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Role of *Bacillus subtilis BE-L21* in enhancing the heat tolerance of spinach seedlings

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Abstract

Owing to cold resistance and a lack of heat resistance in spinach (*Spinacea oleracea* L.), heat is the primary constraint that limits its production in summer. This study examined the auxiliary effects of spinach rhizosphere microbes on improving the heat resistance of spinach. A strain isolated from the rhizosphere soil of heat-stressed spinach was identified as *Bacillus subtilis* and designated *B. subtilis* BE-L21. It produces indoleacetic acid, amylase, and protease and solubilizes phosphorus. Further research revealed that spinach seedlings inoculated with this strain of *B. subtilis* had increased content of soluble protein, soluble sugar, and proline that adjusted their osmotic potential. The reducing content of malondialdehyde showed alleviated irreversible damage of spinach plants under heat stress. Also the increased activities of antioxidant enzymes peroxidase, superoxide dismutase, and catalase enhanced the heat resistance of spinach. The results indicate that *B. subtilis* BE-L21 can contribute to tolerance of spinach seedlings to elevated temperatures by inducing physiological and biochemical changes in the plant.

Keywords: abiotic stress, heat tolerance, plant-microbe interactions, rhizosphere, spinach.

Introduction

The growth and development of plants primarily depend on the nutrients absorbed and transported by the root system. The area in periphery of the plant root that is about few millimeters is considered to be the site at which the most intensive exchange of substances between microorganisms and the plant root takes place. Plants and microorganisms maintain the relationship of mutual adaptation and symbiosis in the rhizosphere soil that are the result of long-term evolution. The microorganisms convert some key nutrients to a state that enables the plants to absorb and utilize them. In addition, plants secrete some secondary metabolites that are available for microorganisms (Van der Heijden and Hartmann 2016, Fitzpatrick *et al*. 2018, 2020, Cordovez *et al*. 2019). The interplay between the plants and rhizosphere microorganisms is one of the most common interactions (Xiong *et al*. 2021).

Certain soil bacteria can interact with plants and promote their growth, and this group of plant growth-promoting rhizobacteria (PGPR) can act by several mechanisms (Company *et al*. 2005). Plant inoculated with PGPR and other microbes can have improved tolerance towards abiotic stresses, such as high temperature, drought, and salinity (Yang *et al*. 2009, Jiménez Bremont *et al*. 2014, Hartman and Tringe 2019, Wang *et al*. 2021). A number of researchers have reported the use of PGPR to enhance tolerance to heat stress, *e.g.*, in wheat (Abd El-Daim *et al*. 2014, Ashraf *et al*. 2019), rice (Shrivastava 2013), cotton (Nehra *et al*. 2016), and soybean (Khan *et al*. 2020). PGPR synthesize plant hormones, *e.g.*, indoleacetic acid (IAA), which plays a vital role in the growth and development of plants. At the same time, IAA can mediate the tolerance of plants to heat stress through the activation of antioxidant enzymes, gene expression, synthesis of proline, and accumulation of photosynthetic pigments

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Abbreviations: CAT - catalase; f.m. - fresh mass; HS - heat stress; HTC - heat-tolerance coefficient; IAA - indoleacetic acid; MDA - malondialdehyde; POD - peroxidase; PGPR - plant growth-promoting rhizobacteria; Pro - proline; SOD - superoxidase; SP - soluble protein; SS - soluble sugar.
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(Siddiqui *et al*.2017, Khan *et al*. 2020, Park et al. 2017). In addition, the heat shock proteins can be increased to improve the tolerance of plants to heat stress (Ali *et al*. 2009).

Spinach (*Spinacia oleracea* L.), an annual, biennial herb of the *Chenopodiaceae* family, is native to the temperate regions. This crop has advantages, such as a short growth cycle, high multiple cropping index, and enormous nutritional value. Spinach is widely grown in the northern and southern regions of China. It is one of the green leafy vegetables that is the most resistant to cold. However, it is very sensitive to high temperatures (Yan *et al*. 2016) and shows a low germination rate and poor growth under hot weather during the summer in southern China, which severely limits its yield and quality. Thus, research on how to improve the heat resistance of spinach is considered to be a hot topic. Spinach responds to heat stress through several mechanisms (Zhao *et al*. 2018, Li *et al*. 2019). The plant-microbe interactions can enhance plant growth. The interactions between spinach and microorganisms and their role in improving the heat resistance of spinach have not been intensively studied. This study is expected to stimulate new ideas for the heat resistance of spinach and lay the groundwork to grow spinach more extensively.

Materials and methods

Heat stress of spinach and isolation of heat-resistant microorganisms: 15-d-old spinach (*Spinacia oleracea* L.) seedlings were exposed to constant irradiance of 120 µmol m^{-2} s^{-1} and temperatures of 35, 40, and 45°C for 3 h a day for 15 d to isolate heat-resistant bacteria from their roots. A total of 10 g of the rhizosphere soil from spinach that grew well under heat stress was collected and placed in a cool and ventilated place to dry. The soil was transferred to a sterilized conical flask with glass beads, and 90 cm^3 of sterile water was added to prepare a soil suspension, which was heated in an 80°C water bath for 15 min. In order to fully separate soil and microorganisms, the conical flask was placed in a thermostatic oscillator, shaken at 120 rpm, and was enriched and cultured for 24 h at 50°C. The soil suspension was diluted and spread on Lysogeny Broth (LB) media. The plates were transferred to a 50 °C thermostatic incubator and cultured upside down for 24 - 48 h. The colony characteristics were observed, and colonies of different shapes, colors, and sizes were repeatedly streaked on LB solid media, transferred to a 50°C thermostatic incubator, cultured upside down for 24 h, and continuously separated and purified until the colors, sizes, and shapes of the colonies on the medium were exactly consistent. At this time, they were assigned numbers and stored at -80ºC with glycerol for potential use.

Morphological identification of heat-resistant microorganisms in the rhizosphere: The isolated strains were inoculated on LB solid media by plate streaking and then cultured in a 37°C thermostatic incubator. The shape, color, size, wrinkle, uniformity of edge, bulges, and wetness of single colonies were observed and noted.

Molecular biological identification of heat-resistant microorganisms in the rhizosphere: The DNA from the strain isolated was extracted using a bacterial genome DNA extraction kit (*CWBIO*, *Cowin Biosciences*, Cambridge, MA, USA) according to the manufacturer's instructions. Primer F: 5'-AGAGTTTGATCCTGGCTCAG-3' was the upstream primer and primer R: 5'-AAGGAGGTGATCCAACC-3' was the downstream primer of the 16S rDNA that was used for amplification. The PCR reaction conditions were as follows: pre-denaturation at 94°C for 2 min, denaturation at 94°C for 30 s, annealing at 59°C for 30 s, extension at 72°C for 30 s, amplification for 35 cycles, and storage at 4°C after a 10 min hold at 72°C. The PCR amplification products were isolated using 1 % (m/v) agarose gel electrophoresis. The 1 500 bp clear bands obtained were sequenced by *Tinge Biotechnology Company* (Harbin, China).

Screening of the growth-promoting strains of heat-resistant microorganisms: The production of indolyl acetic acid (IAA) was determined by the Salkowski colorimetric technique as described by Luo *et al*. (2018). The strains were inoculated into liquid media that contained 2 mg cm^{-3} L-tryptophan and cultured at 50 °C and 120 rpm for 3 d. The culture was centrifuged at 8 815 *g* for 10 min, and 100 mm^3 of supernatant and 200 mm^3 of Salkowski colorimetric solution were taken and mixed onto a white ceramic plate with non-inoculated LB liquid media that was used as the blank control. The mixture was incubated for 25 min in the dark at room temperature. The appearance of pink color indicated the presence of IAA, and the strain was designated as positive for IAA production. If there was no change of color, the strain could not produce IAA and was designated as negative for IAA production. The absorbance of the positive strain was determined at 530 nm. The IAA concentration of each strain was calculated in µg cm^{-3} from the standard curve.

The solubilization of phosphate was measured using Yang's transparent circle technique (Yang *et al*. 2018). Diameter D of the transparent circle and diameter d of the colony of strains that produced the transparent circle were measured to calculate the ratio R (D/d) of the transparent circle diameter to the colony diameter. Quantification of the level of phosphate solubilization of each strain was based on the R value. The strains were inoculated on Pecoski's (PKO) solid medium using the point inoculation technique. Three replicates of each strain were cultured upside down at 50 °C for 3 d to determine whether a transparent circle appeared around the colony. The solubilization index based on colony diameter as an indicator of phosphate solubilization was positive in the isolate *Busuttil's BE-L21*.

The presence of amylase was determined by Scherling's transparent circle method (Scherling *et al*. 2009). The ratio R (D/d) of the transparent circle diameter to the colony diameter was measured and served as the indicator of whether the strain could produce amylase. Three replicates of each strain were inoculated on the LB solid medium that

contained 0.2% soluble starch using the point inoculation technique and cultured upside down in a 50°C thermostatic incubator for 5 d. Iodine was dripped onto the medium. The appearance of a transparent circle around the colony indicated the production of amylase. Strains that did not produce this circle were considered to be negative for amylase production.

The production of protease was also determined by Scherling's transparent circle method as described above. Three replicates of each strain were inoculated on skim milk agar (SMA) for 2 d and observed to determine whether a transparent circle appeared around the strain. The appearance of a transparent circle indicated the production of protease.

Effects of rhizospheric microorganisms on the heat resistance of spinach seedlings: Sterile tweezers were used to carefully insert plant spinach seeds in sterile cultivation media (humus soil:*Vermiculite* 2:1). Twenty spinach seeds were planted per pot, and there were three replicates. The seeds were cultivated under a 12-h photoperiod, irradiance of 120 μmol m^{-2} s^{-1}, temperatures of 24/18°C (day/night) and 50% humidity in a greenhouse. Freshly diluted bacterial culture was inoculated to each pot, while autoclaved double distilled water was used for control spinach plants. When the spinach seedlings had 4 - 5 true leaves, they were transferred to a thermostatic incubator for heat stress treatment at 40°C with a 12-h photoperiod. The seedlings were treated for 0, 6, 12, 24, and 36 h. There were four experimental groups and three biological replicates for each group. The treatments included a control (Group 1), heat stress treatment (HS) (Group 2), isolated heat-resistant strains (BE-L21) (Group 3), and isolated heat-resistant strains and heat stress treatment (BE-L21+HS) (Group 4). After heat stress, the spinach seedlings were carefully removed from the sterilized cultivation substrate. The surface of the spinach seedlings was quickly washed with RNase-free water, wiped dry, and then transferred and stored at -80°C for the subsequent determination of physiological and biochemical indicators.

Determination of physiological and biochemical indicators: The content of osmotically active substances were measured using Coomassie brilliant blue for soluble protein (SP) and sulfuric acid-anthrone for soluble sugar (SS) (Wang *et al*. 2010). The content of proline (Pro) was measured using the ninhydrin method (Wang *et al*. 2010). The content of malondialdehyde (MDA) was determined using the thiobarbituric acid method (Lee *et al*. 2007, Wang *et al*. 2010). The activities of antioxidant enzymes were measured using guaiacol as the substrate for peroxidase (POD), the nitroblue tetrazolium (NBT) photochemical reduction method for superoxide dismutase (SOD), and UV absorption for catalase (CAT) (Yin *et al*. 2017).

Statistical analysis: The result were obtained from at least three biological replicates, and each experiment was repeated at least three times. Data were expressed as means ± standard deviations (SDs). The data were processed by *Microsoft Excel 2010* (Redmond, WA, USA), and an analysis of variance (*ANOVA*) was performed on the plant physiological characteristics using *SPSS 21.0* (*IBM*, Armonk, NY, USA). The significance of differences was assessed by Fisher's least significant differences (LSD) test. For figures *GraphPad Prism 5.0* software (San Diego, USA) was used.

Results

A strain of microorganism was screened in the rhizosphere soil after spinach had been subjected to heat stress. Its colony appeared to be round, creamy white, small, and moist, with a smooth surface, irregular edges, and no protrusions, and was identified as a Gram-positive bacterium by Gram staining. A sequence analysis of 16S rDNA and a comparison of 16S rDNA genetic evolution with 15 species of bacteria identified the closest phylogeny as that of *Bacillus subtilis* (Fig.1). Thus, this bacterium was designated as *B. subtilis* BE-L21.

In this study, the ability of BE-L21 to produce IAA was determined using Salkowski colorimetry, which resulted in the production of pink color, suggesting that BE-L21 can produce IAA. The concentration of secreted IAA was determined to be 12.29 ± 0.80 μg cm^{-3} using a standard curve (y = 0.028x - 0.0392, R^2 = 0.9942).

The phosphate solubilization of BE-L21 was also determined, and a transparent circle was formed on the PKO agar plate, suggesting that such strain can solubilize phosphate. The R value was identified as 2.06 ± 0.18, showed the capability for phosphate solubilization (Fig. 2).

Plant growth promoters can secrete extracellular enzymes, including amylase and protease. In this study, incubation with LB solid media that contained 0.2 % soluble starch was not stained blue with iodine, suggesting that the strain had amylase activity. The R value was identified as 2.27 ± 0.16, which indicated that the strain produced amylase (Fig. 2).

For the determination of protease, a transparent circle was formed on the skim milk plate, suggesting that the strain produced protease (the R value was identified as 3.13 ± 0.58) (Fig. 2).

The effects of heat stress on the content of soluble protein (SP) in spinach seedlings are shown in Fig. 3*A*. The content of SP in spinach seedlings decreased and then increased over time when subjected to a single heat stress condition. The minimum value appeared at 6 h, and the content of SP at that time was 2.19 ± 0.18 mg g^{-1}(f.m.). When the spinach was subjected to heat stress in the presence of BE-L21, the content of SP in spinach seedlings decreased and increased over time. The minimal value appeared at 6 h, and the content of SP at that time was 5.21 ± 0.31 mg g^{-1}(f.m.). The maximum value appeared at 36 h, and the content of SP at that time was 10.29 ± 0.56 mg g^{-1}(f.m.).

Comparing of Group 1 (control) and Group 3 (BE-L21), the content of SP of spinach seedlings in Group 3 was 1.41-fold of that in Group 1. The results showed that the addition of BE-L21 could increase the content of SP in

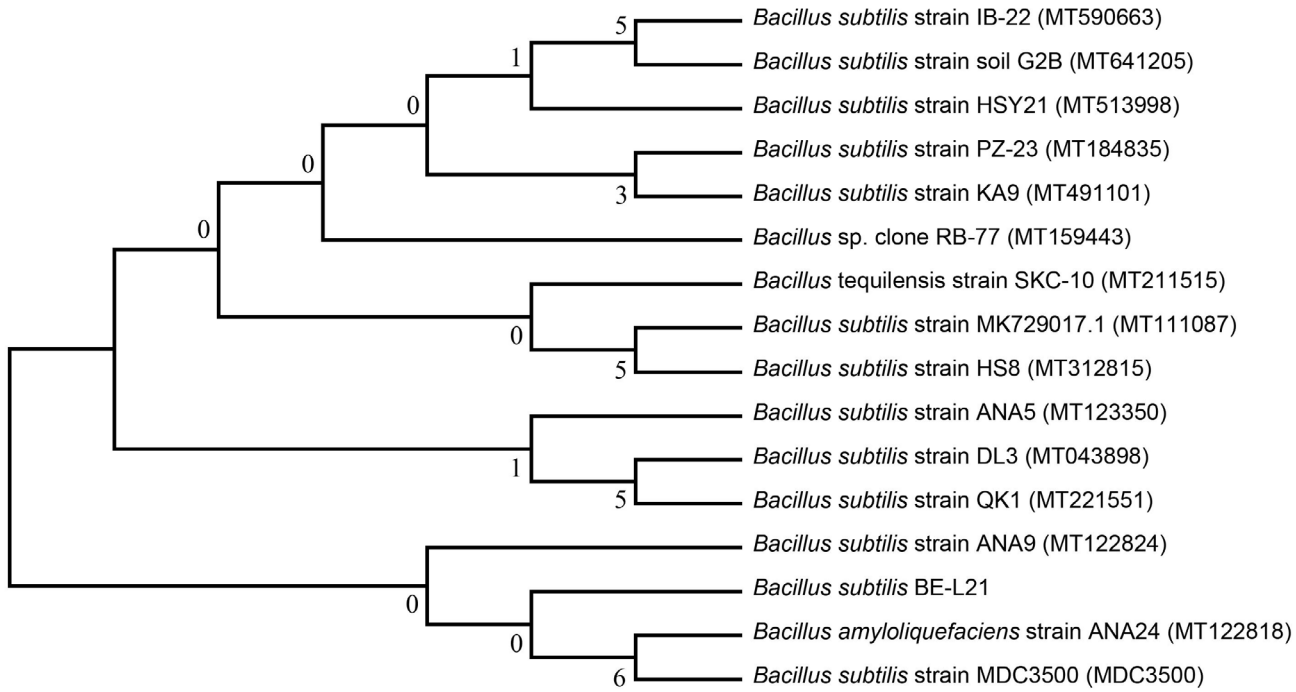

Fig. 1. Phylogenetic tree based on 16S rDNA sequences. The tree was generated by *MEGA 5.0* software using the neighbor-joining method following *Clustal X*. The scale bar indicates an evolutionary distance of 0.01 amino acid substitution per position in the sequence. Bootstrap values are indicated (500 replicates). Accession No. MT590663 (*Bacillus subtilis* strain IB-22), MT641205 (*Bacillus subtilis* strain soil G2B), MT513998 (*Bacillus subtilis* strain HSY21), MT184835 (*Bacillus subtilis* strain PZ-23), MT491101 (*Bacillus subtilis* strain KA9), MT159443 (*Bacillus sp.* clone RB-77), MT211515 (*Bacillus tequilensis* strain SKC-10), MT111087 (*Bacillus subtilis* strain MK729017.1), MT312815 (*Bacillus subtilis* strain HS8), MT123350 (*Bacillus subtilis* strain ANA5), MT043898 (*Bacillus subtilis* strain DL3), MT221551 (*Bacillus subtilis* strain QK1), MT122824 (*Bacillus subtilis* strain ANA9), MT122818 (*Bacillus amyloliquefaciens* strain ANA24), MT534524 (*Bacillus subtilis* strain MDC3500).

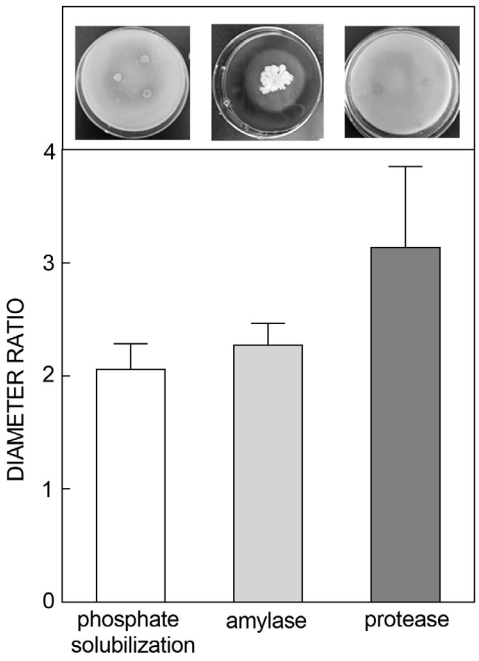

Fig. 2. Detection of phosphorus solubilization, amylase production, and protease production in *BE-L21*. Means ± SDs ($n = 3$).

spinach seedlings. Comparing Group 1 and Group 2 (HS), the content of SP of spinach seedlings in Group 2 was found to be 0.37 - 0.93 folds of that in Group 1. Except for 36 h of heat stress, in the other time points SP decreased significantly.

A comparison of Group 2 (HS) and Group 4 (BE-L21+HS) revealed that the content of SP in the spinach seedlings in Group 4 was 1.32- to 2.38-fold of that in Group 2 (HS) with the maximum value appearing at 36 h of HS. Thus, the addition of BE-L21 significantly increased the content of SP in the spinach seedlings when subjected to heat stress.

The effects of heat stress on the content of SS in spinach seedlings are shown in Fig. 3*B*. The content of SS in spinach seedlings decreased and then increased over time when subjected to a single heat stress. The minimum value appeared at 6 h, and the content of SS at that time was 0.57 ± 0.03 μg g^{-1}(f.m.). Under heat stress and treatment with BE-L21 the content of SS in spinach seedlings decreased and then increased over time. The minimum value appeared at 6 h, and the content of SS at that time was 0.58 ± 0.06 μg g^{-1}(f.m.). The maximum value of 1.37 ± 0.09 μg g^{-1}(f.m.) appeared at 36 h. The content of SS of the spinach seedlings in Group 3 (BE-L21) was 1.40-fold of that in Group 1 (control), and in Group 4 (BE-L21+HS) it was 1.01- to 1.79-fold of that in Group 2 (HS). This indicated that the addition of BE-L21 under heat stress increased the content of SS in spinach seedlings.

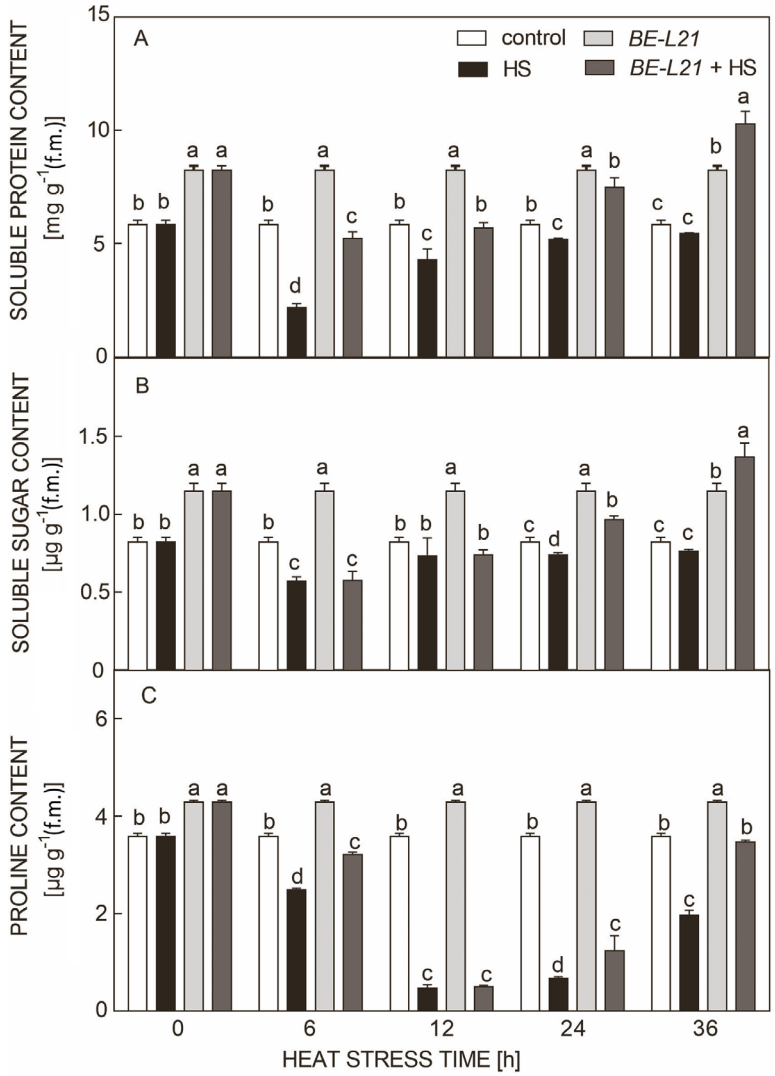

Fig. 3. The accumulation of osmolytes in spinach seedlings in not stressed (control), heat stressed (HS), treated with *B. subtillis BE-L21*, and heat stressed with *BE-L21* (BE-L21+HS). *A* - soluble protein, *B* - soluble sugar, *C* - proline. The values were determined after the plants had been subjected to heat stress at 40°C for 0, 6, 12, 24, and 36 h. Means ± SDs (n = 3). The different small letters indicate a significant difference ($P < 0.05$) between the different treatments.

The effects of heat stress on the content of Pro in spinach seedlings are shown in Fig. 3*C*. The content of Pro in spinach seedlings decreased and then increased over time when subjected to a single heat stress. The minimum value appeared at 12 h, and the content of Pro at that time was 0.46 ± 0.07 μg g^{-1}(f.m.). The maximum value appeared at 6 h, and the content of Pro at that time was 2.48 ± 0.04 μg g^{-1}(f.m.). The content of Pro in spinach seedlings that were subjected to heat stress and treated with BE-L21 increased to maximum of 3.47 ± 0.04 μg g^{-1}(f.m.) at 6 h and then decreased and the minimum value of 0.50 ± 0.02 μg g^{-1}(f.m.) appeared at 12 h.

The effects of heat stress on the content of MDA in spinach seedlings are shown in Fig. 4. Compared with Group 1, MDA was significantly increased by 1.68-fold at 24 h under heat stress with (Group 2). Compared with Group 2 (HS), MDA was significantly decreased by 0.21-fold at 36 h in Group 4 (BE-L21+HS).

The effects of heat stress on the activity of POD in spinach seedlings are shown in Fig. 5*A*. The activity of POD in spinach seedlings decreased and then increased over time when the plants were subjected to a single heat stress. The minimum value appeared at 12 h, and the activity of POD at that time was 195 ± 2.89 U g^{-1}(f.m.) min^{-1}. When subjected to heat stress and treated with BE-L21, the activity of POD in spinach seedlings decreased and then increased over time. The minimum value appeared at 12 h, and the activity of POD at that time was 233.33 ± 1.67 U g^{-1}(f.m.) min^{-1}. The activity of POD was decreased by 0.77 - 0.92-fold after heat stress. Inoculation with the BE-L21 increased POD activity 1.19 - 1.71-fold.

The effects of heat stress on the activity of SOD in spinach seedlings are shown in Fig. 5*B*. The activity of SOD in the spinach seedlings decreased and then increased over time when the plants were subjected to a single heat stress. The minimum value appeared at 12 h, and the activity of SOD at that time was 180.12 ± 7.30 U g^{-1}(f.m.).

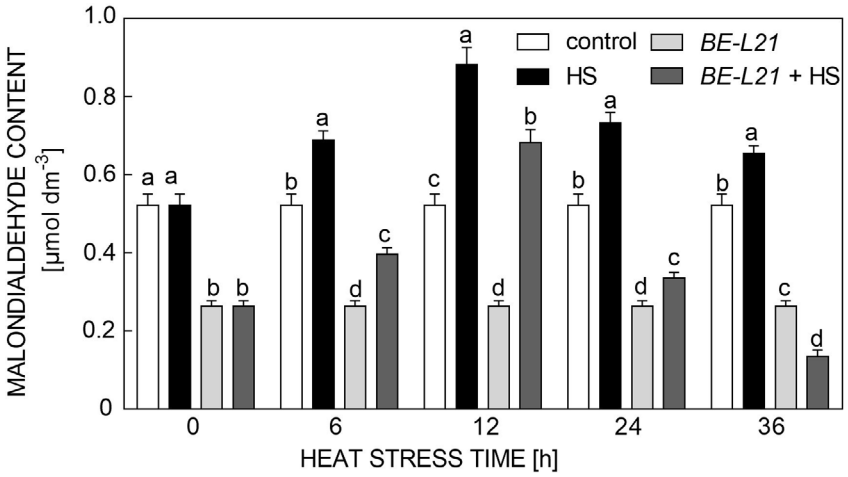

Fig. 4. Content of malondialdehyde in spinach seedlings that were not heat stressed (control), heat stressed (HS), treated with *BE-L21*, and heat stressed with *BE-L21* (*BE-L21*+HS). The values were determined after the plants had been subjected to heat stress at 40°C for 0, 6, 12, 24, and 36 h. Means ± SD (n = 3). The different small letters indicate a significant difference ($P < 0.05$) between the different treatments.

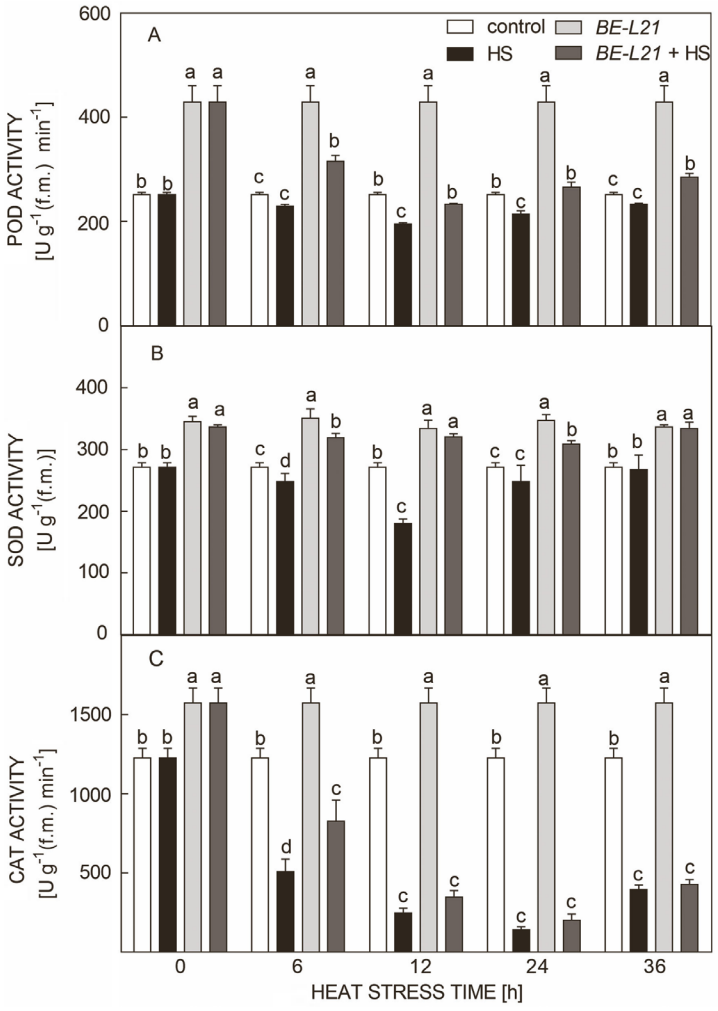

Fig. 5. The effect of plant growth promoting rhizobacteria on POD activity (*A*), SOD activity (*B*), and CAT activity (*C*) under unstressed (control), heat stressed (HS), treatment with *BE-L21* bacteria, and heat stressed with *BE-L21* bacteria. The values were determined after the plants were treated with heat stress at 40°C for 0, 6, 12, 24, and 36 h. Means ± SDs (n = 3). The different small letters indicate a significant difference ($P < 0.05$) among different treatments. CAT - catalase, POD - peroxidase; SOD - superoxide dismutase.

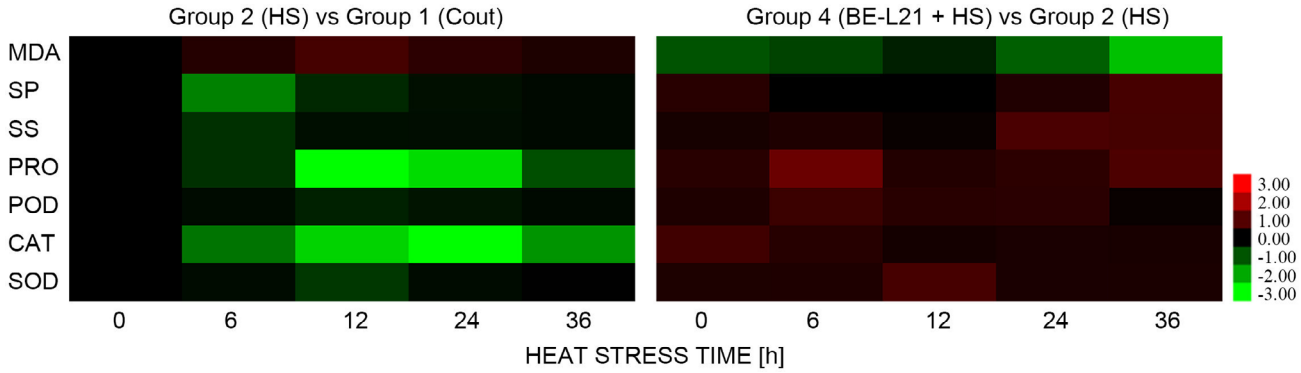

Fig. 6. Comparison of physiological data of spinach seedlings under different treatments for 0, 6, 12, 24, and 36 h. The rows represent individual physiological data. The increased or decreased data were indicated in red or green, respectively. The color intensity increases with increasing abundance, as shown in the scale bar. The scale bar indicates log (base2) transformed relative physiological data abundance ratios ranging from -3.00 to 3.00.

Table 1. Heat-tolerance coefficient of individual parameters (soluble protein, soluble sugar, malondialdehyde, proline, peroxidase, superoxid dismutase, and catalase) of spinach seedlings.

Treatment	Time [h]	SP	SS	MDA	Pro	POD	SOD	CAT	Mean
HS	6	0.374	0.693	1.319	0.695	0.914	0.914	0.413	0.760
	12	0.734	0.893	1.690	0.130	0.777	0.664	0.201	0.727
	24	0.887	0.901	1.405	0.188	0.854	0.914	0.114	0.752
	36	0.932	0.931	1.254	0.549	0.927	0.987	0.321	0.843
HS+ *BE-L21*	6	0.892	0.701	0.760	0.898	1.257	1.177	0.674	0.908
	12	0.973	0.901	1.308	0.140	0.929	1.181	0.283	0.816
	24	1.284	1.177	0.642	0.345	1.060	1.138	0.163	0.830
	36	1.760	1.663	0.257	0.970	1.135	1.233	0.348	1.052

When subjected to heat stress and treated with BE-L21, the activity of SOD in spinach seedlings was found to decrease and then increase over time. The minimum value appeared at 24 h, and the activity of SOD at that time was 308.77 ± 6.07 U g^{-1}(f.m.). The activity of SOD was significantly decreased by 0.66 - 0.91-fold after heat stress. Inoculation with BE-L21 markedly increased it to 1.25 - 1.77-fold.

The effects of heat stress on the activity of CAT in the spinach seedlings are shown in Fig. 5C. The activity of CAT in the spinach seedlings decreased and increased over time when the plants were subjected to a single heat stress. The minimum value appeared at 24 h, and the activity of CAT at that time was 140.0 ± 10.00 U g^{-1}(f.m.) min^{-1}. When subjected to heat stress and treated with BE-L21, the activity of CAT in the spinach seedlings decreased and then increased over time. The minimum value appeared at 24 h, and the activity of CAT at that time was 200.0 ± 8.67 U g^{-1}(f.m.) min^{-1}. The activity of CAT was significantly decreased by 0.11 - 0.41-fold after heat stress. Inoculation with BE-L21 increased it 1.08 - 1.63-fold.

Comparison of all data between Group 1 (control) and Group 2 (HS) and between Group 2 (HS) and Group 4 (BE-L21+HS) is in Fig. 6. Compared to Groups 1, content of SP, SS, Pro and activities of POD, CAT, and SOD decreased in Group 2. However, except MDA, content of SP, SS, Pro and activities of POD, CAT, and SOD were higher in Group 4 (BE-L21+HS) than in Group 2. This indicated that the addition of BE-L21 to plants subjected to heat stress enhanced the heat tolerance of spinach seedlings.

Heat tolerance coefficient = (the average measured value under heat stress/the average measured value of the control group) × 100%. The variation in heat-tolerance coefficient can reflect the tolerance of a plant subjected to heat stress (Table 1). Under heat stress (Group 2), all the indices of spinach seedlings treated with BE-L21 (Group 4) were changed: SP, MDA, and CAT decreased and then increased; Pro and POD increased and then decreased, and SS and SOD increased. These changes suggest that spinach seedlings could resist heat due to plant-microbe interactions.

The range of variation of the heat-tolerance coefficient with respect to the indices of both types of spinach seedlings enabled the ranking of the sensitivity of each single index to heat stress in descending order: SS>Pro>SP>MDA>SOD>POD>CAT. Of all the indices, SS and Pro were more sensitive to heat stress, but CAT was less sensitive (Table 1). According to the heat-tolerance coefficient of spinach seedlings (Table 1), the mean value of heat-tolerance coefficient of each time point in Group 4 (BE-L21+HS) was higher than that in Group 2 (HS),

suggesting that the addition of heat-tolerant strain BE-L21 can result in plant-microbe interactions that improve the heat tolerance of spinach seedlings subjected to heat stress.

Discussion

At present, there is increasing attention on PGPR as an alternative method to alleviate heat stress in plants. We isolated and identified thermotolerant *B. subtilis* BE-L21 as a bacterium that could produce biologically active metabolites, such as IAA, amylase, protease, and help to phosphorus solubilization. In this study, we used spinach and *B. subtilis* BE-L21 to test plant-microbial interaction under heat stress. By measuring physiological and biochemical indexes in 4 groups of differently treated spinach plants, the role of PGRR in adaptation to heat stress was elucidated.

Compared to Group 1 (control), SP, SS, and Pro content was increased by adding *B. subtilis* BE-L21. Positive effects on growth have also been shown in studies of *Oryza sativa*, *Triticum aestivum*, and *Zea mays* (Bashan *et al*. 2006, Adesemoye *et al*. 2008, Othman and Panhwar 2014, Orozco-Mosqueda *et al*. 2018, Khan *et al*. 2020).

Under heat stress, the accumulation of Pro and SS is beneficial to antioxidant and subcellular structure stabilization (Wahid and Close 2007, Hayat *et al*. 2012). Studies on spinach (Li *et al*. 2019) and *Nicotiana tabacum* (Cvikrova *et al*. 2012) have shown that the heat stress-induced accumulation of soluble sugars and proline plays an important role in heat-stress tolerance. In this study, the content of Pro and SS was increased in Groups 4 (BE-L21+HS) compared with Group 2 (HS). These results suggest that *B. subtilis* BE-L21 can help spinach respond to heat stress by accumulation of osmoprotectants and non-enzymatic antioxidants.

Under heat stress, the reactive oxygen species (ROS) in the plant cells extensively accumulate and cause the peroxidation of membrane lipids, which results in oxidative stress. The heat resistance of plant would be enhanced by inducing changes in the activities of antioxidant enzymes. In this study, the addition of *B. subtilis* BE-L21 improved the heat resistance of spinach seedlings under heat stress by enhancing the activities of POD, SOD, and CAT. Ali *et al*. (2009) isolated a heat-resistant *Pseudomonas* strain AKM-P6 from the rhizosphere that promoted growth of pigeon peas under semiarid conditions in India. They found that root and aboveground biomass of the inoculated seedlings was significantly higher than that of the uninoculated control.

The variation in heat-tolerance coefficient can reflect the tolerance of a plant subjected to heat stress conditions. Compared with Group 1 (control), all the indices of spinach seedlings subjected to heat stress changed to varying degrees. Comprehensive analysis of the heat-tolerance coefficient of spinach seedlings showed that the mean value of heat-tolerance coefficient in each time point in Group 4 (HS + BE-L21) was higher than that in Group 2 (HS). These results indicated that the addition of heat-tolerant strain BE-L21 improved the heat tolerance of spinach seedlings under heat stress. Abd El-Daim *et al*. (2014) applied short heat stress treatments on the seeds and seedlings of wheat (cvs. Olivin and Sids1) following treatment with *Bacillus amyloliquefaciens* UCMB5113 or *Azospirillum brasilense* NO40. The results showed that the improvement of heat resistance of the wheat cultivars appeared to be associated with a decrease in the production of ROS. At the same time, there were some slight changes in metabolite production.

In summary, we explored the interaction between spinach and microorganisms to improve the heat resistance of spinach seedlings. Future studies should be done on *B. subtilis* BE-L21 strain and how the increased heat resistance observed in this study translates to plant viability. This study is expected to generate novel ideas for the heat resistance of spinach and pave the way for the extensive cultivation of spinach.

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