bacterial isolate was adjusted to an OD of 0.2, 0.4, 0.8 and 1.0 separately and used in assay in combination with different seed incubation times of 30 min, 1 h, 3 h and 6 h. Based on this screening, among all the tested isolates only one potential isolate designated as Ph-04 was selected for further study based on its ability to consistently promote germination of wheat seeds under high temperature. Further, we found an OD of 0.8 and an incubation period of 30 min as optimum for bacterial treatment of wheat seeds with Ph-04 in our study.

### 2.3. 16S rRNA sequencing and phylogenetic analysis

The selected isolate was identified using 16S rRNA gene sequencing. Briefly, genomic DNA of bacteria was extracted using standard methods and used as a template for amplifying the 16s rRNA gene. Polymerase chain reaction was performed using DreamTaq DNA Polymerase (Thermo Scientific) and universal 16S rRNA gene primers 16S-27F (5'-AGGTTGATCMTGGCTCAG-3') & 16S-1492R (5'-GGA- TACCTGTAGCAGCTT-3'). PCR conditions were as follows: 95 °C for 5 min (1 cycle); 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min (30 cycles); and 72 °C for 5 min (1 cycle). The PCR product was run on a 1% agarose gel, and the amplification product was gel eluted using a GeneJet gel extraction kit (Thermo Scientific). The purified PCR product was sequenced through Sanger sequencing technology commercially at Eurofins. The sequence was matched against NCBI database of 16S rRNA sequences. For phylogenetic analysis, reference sequences of closely related *Bacillus* species were downloaded from NCBI and used for phylogenetic tree construction. FastME/OneClick tool (ngphylogeny.fr) was used for multiple-sequence alignment, alignment curation, and the phylogenetic tree inference. The phylogenetic tree was visualized using Interactive Tree of Life (Lemoine et al., 2019; Letunic and Bork 2021).

### 2.4. Thermotolerance of selected bacteria

Ph-04 was grown in LB broth media at 28 °C, in a rotatory shaker incubator at 150 rpm for 16 h. Bacterial culture OD_{600} was adjusted to 1.0 using LB broth, serially diluted and plated on LB agar plates. Bacterial plates were incubated at 30 °C, 35 °C, 40 °C, and 45 °C respectively for 48 h and colony forming units per milliliter (CFU ml^{-1}) was calculated.

### 2.5. Plant growth promotion (PGP) trait assays

Indole acetic acid (IAA) and gibberellic acid (GA) production was determined according to Banerjee et al. (2019). Phosphate solubilization activity of bacterial isolate was assessed by growing on Pikovskaya agar (Hismedia). After spot inoculating on Pikovskaya's agar, plates were incubated for seven days at 28 °C. A clear halo zone around the bacterial colony on Pikovskaya's medium was considered positive for phosphate solubilization. Siderophore production assay was performed using Chrome Azolol S (CAS) agar medium (Khan et al., 2020). HCN production was estimated according to Slama et al. (2019) on HCN medium (nutrient agar supplemented with 4.4 g L^{-1} of glycerine). ACC deaminase production was confirmed by growth on minimal media containing ACC as sole nitrogen source according to Glick et al. (1994). Biofilm growth in glass tubes was determined according to Haney et al. (2018).

### 2.6. Bacterial treatment of seeds

Bacteria streaked freshly from glycerol stock on a LB agar plate was used in all experiments. Ph-04 bacterial strain was grown in 100 mL of sterilized LB broth at 28 °C for 16 h under continuous shaking. The culture was centrifuged, and the pellet was washed and resuspended in sterile Milli-Q water under aseptic conditions. Prior to seed treatment, bacterial suspension was diluted as necessary to achieve an optical density of 0.8 at 600 nm. Surface sterilized wheat seeds were incubated in bacterial suspension for 30 min, blotted dried and subsequently used in all experiments. Another set of wheat seeds incubated in sterile water for 30 min served as a control/mock treatment.

### 2.7. Germination percentage and etiolated seedling growth assay in dark

Bacterial treated seeds and control/mock treated seeds were placed on sterile water agar plates and incubated at 25 °C, 37 °C and 40 °C respectively in the dark. The germination percentage was calculated by counting the number of seeds with a radicle after 4 d. Growth of etiolated seedlings at increasing temperature was analyzed by measuring the length of coleoptile/shoot, radicle and seminal roots after four days of growth in the dark at defined temperatures. A total of ≥ 60 seeds were used per each experiment and the experiment was repeated three times.

### 2.8. Heat stress assay of light-grown seedlings

Bacterial treated seeds and control/mock treated seeds were germinated in pots containing sterilized soil and grown in a plant growth chamber at 28 °C under a photoperiod light of 12 h/12 h (L/D). For heat stress assay, seven-day-old seedlings were transferred to, and maintained at 42 °C continuously for 6 h and transferred back to 28 °C. Seedling survival and recovery after heat stress was observed by allowing the plants to recover at 28 °C for another five days. Plants growing continuously at 28 °C served as unstressed control. Leaf samples were collected from unstressed and heat-stressed plants immediately after heat stress and after recovery, frozen in liquid nitrogen and stored at -80 °C for subsequent use.

### 2.9. Evans blue staining and electrolyte leakage assays

Heat-induced membrane damage was assessed by Evans blue staining and electrolyte leakage experiments. Briefly, control and heat-stressed leaves were cut into 2 cm long pieces and submerged in 0.25% (wt/vol) Evans blue solution for 30 min under continuous agitation. The stained leaf segments were washed repeatedly with Milli-Q water to remove excess and unbound stain, stored in 10% glycerol solution for subsequent use.

For measurement of electrolyte leakage, leaves were washed with deionized water and cut into 2 cm uniform pieces. Four leaf segments from random plants (of the same group) were suspended in 7 ml of deionized water and incubated at 50 °C for 1 h followed by incubation at 28 °C for 10 min, and the electrical conductivity (EC_{initial}-Heat treated) was measured using a conductivity meter (Eutech). The ion leakage from leaf segments without heat treatment (EC_{initial} of the untreated sample) was determined after 70 min incubation at 28 °C. To measure the total conductivity (EC_{total}) of both heat-treated and untreated samples, the tubes with leaf segments were incubated in a water bath at 98 °C for 15 min and then cooled for 15 min at 28 °C. Percentage of ion leakage was estimated using the formula: 100 (Rt-Ro/1-Ro), where Rt = EC_{initial}/EC_{total} for the heat-treated sample, and Ro = EC_{initial}/EC_{total} for the untreated sample.
2.10. DAB staining

Histochemical staining of H$_2$O$_2$ was performed using 3,3 Dia-
ninobenzidine (DAB) following the method of Xing et al. (2013) with
slight modifications. Leaf segments were stained with DAB solution (1
mg/ml, pH 5.5) for 12 h and subsequently boiled in 95% ethanol for 15
min to remove the chlorophyll and stored in 50% glycerol for micro-
scopic observation. In the presence of H$_2$O$_2$, DAB forms reddish-brown
precipitates.

2.11. Determination of H$_2$O$_2$ content

Hydrogen peroxide content was determined according to Velikova
et al. (2000). Leaf samples (500mg) were homogenized with 0.1% (w/v)
trichloroacetic acid (5 ml) in an ice bath, and the homogenate was
centrifuged at 12000 rpm for 15 min. Reaction mixture contained 10 mM
potassium phosphate buffer (pH 7.0) (0.5 ml), 1 M KI (1 ml), supernatant
(0.5ml) and incubated in the dark for 1 h. Absorbance was measured at
390 nm using a spectrophotometer (Shimadzu, Japan), and the content of
H$_2$O$_2$ was calculated using a standard curve with known concentra-
tions and expressed as μmoles g$^{-1}$.

2.12. Proline content

Quantification of proline was carried out using colorimetric method
described by Abraham et al. (2010). Approximately 100 mg of leaf tissue
was homogenized in 3% sulfo salicylic acid. The homogenate was
centrifuged at room temperature at 12,000 g for 5 min. 100 μL from the
supernatant was added to the reaction mixture containing 100 μL of 3%
sulfo salicylic acid, 200 μL of glacial acetic acid, 200 μL of acidic ninhy-
drin. The mixture was incubated at 96 °C for 60 min, and the reaction was
terminated by immediately cooling on ice. Samples were extracted using
toluene, and the absorbance was read at 520 nm using toluene as a refer-
ence. Proline concentration was determined using a standard curve and
calculated on fresh weight basis.

2.13. Catalase (CAT) and ascorbate peroxidase (APX)

Catalase (CAT) and ascorbate peroxidase (APX) activity was determi-
ned as previously described by Elavarthi and Martin (2010). Catalase activity
was followed as a decrease in absorbance at 240 nm upon
decomposition of H$_2$O$_2$. Extinction coefficient of H$_2$O$_2$ (40 mM$^{-1}$ cm$^{-1}$ at
240 nm) was used to calculate the catalase activity and expressed as
micromoles of H$_2$O$_2$ g$^{-1}$ FW. APX activity was assayed by measuring the
rate of decrease in absorbance of ascorbate at 290 nm. The extinction
coefficient of 2.8 mM$^{-1}$ cm$^{-1}$ for reduced ascorbate was used in calculat-
ing the enzyme activity and expressed as micromoles of ascorbate
peroxidase per minute per gram fresh weight.

2.14. Relative water content (RWC)

Fresh leaves from randomly selected plants were collected, fresh
weight was measured immediately and sealed in plastic bags. Water
(10ml) was added to immerse the leaves, and sealed plastic bags were
placed at room temp for 4 h. After blot drying with paper towels, leaf
turgid weight (TW) was measured. Finally, leaf samples were dried in a
hot air oven at 60 °C for 3−4 days; after that dry weight (DW) of the
sample was recorded. RWC (%) was calculated from following equation:
RWC = (FM – DM)/(SM – DM) × 100. Where FM is leaf fresh mass, DM is
dry mass of leaves after drying, and SM is the turgid mass of leaves.

2.15. Total chlorophyll

Total chlorophylls were extracted with 80% acetone and estimated by
the method of Arnon (1949). The optical density (OD) was taken at 645
and 663 nm by a spectrophotometer (Shimadzu, Japan) with 80% acetone serving as blank.

2.16. Statistical analyses

ANOVA, followed by means comparison using Turkey and Bonferroni
tests, were performed to determine significance levels. OriginPro 8.5 was
used for statistical analyses and plotting graphs.

3. Results

3.1. Isolation and selection of heat stress tolerance promoting bacteria from
etiolated seedlings of P. hysterophorus

Skotomorphogenesis (etiolated growth) is a critical developmental
stage on which the establishment and the survival of the seedling depend
upon. Bacteria associated with the skotomorphogenic growth of
P. hysterophorus were obtained by plating the aqueous extract of etiolated
seedlings of P. hysterophorus on different culture media. A total of 21
bacterial isolates were preliminarily screened for their ability to promote
germination under high temperature in wheat. Though 11 of the 21
isolates showed growth promotion of etiolated wheat seedlings at opti-
 mum temperature, only one isolate designated as Ph-04 (for Parthenium
hysterophorus isolate -04) was found to promote germination under
increasing temperatures, and this isolate has been selected for further
studies. For the molecular identification of the Ph-04, the 16s rRNA gene
was amplified and sequenced. The sequence of the 16s rRNA gene
was submitted to Genbank under the accession number MZ413335. Based
on the sequence identity and phylogenetic analysis of the 16s rRNA
sequences, Ph-04 is closely related to Bacillus paramycoides, a recently re-
ported novel species within the Bacillus cereus group (Figure 1a) (Liu
et al., 2017). Interestingly, in our phylogenetic analysis of 16s rRNA
sequences, a recently reported (Khan et al., 2020) plant growth and
thermotolerance promoting Bacillus cereus SA1 strain also formed a
separate clade from Bacillus cereus and appeared to be closely related to
one of the novel species within the Bacillus cereus group of bacteria
(Figure 1a). Thermotolerance analysis of Ph-04 strain showed that the
isolate grows normally between 30 °C to 40 °C, and can tolerate up to 45
°C (Figure 1b). Further, analysis of plant growth promoting traits showed
that the Ph-04 is positive for indole acetic acid, gibberellic acid and
1-aminocyclopropane-1-carboxylate (ACC) deaminase (ACCD) produc-
tion and negative for phosphorous solubilization and hydrogen cyanide
(HCN) production (Table 1).

3.2. Ph-04 induces germination, and etiolated seedling growth in wheat
under high temperature

To study the heat tolerance promoted by Ph-04, we first analyzed the
ability of the bacteria to promote germination at a high temperature of 37
°C compared to the germination at an optimum temperature of 25 °C.
Control/mock-treated seeds did not germinate at a higher temperature of
37 °C, whereas the percentage of seed germination in Ph-04 treated seeds
was almost similar at 25 °C and 37 °C respectively (Figure 1c, d). Further,
Ph-04 treated seeds also exhibited germination at 40 °C as well, sug-
gesting that Ph-04 can induce germination even at an elevated tempera-
ture of 40 °C (Figure 1c, d).

Since we have isolated the Ph-04 from etiolated seedlings of
P. hysterophorus, we next investigated its effect on etiolated seedling
growth at increasing temperatures in wheat. Further, etiolated seedling
growth at high temperatures also simulates the skotomorphogenic
growth at high soil temperatures. At 25 °C, Ph-04 treated seedlings
exhibited significantly enhanced coleoptile, radicle, and seminal root
growth compared to mock-treated seedlings (Figure 1e, f). Though there
is a temperature-dependent inhibition of growth observed in Ph-04
treated etiolated seedlings, yet they exhibited significant growth of
coleoptile, radicle and seminal roots at 37 °C and at 40 °C compared to
control/mock treatment (Figure 1f, g, & h). On the contrary, control/mock-treated seeds failed to germinate even at 37 °C with no emergence of either coleoptile or radicle (Figure 1c, g, & h), confirming that the Ph-04 strain can promote germination and etiolated seedling growth of wheat under increased temperatures.

3.3. Ph-04 treatment protects light-grown wheat seedlings from heat stress

After confirming the effect of Ph-04 on skotomorphogenic growth of etiolated seedlings at high temperatures, we next asked whether the protection conferred by Ph-04 is only at the developmental stage of seed germination.

Figure 1. Ph-04 bacterial isolate: enhances wheat germination and etiolated seedling growth at normal and high temperatures. a) 16S rRNA gene sequence based phylogenetic analysis of Ph-04. 16S rRNA reference gene sequences of closely related species within the Bacillus cereus group were used. b) Thermotolerance of Ph-04 strain. Bacterial culture was serially diluted and plated on LB agar plates, incubated at 30 °C, 35 °C, 40 °C, and 45 °C respectively for 48 h and CFU/ml was calculated. c) Control/mock and Ph-04 treated wheat seed germination at 25 °C, 37 °C and 40 °C. Pictures were taken after 4d of incubation in dark. d) Percentage of seed germination of control/mock and Ph-04 treated wheat seeds at 25 °C, 37 °C and 40 °C. n ≥ 60, Error bars represent ±SD. e) Etiolated seedling phenotype of control/mock and Ph-04 treated wheat seedlings growing at 25 °C, 37 °C and 40 °C. n ≥ 60 seedlings. ***p < 0.001, ****p < 0.0001. Error bars represent ±SD.
etiolated seedlings or during the growth of light-grown autotrophic plants as well. To test the effect of Ph-04 treatment on heat stress tolerance of light-grown plants, seven-day-old seedlings of mock-treated and Ph-04 treated seedlings growing at 28 °C were exposed to 42 °C continuously for 6 h and observed the phenotype. Mock-treated plants started to wilt within 3 h and by 6 h completely drooped (Figure 2a). Whereas, Ph-04 treated plants did not wilt or droop even after 6 h of exposure to high temperature, suggesting that Ph-04 can confer heat tolerance not only in etiolated seedlings stage in the dark but also in light-grown plants (Figure 2a).

3.4. Enhanced membrane stability exhibited by Ph-04 treated plants under heat stress

Since Ph-04 treated plants did not wilt or droop at high temperature compared with controls, we reasoned that there must be a difference in membrane stability between the plants. Evans blue was used to determine any increase in membrane damage and permeability immediately after heat stress treatment in mock and Ph-04 treated plants. Cells whose membranes are intact will not take up the Evans blue dye. Under unstressed conditions at 28 °C, no blue coloration was observed in the leaves of all samples (Figure 2b). However, after exposure to heat stress at 42 °C, mock treated plant leaves exhibited strong accumulation of blue color in the leaf tissues, whereas little to no blue coloration was observed in Ph-04 treated plants (Figure 2b). Quantification of membrane damage by measuring the intake of Evans blue per gram fresh weight also showed that membrane damage is significantly less in Ph-04 treated plants compared to the controls. Slightly higher Evans blue content observed in leaves at 28 °C was due to the damage of cells and subsequent uptake of stain when leaves were cut into small pieces for the experiment, and hence it is uniform among the samples. Both qualitative and quantitative assessment of membrane damage with Evans blue show that the membrane integrity of cells was maintained even at a high temperature of 42 °C in Ph-04 treated plants. To further confirm the enhanced membrane stability of Ph-04 treated wheat seedlings under elevated temperature, we performed an electrolyte leakage experiment, which showed that ion leakage is significantly lower in Ph-04 treated plants compared with the control/mock.

3.5. Histochemical staining and accumulation assay for H₂O₂ after heat stress

To understand the reason for the differences in membrane damage between Ph-04 treated and the control plants, we looked at the agents responsible for membrane damage during heat stress. H₂O₂ is one of the Reactive Oxygen Species (ROS) that is generally produced during regular metabolism and can cause oxidative damage to cellular components and the membrane lipids in plant cells at high concentrations. We first analyzed the accumulation of H₂O₂ in heat-stressed and unstressed plant leaves immediately after heat stress using DAB staining (Figure 3a). Strong staining was observed in mock treatment compared to Ph-04 treatment suggesting greater H₂O₂ accumulation in control sample upon heat stress (Figure 3a). At the same time, unstressed plants of mock and Ph-04 treatments at 28 °C did not show any observable difference in DAB staining.

We further quantified the H₂O₂ in plants exposed to heat stress and plants growing at optimum conditions. Interestingly even among plants growing at 28 °C, there were significant differences in the H₂O₂ content between mock-treated and Ph-04 treated plants (Figure 3b). Plants from mock treatment, upon exposed to heat stress showed a two-fold or higher increase in H₂O₂ content compared with unstressed plants, and showed significantly higher H₂O₂ levels than Ph-04 treated plants (Figure 3b). Though the H₂O₂ accumulation slightly increased in Ph-04 treated plants after heat stress, albeit only to a lesser extent than mock/control.

3.6. Ph-04 treated plants exhibit enhanced accumulation of proline, increased activity of CAT and APX

To understand the phenomenon behind the reduced accumulation of H₂O₂ in heat stressed Ph-04 treated plants, and Ph-04 mediated heat stress tolerance, we analyzed the accumulation of proline, and measured the activity of CAT and APX enzymes.

Proline accumulation in response to environmental stress is a common phenomenon observed in plants under oxidative stress. Under high temperatures, proline can alleviate osmotic stress induced by heat. We found that in Ph-04 treated plants exhibited constitutively higher basal proline levels compared to mock treated plants. In Ph-04 treated plants exposed to heat stress, proline content has increased by several fold over unstressed plants and was significantly higher than control (Figure 3c).

We next studied the enzymatic activity of CAT and APX, which play an essential role in H₂O₂ detoxification. Interestingly higher basal activity of both CAT and APX was observed in Ph-04 treated plants under unstressed conditions, which was significantly higher than control plants (Fig. 3d, e). Under heat stress, significantly higher activity levels of both CAT and APX were observed in plants of Ph-04 treatment compared to mock treatment.

3.7. Higher survival percentage and recovery post heat stress in Ph-04 treated plants

We next monitored the recovery of plants post heat stress treatment by moving the plants after heat stress to an optimum temperature of 28 °C and allowed them to recover for a period of five days. The percentage of seedling survival, morphological and physiological differences was recorded after recovery time. There was no significant difference in the percentage of survival between heat-stressed and unstressed plants in Ph-04 treatment (Figure 4a). Whereas plants from mock treatment exhibited a significant decrease in plant survival between unstressed and heat-stressed plants (Figure 4a). Upon morphological observation of survived plants, it was found that many of the mock treated plants exhibited heat burn and curling at the tip of the leaves, whereas no such damage was observed in Ph-04 treated plants (Figure 4b). Since high-temperature stress can induce the degradation of chlorophyll and influence the relative water content (RWC) of plants, we analyzed the RWC (%) and chlorophyll content in heat-stressed plants after recovery. RWC (%) and total chlorophyll content analysis showed a significant decrease in control plants recovered from heat stress compared to unstressed plants. Whereas no such decrease was observed in Ph-04 treated plants (Fig. 4c, d).

4. Discussion

Extreme climate, especially high temperatures can severely reduce wheat yield. We have screened some potential isolates of vertically transmitted bacteria of P. hysterophorus for their ability to confer heat stress tolerance in wheat. One isolate designated as Ph-04 has been selected for its ability to confer high temperature tolerance in wheat and was studied further. Phylogenetic analysis shows that Ph-04 is closely...
related to *Bacillus paramycoides*, a novel species within the *Bacillus cereus* group (Liu et al., 2017). Any increase in air temperature due to climate change will lead to an increase in soil temperature. High soil temperature results in reduced seed germination or reduced seedling vigour and can cause damage to the emerging radicle and plumule. In wheat, greater sowing depth could be required when planting the seeds in dry soil to avoid dehydration (Rebetzke et al., 2016). The length of a coleoptile is considered an important factor in the effective establishment of cereal crops (Rebetzke et al., 2016). When sown deeper or under warmer soil conditions, coleoptile emergence may be delayed or reduced, leading to poor establishment and yield of the crop (Rebetzke et al., 2007). It has been shown that the mean coleoptile length of wheat was drastically reduced with increasing soil temperature (Rebetzke et al., 2016). Enhanced growth of coleoptile and root system was observed in Ph-04 treated seedlings under elevated temperatures compared to mock treatment makes it a potential isolate to overcome the adverse effect of future increasing soil temperatures.

Heat stress directly influences membrane permeability by affecting the fluidity of the membrane and indirectly by producing ROS which acts on membrane lipids, together causing electrolyte leakage and reduced viability of cells. Hence membrane stability and integrity have been correlated with heat stress tolerance (Wahid et al., 2007). Evans blue, having a large molecular mass of 961 g mol$^{-1}$ can only penetrate to the cytosol of cells with enhanced plasma membrane permeability (Huve et al., 2011). Upon heat stress treatment of light-grown plants, Evans blue penetration is relatively very less in Ph-04 treated plants than in control. Electrolyte leakage as a measure of membrane damage has been used to evaluate the thermotolerance in wheat genotypes against heat stress (Blum and Ebercon 1981). Enhanced membrane integrity, evident from reduced electrolyte leakage in Ph-04 treated plants, may explain the observed thermotolerance.

Abiotic stresses such as heat, drought and salinity cause an increase in the level of reactive oxygen species (ROS), including H$_2$O$_2$ and collectively cause oxidative stress. H$_2$O$_2$ has a long half-life compared to other ROS members, and low levels of H$_2$O$_2$ acts as a signaling molecule whereas, greater accumulation of H$_2$O$_2$ leads to membrane lipid peroxidation and cellular toxicity and death (Das and Roychoudhury 2014). When 8d-old wheat seedlings were given heat shock for 2 h at 30 °C, 35 °C, and 40 °C, a gradual increase in the accumulation of H$_2$O$_2$ was observed with highest accumulation observed at 40 °C (Kumar et al., 2012). H$_2$O$_2$ accumulation in populations of ryegrass showed a linear relationship with the extent of physiological damage, and tolerant ryegrass populations have significantly lower accumulation of H$_2$O$_2$ under heat stress (Soliman et al., 2011). In our study, reduced H$_2$O$_2$ accumulation after heat stress in Ph-04 treated plants could explain the minimum membrane damage and enhanced heat stress tolerance compared to control plants. The level of H$_2$O$_2$ accumulation upon heat stress appears to be directly proportional to the observed heat stress tolerance and sensitivity in Ph-04 and mock-treated plants, respectively.

In higher plants, osmoprotectants such as proline, which also acts as a non-enzymatic antioxidant, accumulates as an adaptive mechanism in response to various stress conditions, including high-temperature. Apart from maintaining osmotic balance, osmoprotectant like proline is necessary for maintaining the stability and integrity of enzymes, membranes and buffering the cellular redox potential under high-temperature stress. In our study, a higher basal level of proline was observed in Ph-04 treated seedlings growing at 28 °C, which was significantly higher than

**Figure 2.** Ph-04 protects light grown wheat plants from heat stress. a) Phenotypes of seven-day old control/mock and Ph-04 treated wheat plants growing at 28 °C (Upper panel) and plants after 6h of high temperature exposure at 42 °C (lower panel). b) Evans blue staining of leaves showing loss of membrane integrity. Illustration of Evans blue staining before and after heat stress. c) Quantitative assessment of the degree of Evans blue uptake. Data is from three biological replicates. **p < 0.01, Error bars represent ±SD. Little uptake of Evans blue in Ph-04 treated plants can be observed under heat stress, suggesting greater membrane thermostability. d) Electrolyte leakage assay to evaluate the membrane stability of control/mock and Ph-04 treated wheat plants. Ten-day old seedlings were used. Reduced ion leakage from Ph-04 treated plants can be observed compared to the controls. **p < 0.01. Error bars represent ±SD.
the mock treated plants. Upon heat stress treatment, the proline levels were also increased in plants of mock treatment. However, Ph-04 plants exhibited severalfold higher proline levels than control plants. Several studies supported the protective role of proline against heat stress in wheat. For example, Ahmed and Hasan (2011) studied the effect of high temperature stress on proline accumulation in twenty wheat genotypes at seedling stage. Proline content was similar in both heat tolerant and susceptible genotypes at 25 °C (unstressed condition), however upon exposure to heat stress at 35 °C, a relatively higher accumulation of proline was observed in heat tolerant genotypes (Ahmed and Hasan 2011). Similar, higher proline content was also observed by Katakpara et al. (2016) in heat tolerant wheat genotypes and the authors proposed proline content as a biochemical marker for screening wheat genotypes for heat stress tolerance. Interestingly exogenous supplementation of proline also confers enhanced tolerance to heat stress as observed in chickpea by reducing membrane damage, cellular injury and modulating antioxidant levels (Kaushal et al., 2011).

Increased activity of antioxidant enzymes and lower oxidative damage was observed in some heat-tolerant genotypes of wheat (Sairam et al., 2000; Awasthi et al., 2015). Both catalase (CAT), ascorbate peroxidase (APX) are involved in scavenging H₂O₂. Combined activity of APX and CAT regulates steady-state concentration of H₂O₂ in leaves, thereby determining the magnitude of oxidative damage (Dash and Mohanty 2002). Heat stress tolerance in different wheat genotypes estimated by their ability to sustain photosynthetic efficiency under heat stress, is correlated with capability of APX and CAT to scavenge H₂O₂ co-operatively (Dash and Mohanty 2002). Previous studies on PGPR dependent heat tolerance in wheat also showed upregulation of CAT and APX in bacterial primed plants upon heat stress but not under unstressed conditions (Sarkar et al., 2018). Whereas, in our study Ph-04 treated plants exhibited higher basal activity levels of CAT and APX in unstressed plants apart from exhibiting higher activity upon heat stress. A recent report describing the root endophyte mediated thermotolerance in wheat and Arabidopsis showed that constitutive H3K4me3 modification of heat stress memory genes, including the APX2 gene, was responsible for the thermotolerance (Shekhawat et al., 2021). An increase in proline content and APX activity levels was observed during Bacillus cereus SAI strain mediated heat stress tolerance in soybean, where SAI treated soybean
plants exhibited several folds higher APX activity levels and proline content over untreated control plants under heat stress (Khan et al., 2020). Increased levels of CAT activity and proline content were also observed in Bacillus cereus mediated mitigation of heat stress in tomato (Mukhtar et al., 2020). Proline plays a crucial role in maintaining the protein structure, scavenges ROS and protects the tissue from heat. Further, CAT and APX are important antioxidant enzymes involved in scavenging H$_2$O$_2$ thereby reducing the heat-induced oxidative stress and damage. Thus, having a higher proline level, increased activity of CAT and APX in Ph-04 plants might have protected the plants from heat stress by removing the excess accumulation of H$_2$O$_2$ and maintaining membrane thermostability. Relative water content (RWC) and total chlorophyll content are crucial physiological parameters that reflect the tolerance of plants against heat stress. In our study higher percentage of Ph-04 treated plants survived heat stress and exhibited better physiological parameters post heat stress recovery than mock-treated plants.

5. Conclusions

Treatment of wheat seeds with the bacterial isolate Ph-04 improved germination and etiolated seedling growth at high temperatures. Ph-04 treatment also protected the light-grown wheat seedlings from heat stress-induced membrane damage. Hight-temperature induced accumulation of H$_2$O$_2$ is also significantly reduced in Ph-04 treated plants than control plants. Higher accumulation of proline and increased activity of antioxidant enzymes CAT and APX under heat stress may have protected Ph-04 treated seedlings from high-temperature mediated oxidative damage. Further work is needed to understand the molecular mechanism behind Ph-04 mediated heat stress tolerance in wheat. Plant adaptive mechanisms against heat stress are under multigene control, and hence manipulating these at a molecular level using genomic tools is difficult and time-consuming. Plant-associated microbes that can induce these adaptive mechanisms in non-native host plants, as evident from our study, can improve plant fitness under harsher environments. Our results support that invasive weed can be a good source for identifying beneficial microorganisms suitable for employing in climate resilient agriculture.

Declarations

Author contribution statement

Ankita Dubey, Kundan Kumar: Performed the experiments.
Tantravahi Srinivasan: Contributed reagents, materials, analysis tools or data.
Anil Kondreddy: Conceived and designed the experiments; Analyzed and interpreted the data.
Koppolu Raja Rajesh Kumar: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data associated with this study has been deposited at NCBI under the accession number MZ413335.
Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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