biologia plantarum
an international journal for experimental botany

BIOLOGIA PLANTARUM (2023) 67: 213-223 DOI: 10.32615/bp.2023.022

Gene expression and biochemical profiling in the mitigation of heat stress in common bean using *Bacillus subtilis*

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Abstract

The present work aimed to evaluate the effect of heat stress on common bean (*Phaseolus vulgaris* L.) genotypes during the reproductive phase as a function of the inoculation of plants with *Bacillus subtilis*. The treatments were established by inoculating two strains of *B. subtilis* (AP-3 and AP-12) and a control. The plants were subjected to heat stress when they reached the reproductive stage, with an increase in temperature to 28/33°C. The duration of the stress period was ten days. Flowering, biochemical, and gene expression evaluations were performed. There was the interaction of *B. subtilis* AP-3 with the bean cultivar IAC-Imperador, reducing flower abortion, promoting the formation of new flower buds, and increasing the content of proline and guaiacol peroxidase activity in plant tissues. However, there was a reduction of transcription of genes encoding the 1-carboxylic acid-1aminocyclopropane oxidase and ethylene response factors and an increase of the Δ^1-pyrroline-5-carboxylate synthetase1 gene. These results suggest that *B. subtilis* may modulate some metabolic pathways in response to high-temperature stress during the reproductive phase of the common bean. This also confirms that *Bacillus* strains represent a useful option to moderate abiotic stresses.

Keywords: gene expression, *Phaseolus vulgaris* L., plant growth-promoting rhizobacteria, stress tolerance.

Introduction

As a short-cycle crop, the common bean (*Phaseolus vulgaris*) is often subjected to environmental variations (Hoffmann *et al*. 2007). Recent climate modelling studies estimate that the increase in global temperature will cause negative and gradual impacts on the crop until the year 2050, leading to a drastic reduction in the cultivated area (CGIAR 2015). High temperatures (> 30°C during the day and/or > 20°C at night) during reproductive development reduce bean productivity, affecting flowering and, therefore, pod production and grain filling, which leads to restriction of summer bean cultivation in tropical regions (Rainey and Griffiths 2005, Bita and Gerats 2013).

Among bean genotypes, we can find cultivars with higher or lower tolerance to heat stress (Silva *et al*. 2020). Elevated temperatures cause biochemical, physiological, molecular, and morphological changes, and their effects may be related to the reduction of photoassimilates, the decrease in metabolic activity, and the imbalance of plant hormones (Rainey and Griffiths 2005, Saini *et al*. 2022). Due to heat stress, plants can suffer oxidative damage, which can be seen in the enzymatic and non-enzymatic responses (Mittler 2002, Baxter *et al.* 2014, Chandra *et al*. 2018). At the molecular level, heat stress can trigger changes that include the alteration of genes involved in physiological protection. Some of these genes are responsible for the expression of osmoprotectants,

Received 1 July 2022, *last revision* 15 May 2023, *accepted* 16 May 2023.
Abbreviations: ACC - 1-carboxylic acid-1-aminocyclopropane; ACCS - ACC synthase; ACO - ACC oxidase; ERF - ethylene response factors; GPX - guaiacol peroxidase; HSP - heat stress protein; HSR - heat stress responsive; P5CS1 - Δ^1-pyrroline-5-carboxylate synthetase1; PGPR - plant growth-promoting rhizobacteria; ROS - reactive oxygen species; SPAD - soil plant analysis development.
Acknowledgements: This study was funded by the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP Grant 2017/16202-2 and 2021/10823-0). We appreciate the collaboration of Dr. Valdemiro Pereira de Carvalho Júnior, Universidade Estadual Paulista (UNESP) in the analysis of ethylene. Fabio F. Araujo thanks CNPq for their fellowship of research.
Conflict of interest: The authors declare that they have no conflict of interest.

such as proline (Ghosh *et al.* 2021), other are heat stress-responsive (*HSR*) genes such as chaperones and ROS scavengers (Ding *et al.* 2020), and the transcription factors including heat shock factors (*HSF*), *WORK*, *MYB*, *NAC*, *DREB*, and *bHLH* that regulate the expression of heat shock proteins (HSPs), and stress-induced proteins (Haider *et al.* 2022).

HSFs and other molecular chaperone proteins that play a specific role in proteostasis regulation in plant cells at high temperatures directly target HSPs (Zhou *et al.* 2022). HSP90 and HSP70 are negative regulators of heat stress transcription factors (HSFs), which are necessary for heat stress responses such as activating a set of heat stress genes encoding molecular chaperones (Hahn *et al.* 2011). Chaperones associated with accessory proteins, such as kinases, ubiquitin ligases, and transcription factors, are required to guide correct protein folding and maintain protein stability, especially under stressful conditions, and they are thus essential for cell survival (Haase and Fitze 2016). *Arabidopsis thaliana* has been used as a model to describe the roles of HSP90 under stress conditions, including binding damaged proteins and guiding them for refolding or degradation, regulation of heat-shock gene induction, and alteration of gene expression system (Kozeko 2019). HSP90 also mediated the phosphorylation and activation of some MAP kinases that, under heat stress, are reduced, affecting the stomatal differentiation and consequently the plant development (Samakovli *et al.* 2020).

Ethylene is one of the hormones involved in plant stress responses, especially due to abiotic factors such as drought, flooding, high temperatures, *etc*. Ethylene is formed from the conversion of S-adenosyl-methionine (SAM) into 1-carboxylic acid-1-aminocyclopropane (ACC) under the action of ACC synthase (ACCS) (Adams and Yang 1979). This hormone is capable of activating the plant's defenses, such as the production of phytoalexins (Fan *et al.* 2000), in the same way, that it is essential for fruit ripening and senescence (Vilas Boas 2002).

The plant rhizosphere is the natural habitat of several species of rhizobacteria that have the potential to improve plant development and growth, in addition to contributing to mediate biological activities (Hassan *et al.* 2019). Plant growth-promoting rhizobacteria (PGPR) have been extensively studied in recent decades, showing promising results for agricultural crops (Araujo 2008, Kumar *et al.* 2011, Mendis *et al.* 2018, Ayuso-Calles *et al.* 2021). Inoculation with PGPR can increase plant tolerance to different abiotic stresses (Goswami and Deka 2020). Among the PGPR genera found in the rhizosphere, the genus *Bacillus* is characterized as a biocontrol agent and biofertilizer, which induces systemic resistance in plants (Guo *et al.* 2019). In addition, it contributes positively to the development and growth of the plant (Lastochkina 2019). Research shows the existence of *Bacillus* species that helped in the growth and development of some crops under water and salt stresses, such as soybean (El-Esawi *et al.* 2018), pepper (Wang *et al.* 2018), and wheat (Ibarra-Villarreal *et al.* 2021). *Bacillus* strains can produce ACC deaminase causing a “cascade effect” such as reduced ethylene content and oxidative cell damage, benefiting the plant's developmental process under unfavorable environmental conditions (Misra and Chauhan 2020, Singh *et al.* 2021).

Thus, the present study aimed to evaluate the potential of *B. subtilis* in the modulation of gene expression and biochemistry to relieve the stress caused by the increase in temperature, during the flowering of common bean (*Phaseolus vulgaris* L). The results obtained in this study can generate new points of view on the role of *Bacillus subtilis* in the regulatory and metabolic mechanisms of common beans cultivated under high temperature.

Materials and methods

Experimental design: The experimental design applied was the factorial (3 × 2) completely randomized, composed of three treatments: *Bacillus subtilis* lineage AP-3, *B. subtilis* lineage AP-12, control treatment (no *B. subtilis* inoculation) and two bean cultivars: IAC-Imperador (early cycle) and TAA-Dama (intermediate cycle), with six replications. The experimental arrangement consisted of plastic pots with a capacity of 3 L. The soil used for cultivation was the commercial substrate *Carolina* (700 g per pot). The seeds were disinfected in sodium hypochlorite solution (2%) diluted in water and then rinsed in running water. Subsequently, they were treated at a dose of 250 mL for 50 kg of seeds, with the commercial liquid inoculant for common bean (*Phaseolus vulgaris* L.), composed of *Rhizobium tropici* (SEMIA 4077 and SEMIA 4088). The plants were grown in a plant growth chamber (*Fitotron*, *Weiss Technik*, London, UK), with controlled temperature, humidity, and photoperiod. The radiation emission was through fluorescent lamps that provided a total of 350 W m^{-2}. The photoperiod was 16 h with an irradiance of 300 µmol m^{-2} s^{-1}. The initial temperature of cultivation was adjusted to 20°C at night and 25°C day and the relative humidity was set at 60%. The plants were grown for 55 d.

Selection of *B. subtilis* strains and application in cultivars: To select a strain to be used in the research, we evaluated seven strains of *B. subtilis* belonging to the bacterial culture collection of the Soil Microbiology Laboratory of the University of West Paulista, located in Presidente Prudente, SP, Brazil. In this, one criterion of higher and lower activity of ACC deaminase *in vitro* was used, according to Lucon *et al.* (2008). The growth capacity of the strains in the presence of ACC was verified in a liquid medium incubated at 48 to 27°C, under constant agitation. Strain *B. subtilis* AP-12 was selected, with high growth performance, and the AP-3 strain was selected due to its role in minimizing the effects caused by abiotic stress and promoting plant growth in previous studies (Araújo *et al.* 2005, Lima *et al.* 2019). *Bacillus* species were grown in Petri dishes with a solid nutrient agar culture medium (3 g L^{-1} yeast extract, 5 g L^{-1} peptone, and 20 g L^{-1} agar-agar). The plates were kept in a bacterial oven for 48 h at 32°C. Then, bacterial colonies were about 100 g and were immersed in 40 mL of saline water ($MgSO_4$ + 7 H_2O,

0.01 M). Subsequently, the solution was stirred in *Vortex*™ for total dispersion and the bacterial solution was diluted. At the time of sowing, 0.1 mL of a solution containing 1.0×10^9 cells per mL of each of the *Bacillus* species was applied to the seeds. The pots were stored in the *Fitotron*® and irrigated periodically. After seedling emergence, thinning was performed, leaving only one seedling per pot.

Imposition of heat stress and collection parameter: The temperature increase in bean plants started during the reproductive stage of the plant (R5 to R7), characterized by the development of flower buds up to pod formation. The temperature inside the *Fitotron*® was raised from 20/25°C to 28/33°C night/day for ten days. In the other phenological stages of development, the temperature was maintained at 20/25°C. All leaf samples collected before (0 h) during (96 h) and after (240 h) the heat stress period were frozen in liquid nitrogen and stored in a freezer at -80°C until genetic and enzymatic analyses.

Evaluation of flower buds: Plant flowering was evaluated individually in each pot, quantifying the total number of flowers emitted during the R5 phenological stage, before (0 h), during (96 h), and at the end (240 h) of heat stress. To facilitate quantification, the flower buds were marked and monitored daily throughout the period in which the temperature was maintained. At the end of the study, the percentage of floral abortion was estimated, based on the bud count during the entire stress period.

Chlorophyll determination: The protocol for the quantification of the relative content of total chlorophyll in the leaves was established before (0 h), during (96 h), and at the end (240 h) of the period of temperature increase. The non-destructive method was used, using the portable digital chlorophyll meter (*ClorofiLOG* model *CFL 1030*, *Falker*, Porto Alegre, RS, Brazil). The evaluation was performed on a leaflet of two trifoliate leaves inserted in the middle third of the main stem of the plant. The procedure was performed on each pot. The unit of measurement for chlorophyll expressed by this equipment is the SPAD index (Silveira *et al.* 2003).

Determination of proline content and guaiacol peroxidase (GPX) activity in leaves: To determine the content of the amino acid proline in the leaf tissue, the methodology described by Bates *et al*. (1973) was used. The enzymatic extraction of the enzyme GPX (EC 1.11.1.7) was performed according to Araújo *et al.* (2005). Both determinations started with frozen leaf material, preserving the integrity of the sample. The analyses were performed during the R5 stage, before (0 h), during (96 h), and after (240 h) the high-temperature treatment.

Determination of leaf ethylene: To estimate the production of ethylene from the leaf tissue, leaflets were collected from the main stem of each plant during the R5 stage, before (0 h), during (96 h), and at the end (240 h) of heat stress. For the collection, a cylindrical leaf cutter with a diameter of 5 mm was used. Eleven discs were collected from each leaflet, reaching a portion of 200 mg. The discs were placed in 20 mL glass vials and quickly sealed with silicone septa. The samples were analyzed for an average period of 4 h after sealing the vials. The determination of ethylene proceeded according to Makky *et al*. (2014), using the gas chromatograph *GC-2010* (*Shimadzu*, Tokyo, Japan) with a capacity of 10 µL. To identify the exact moment of the reflectance of the ethylene gas, it was necessary to measure a sample containing only the gas. The time required for the detection of ethylene by the equipment was 1.44 min. The spectral signature of ethylene produced in the measurement of each leaf sample was used to calculate the final concentration based on the ethylene gas sample analysis. The time required for the detection of ethylene gas by the equipment was 1.44 min. The peak formed in each sample was quantified and used to calculate the final concentration based on the pure ethylene standard sample.

Total RNA extraction and candidate gene expression analysis: *Primer Express*™ *v. 3.0.1* (*Applied Biosystems*, Massachusetts, USA) was used to design primers specific to the four genes encoding heat shock protein 90 - HSP90 (Phvul.002G083000.1), ACC oxidase - ACO (Phvul.008G214200.1), ethylene response factor (ERF), and Δ^1-pyrroline-5-carboxylate synthetase1 - P5CS1 (Phvul.008G230300.1). Total RNA was extracted using a *PureLinkTM RNA Mini Kit* (*Invitrogen*, Carlsbad, CA, USA), conformed manufacturer's instructions. The integrity of the RNA samples was verified by electrophoresis on a 1.2% (m/v) agarose gel, then treated with *PureLink*™ DNAse set (*Invitrogen*), as recommended by the manufacturer. RNA concentrations and purity of all samples were determined using *NanoDrop® ND-100* (*Thermo Scientific*, Massachusetts, USA), spectrometry at 260 and 280 nm, and ratios 260/280 and 260/230 nm were used. For cDNA synthesis reactions, samples with a ratio above 1.8 were prioritized. One microgram of RNA was used to synthesize cDNA, using *SuperScript IV* reverse transcriptase (*Thermo Fisher Scientific*, Massachusetts, USA), using the manufacturer's protocol. The resulting cDNA from each sample was diluted (1:5) and stored at -20°C until use. RT-qPCR was performed using *StepOnePlus*™ real-time PCR (*Applied Biosystems*), and all the PCR reactions were conducted in a 10 µL reaction volume containing 5 µL of cyanine dye SYBR, 0.4 µL of 10 µM solution of each primer, 1 µL of diluted cDNA, and 3.2 µL of ddH_2O. The RT-qPCR program set the following parameters: 95°C for 2 min, 40 cycles of 95°C for 30 s, and 60°C for 30 s. For each sample, three biological repeats, with three technical replicates, were performed to acquire reliable results. The relative expression of genes was calculated using the method $(1+E)^{-\Delta\Delta CT}$ of Livak and Schmittgen (2001), and the expressions of target genes were normalized with elongation factor 1α - *EF1α* (Phvul.010G124700.1) as the reference gene, the control treatment (without bacteria inoculation) before heat stress for each cultivar was used as calibrator for fold change expression. The amplification efficiency of each pair of primers was estimated using the *LinReg* PCR program.

Data analysis: The multivariate analysis of repeated measures of biochemical and RT-qPCR data was performed using the *SPSS-IBM* software *version 27*, with adjustment in the covariance structure selection model, according to the Greenhouse and Geisser correction method. For the sphericity test, an analysis of variance (*ANOVA*) was performed. The other performances were performed using the *SISVAR* software, applying the *Tukey*'s test to compare averages, and using probability levels of 95%.

Results

In the evaluation of flower buds, the treatment with the *B. subtilis* AP-12 showed a reduction in the floral abortion rate in the first 96 h of heat stress. However, this reduction was not seen after 240 h, showing an increase in the abortion rate (Table 1). On the other hand, with the end of heat stress, an increase of 40% in the number of flower buds of bean plants inoculated with the two strains under study is estimated. Regarding the cultivars, IAC-Imperador seems to be more tolerant to heat increase, expressing a low rate of floral abortion within 120 h, however, the number of flower buds after the increase in temperature was rather high also in cv. TAA-Dama. According to the statistical analysis of the data, there was an interaction between the *B. subtilis* strains and the cultivars in question. In Fig. 1, we can see that the treatment with the AP-3 showed a decrease in the floral abortion rate in cv. IAC-Imperador within the periods of 96 and 240 h of heat stress. Considering the flower buds after the stress phase, we can observe a great effect of two *B. subtilis* isolates on cv. IAC-Imperador (Fig. 2).

The results show that, during the temperature increase (96 h), the cv. IAC-Imperador showed a drop in the chlorophyll content, showing an increase with the AP-12 treatment only after the heat stress of 240 h (Fig. 3*A*). On the other hand, the chlorophyll content in cv. TAA-Dama was maintained throughout the heat period, with no difference due to *B. subtilis* treatment.

As concern the proline content, the high temperature mostly increased it as well as treatments with *B. subtilis* AP-3 and AP-12. In the cv. IAC-Imperador, the proline content after 240 h of heat stress was considerably higher

Table 1. Evaluation of flower abortion and formation of new floral buds in two common bean cultivars inoculated with *B. subtilis* strains (AP-3 and AP-12) exposed to temperature rise during the reproductive period for 240 h. *In italics* – significant differences between treatments; *lowercase letters* – significant differences between means in columns (*Tukey*'s test, $P < 0.05$).

Treatments	Abortion after 96 h [%]	Abortion after 240 h [%]	Buds after 240 h [%]
B. subtilis AP-3	9.91^{ab}	89.3^{a}	24.7^{a}
B. subtilis AP-12	5.07^{b}	92.8^{a}	23.9^{a}
Control	14.1^{a}	94.0^{a}	15.9^{b}
Pr > Fc	*0.0095*	*0.077*	*0.011*
Cultivars			
IAC-Imperador	11.8^{a}	89.7^{a}	19.8^{b}
TAA-Dama	7.55^{a}	94.4^{b}	23.3^{a}
Pr > Fc	*0.065*	*0.0084*	*0.001*

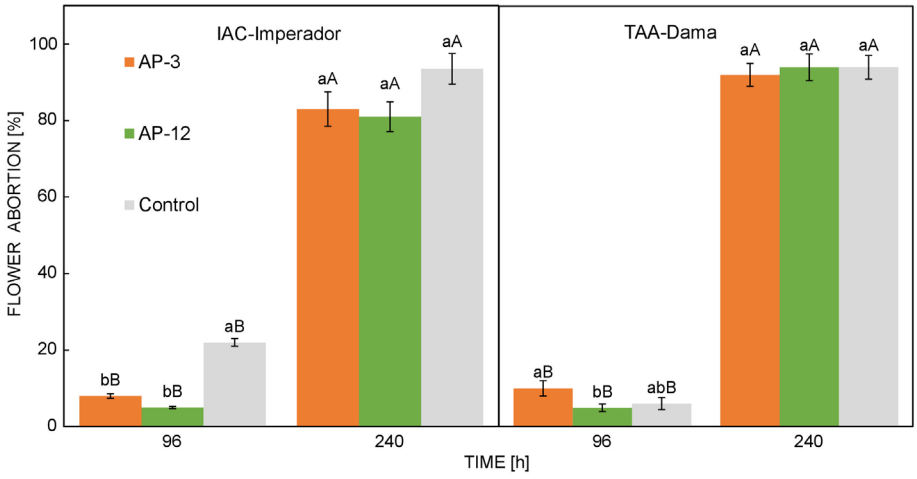

Fig. 1. Percentage of flower abortion after increased temperature during reproductive stage for 96 and 240 h in two common bean cultivars (IAC-Imperador and TAA-Dama) inoculated with *B. subtilis* strains (AP-3 and AP-12). Means ± SDs, $n = 6$. *Different lowercase letter*s represent significant differences between inoculation and *uppercase letters* represent significant differences among the periods of treatment ($P < 0.05$).

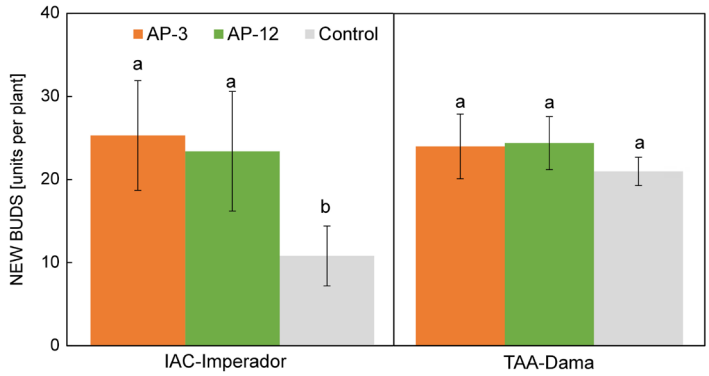

Fig. 2. Number of new buds after heat stress for 240 h in two common bean cultivars (IAC-Imperador and TAA-Dama) inoculated with *B. subtilis* strains AP-3 and AP-12. Means ± SDs, $n = 6$. *Different letters* represent significant differences caused by inoculation ($P < 0.05$).

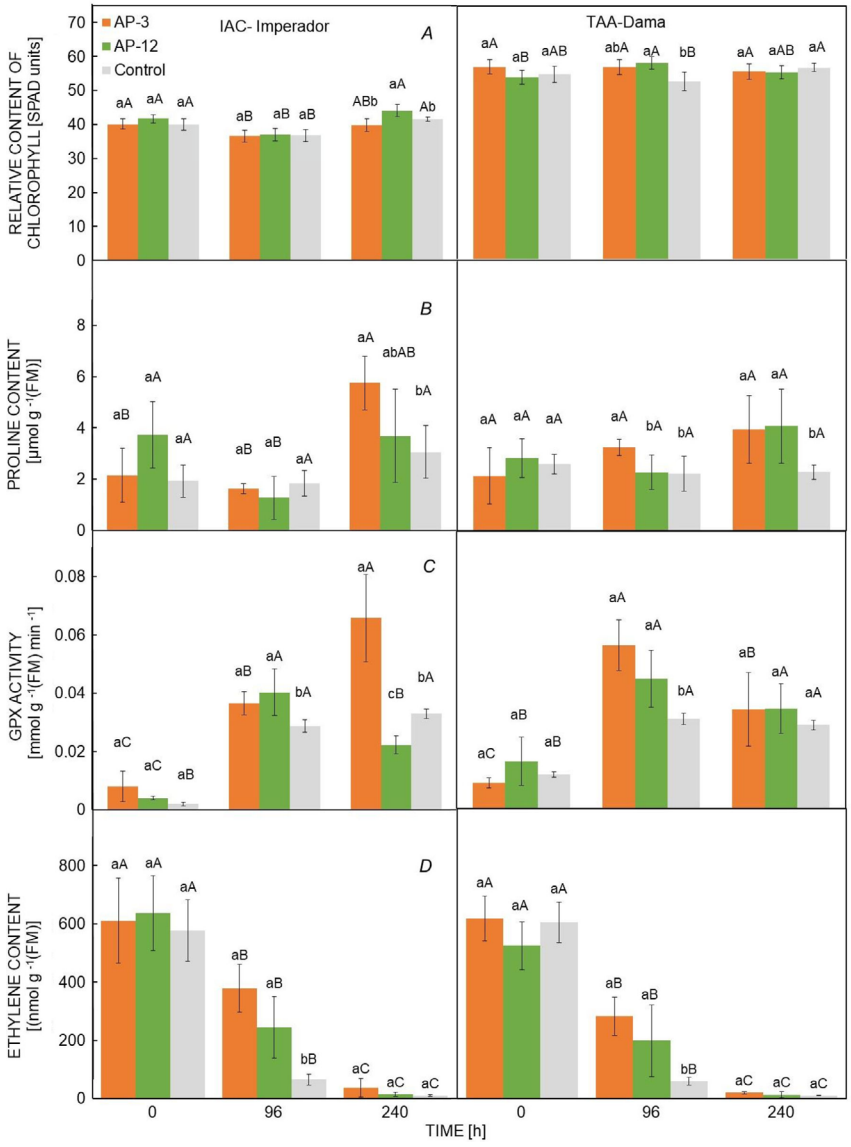

Fig. 3. Changes in relative chlorophyll content (*A*), proline content (*B*), peroxidase activity (*C*), and ethylene content (*D*) in different periods of heat stress during the reproductive stage of common beans inoculated with two *B. subtilis* strains AP-3 and AP-12. Means ± SDs, $n = 6$. *Different lowercase letters* represent significant differences between inoculation and *uppercase letters* represent significant differences among the period's treatments ($P < 0.05$).

in plants with the AP-3. In the cv. TAA-Dama, both strains increased proline content after 240 h of heat stress (Fig. 3*B*).

Evaluating the enzymatic activity of guaiacol peroxidase among the cultivars, we observed that in the cv. IAC-Imperador the enzymatic activity gradually increased in response to the AP-3 treatment during high temperature, indicating greater activity after the stress period (Fig. 3*C*). Regarding the cultivar TAA-Dama, the highest activity of guaiacol peroxidase was after 96 h of heat stress (96 h), especially under the treatment with *B. subtilis* AP-3. Leaf ethylene content decreased during temperature increases in both cultivars. Plants inoculated with *B. subtilis* showed a slower decrease than controls (Fig. 3*D*).

In the current study, four genes have been selected for relative gene expression analysis because of their potential involvement in abiotic stress responses (*HSP90*, *P5CS1*) and ethylene synthesis (*ACO*, *ERF*). Changes in expression were investigated using RT-qPCR (Fig. 4). Concerning the relative expression of genes involved in ethylene synthesis, the two cultivars presented different gene expression profiles for the *ACO* gene. *B. subtilis* inoculation caused an increased expression in cv. IAC-Imperador while reduced expression in cv. TAA-Dama after 96 h of heat stress (Fig. 4*A*). The *ERF* gene expression was higher at 240 h for both cultivars mainly in plants without *B. subtilis* inoculation. In the control plants (without inoculation), the expression was raised at the end of the stress period in both cultivars (Fig. 4*B*). Interestingly, plants that were inoculated with *B. subtilis* strains had reduced expression of genes involved in ethylene synthesis after heat stress (Fig. 4*A*,*B*).

We observed that the expression of the *HSP90* gene in cv. IAC-Imperator was lower after the stress period of 96 h and after 240 h the expression returned to values similar to

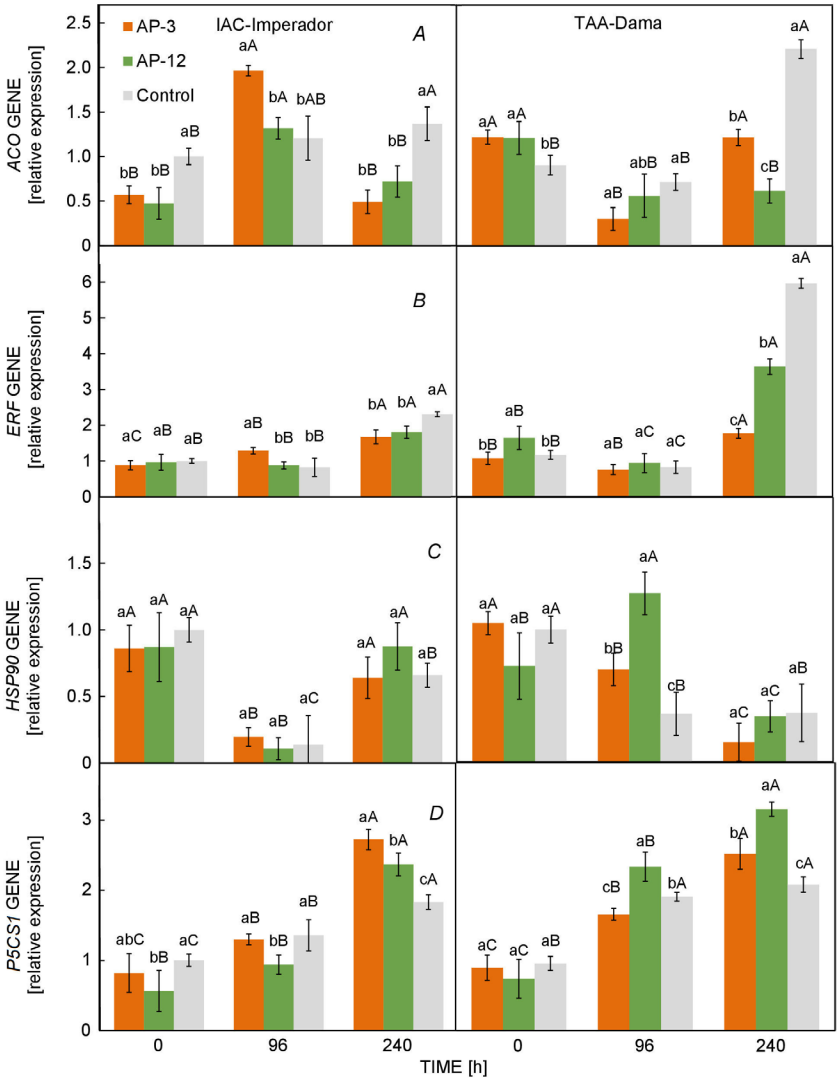

Fig. 4. Relative expression of genes in leaves of two common bean cultivars (TAA-Dama and IAC-Imperador), without and with inoculation of *B. subtilis* (strains AP-3 and AP-12) before (0 h), during (96 h), and after (240 h) heat stress. Means ± SDs, $n = 6$. *Different lowercase letters* represent significant differences between inoculation and *uppercase letters* represent significant differences among the period's treatments ($P < 0.05$).

0 h. On the other hand, the expression of the *HSP90* gene increased in cv. TAA-Dama inoculated with *B. subtilis* after 96 h of heat stress (Fig. 4*C*).

Our results revealed that there was a gradual increase in the expression of the *P5CS1* gene, related to proline synthesis, in both cultivars throughout heat stress (Fig. 4*D*). The inoculation of *B. subtilis* strains also significantly increased the *P5CS1* gene expression at the end of the heat stress period, with emphasis on the AP-12 in the cv. TAA-Dama and AP-3 in the cv. IAC-Imperador. Collectively, these results demonstrate that the genes for ethylene synthesis and stress-related genes were modulated by inoculation of *B. subtilis* strains before, during, and after heat stress.

Discussion

In recent years, *Bacillus* strains inoculation has been tested to mitigate the abiotic stresses in plants (Tiwari *et al.* 2017, Lastochkina 2019, Poveda and González-Andrés 2021). Thus, our data suggest that inoculation of *B. subtilis* in the common bean can attenuate the effect of 96-h heat stress by reducing flower abortion, and consequently triggered an increase in pod formation in the common bean (Table 1, Fig. 2). Comparing the cultivars, it was found that the cultivar IAC-Imperador showed better performance in reducing bud abortion in response to inoculation with *B. subtilis* (Fig. 1). We observed a flower abortion above 90% in all plants after 240 h of heat stress (Table 1). A large proportion of flowers or young pods in the common bean are abscised by the effects of different factors (Peksen 2007). Izquierdo and Hosfield (1981) reported average reproductive abscission 48% in beans. It was also reported that heat stress (42°C for 6 h) in tomatoes in the reproductive stage, reduced the number of fruits by 50% (Mukhtar *et al.* 2020). Other results suggest that deterioration in flower buds in beans under high temperatures together with reduced pollen fertility resulted in reduced yield (Suzuki *et al.* 2001). Tsukaguchi *et al.* (2003) also confirmed that temporary water stress caused the deterioration of flower buds, being one of the factors responsible for damage to the pollen grains.

When evaluating 12 common bean cultivars, Silva *et al.* (2020) concluded that heat stress can damage pollen grain formation responsible for a large loss in crop yield. These authors also included common bean cultivars tolerant to heat stress, such as cv. IAC-Imperador. It was also observed in our study that this cultivar showed great interaction with the *B. subtilis* strains used, revealing the high potential for future studies.

The efficiency of photosynthesis has been a good indicator of plant response to abiotic and biotic stresses (Huang 2006). The SPAD unit has a good correlation with chlorophyll content and so photosynthetic activity in tomatoes and under heat stress, there was an increase in this index in plants (Bhattarai *et al.* 2021). In our study, this increase was slight and only significant after 240 h of heat stress in the cv. IAC-Imperador when inoculated with the *Bacillus* AP-12 strain (Fig. 3*A*).

Heat stress in common beans can simultaneously cause temporary loss of leaf water content, leading the plant to physiological changes similar to that caused by water stress (Tsukaguchi *et al.* 2003). It was also reported that heat stress in *Solanum lycopersicum* in the reproductive stage caused a loss of about 20% in leaf water content (Mukhtar *et al.* 2020). The accumulation of proline in tissues has often been associated with mechanisms to mitigate damage caused by water deficit in plants (Furlan *et al.* 2020, Ghaffari *et al.* 2021). Proline contributes to osmotic adjustment for maintaining cell pressure potential under water deficit conditions (Batool *et al.* 2020). Proline accumulation was considered one of the adaptive mechanisms of plants under heat stress conditions (Yuan *et al.* 2017). Our evidence revealed that the inoculation of *B. subtilis* in common beans under heat stress conditions provided higher proline accumulation in the cv. IAC-Imperador (Fig. 3*B*). Although the *P5CS* gene expression increased in both cultivars after heat stress imposition (Fig. 4*D*) the cv. TAA-Dama accumulated less proline (Fig. 3*B*) possibly due to proline degradation.

The evaluation of osmoprotective compounds such as proline and antioxidant enzymes such as peroxidases (Fig. 3*C*) can help to elucidate the defense mechanisms of the common bean plant against heat stress. During heat stress, an increase in peroxidase activity was detected in a study with strawberries together with a reduction in total protein content (Gulen and Eris 2004). It was found in our study that *B. subtilis* AP-3 provided an increase in GPX activity at the end of the stress period in cv. IAC-Imperador (Fig. 3*C*). In addition, in response to abiotic stresses, plant metabolism is altered, as well as the synthesis of hormones, such as ethylene, which can interfere in processes such as nodulation, leaf senescence, flower abortion, and root growth, among others (Sharma *et al.* 2019, Husain *et al.* 2020). This hormone is formed from 1-carboxylic acid-1-aminocyclopropane (ACC) under the action of ACC synthase (ACCS), and the conversion of ACC to the ethylene molecule occurs by the enzyme ACC oxidase (ACO) (Adams and Yang 1979; reviewed by Husain *et al.* 2020). There are reports in the literature that *B. subtilis* can interfere with the production of ethylene by degrading ACC using the enzyme ACC deaminase (Misra and Chauhan 2020). It is known that co-inoculation in plants with *Rhizobium* and ACC deaminase producing rhizobacteria can alleviate the effects of environmental stress on plant growth by decreasing ethylene, which is known to be an important factor causing reduced plant growth or death (Tittabutr *et al.* 2013).

The *B. subtilis* AP-3 and AP-12 used in the experiments were previously characterized as ACC deaminase producers. The inoculation of *Bacillus* into common beans triggered an increased expression of the *ACO* gene after 96 h of heat stress followed by a decreased expression after 240 h in cv. IAC-Imperador, which is very important for the conversion of ACC into ethylene. This fact was observed in bean plants at the end of heat stress (Fig. 4*A*). However, we noted that the evaluation of leaf ethylene did not correspond with this effect of reducing *ACO*

expression, as there was a decrease in ethylene detection throughout the stress period in all treatments. Although the ethylene synthesis decreased under heat stress, this decrease was less in plants inoculated with *B. subtilis* strains compared to control plants.

Ethylene can be generated by plant tissues under stress conditions and multigene families, like *ACO*/*ERF*, can play an important role in the regulation of plant stress responses (Husain *et al.* 2020, Zhang *et al.* 2021). Additionally, a reduction in the expression of genes related to ethylene production response (ERF) has been found in response to abiotic stress (Husain *et al.* 2020). This same effect of reducing the expression of *ACO* and *ERF* was found in tomatoes subjected to low temperatures and inoculated with ACC deaminase-producing bacteria (Subramanian *et al.* 2015). This reduction in the expression of these genes was also detected in this study, in plants inoculated with *B. subtilis* at the end of the heat stress period (Fig. 4*A*,*B*). *Burkholderia phytofirmans* also characterized by high expression of ACC deaminase favored the adaptation of grape vines to the cold period by reducing the production of ethylene (Theocharis *et al.* 2012). The strain AP-12 of *B. subtilis* selected in our study as having the highest production of ACC deaminase also stood out in reducing flower abortion in common beans during heat stress.

In plants, ethylene is also attributed to the production of hydrogen peroxide and the increase of peroxidases under stress conditions, suggesting its involvement in the induction of resistance (Zhu *et al.* 2016; reviewed by Nazir *et al.* 2020). Heat stress often leads to the accumulation of ROS, such as superoxide radicals and hydrogen peroxide, which cause oxidative damage and disrupt metabolic homeostasis in plants (Khan *et al.* 2020). Plant cells produce H_2O_2 in response to biotic and abiotic stresses (Andrade *et al.* 2018, Bagheri *et al.* 2019, Khan *et al.* 2019).

The improvement in heat stress tolerance for plants inoculated with rhizobacteria may be associated with reduced generation of ROS and greater activity of antioxidant enzymes such as peroxidases and, consequently, less damage to cells (Abd El-Daim *et al.* 2014). Our results will provide relevant information as it was also observed that the inoculation of *B. subtilis* provided increases in peroxidase activity during heat stress, especially in cv. IAC-Imperador (Fig. 3*C*). Khan *et al.* (2020) also observed that the response to heat stress in soybean inoculated with *B. cereus* may be related to the reduction of ROS and the participation of superoxide dismutase and peroxidase.

In addition, the gene transcription patterns obtained from the RT-qPCR experiments combined with biochemical standards in the current study showed that genes studied in our experiments may function in mechanisms that mediate network for multiple stimulus responses and adaptations. Most plants present mechanisms for defense against different types of stresses, and it is known that *HSP90* genes are expressed in response to abiotic stresses (Song *et al.* 2019, Appiah *et al.* 2021).

In literature, the expression pattern of *P5CS* genes affects proline accumulation in plants, and *P5CS* has been the target of study in several plant species (Bagdi *et al.* 2015, Singh *et al.* 2015, Maghsoudi *et al.* 2018). This fact was also confirmed in our work by the expression of the *P5CS1* gene related to proline synthesis in the inoculated plants (Fig. 4*D*).

According to previous studies, the evaluation of ethylene in detached bean leaves under conditions of heat stress and possible occurrence of water stress may not reflect the real situation that happens in the intact plant (Morgan *et al.* 1990, Ferreira 2017). The evaluation of gene expression related to ethylene production may be useful to better elucidate the participation of this hormone in events related to abiotic stresses. The evaluation of ethylene in detached parts of plants has been questioned, as it can often produce contrasting results, considering the trauma of the abscission itself and the loss of water that occurs in the detached part, so the intact plant may express lower values than the detached part (Morgan *et al.* 1990). In this sense, the water deficit also can interfere with the ethylene evaluation results, according to Ferreira (2017), evaluating the physiology of beans, under abiotic stress, also using the detached leaves method, it was observed an increase in the production of ethylene in control plants compared to plants under abiotic stress.

The reduction of damage from heat stress in response to inoculation with other *Bacillus* species was also found in studies with soybean and wheat (Abd El-Daim *et al.* 2019, Khan *et al.* 2020). In addition, recently, Jabborova *et al*. (2020) performed the inoculation of *B. subtilis* into *Triticum aestivum*, subjected to salt stress, and observed significantly increased biomass. In another study, Woo *et al*. (2020) reported that with the inoculation of *B. subtilis* (GOT9), greater tolerance to drought and salt stress was triggered in *Arabidopsis thaliana* and *Brassica campestris*. Thus, the important role played by *Bacillus* strains is evident, mitigating damage to plants caused by environmental stresses and increasing the productivity of important agricultural crops. However, further studies are still needed, at both the physiological, biochemical, and molecular levels for the improvement of heat tolerance through the inoculation of *B. subtilis* strains.

Inoculation with *B. subtilis* may be beneficial in assisting the common bean's biochemical response to temperature increase. In this study, we discovered that inoculating bean plants with *B. subtilis* during heat stress reduced floral abortion and increased bud numbers. This is explained in part by a decrease in the expression of genes involved in ethylene production and an increase in proline content and peroxidase activity. In short, the results obtained should be investigated as soon as possible, beginning with bean cultivation in the field.

Conclusions

Inoculation of *B. subtilis* in common bean cultivars can contribute to reducing the stress caused by high

temperatures. There was good interaction between *B. subtilis* AP-3 and the cv. IAC-Imperador, causing a great reduction in floral abortion, an increase in the content of proline, and guaiacol peroxidase activity in the inoculated plants. There was an increase in the expression of the *P5CS1* gene and a reduction in the expression of *ACO* and *ERF* genes related to ethylene synthesis. It is suggested that *B. subtilis* modulates metabolic pathways in response to the effect of high temperatures in the reproductive period of common bean cultivation and this should be also evaluated under field conditions in the near future.

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