MITIGATION OF *TETRANYCHUS URTICAE* INDUCED OXIDATIVE STRESS IN SOYBEAN PLANTS USING PLANT GROWTH PROMOTING RHIZOBACTERIA

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Abstract. The current study looks at the possibility of enhancing plant tolerance to insect attacks by using plant growth-promoting rhizobacteria *Bacillus subtilis*. Under stress conditions, the metabolism of a plant cell is generally characterized by an elevated formation of reactive oxygen species. However, plants have developed antioxidant protective mechanisms against oxidative stress. The aim of this paper was to determine the biochemical response of inoculated and non-inoculated soybean seedlings (with and without exposure to mites). The results in plants treated with mites have shown a higher lipid peroxidation intensity in non-inoculated plants compared to the inoculated ones. During biotic stress caused by a mite attack, inoculation successfully reduced oxidative stress. Inoculation with *B. subtilis* induced mild stress, which appeared to fortify soybean plants for enhanced defense. Pyrogallol peroxidase and guaiacol peroxidase were especially active in plant roots after inoculation. These results highlight mild oxidative stress caused by *B. subtilis* which might be beneficial to soybean seedlings by enhancing antioxidant defense mechanisms prior to a mite attack. While our findings do not demonstrate direct acaricidal activity, they suggest that PGPR inoculation may enhance plant tolerance to herbivore-induced oxidative stress, offering an indirect strategy to improve crop resilience.
Keywords: *antioxidant enzymes, Bacillus subtilis, biocontrol agents, induced resistance, two-spotted spider mite*

Introduction

Using microbial biocontrol agents to produce higher crop yields, while minimizing the negative influence on the environment at the same time, is a new challenge modern agronomy is facing (Xiang et al., 2012). Plant growth-promoting rhizobacteria (PGPR) are genuine hosts to soil and the plant rhizosphere. PGPR, with the ability to colonize roots, exhibit a significant influence on the biocontrol of plant pathogens (Beneduzi et al., 2012). *Bacillus* species can survive in soil for an extended period of time under harsh environmental conditions, playing a significant role in improving the tolerance to biotic stresses (Hashem et al., 2019) such as plant pathogens (i.e., bacteria, fungi, viruses), plant feeders (nematodes and arthropods), and weeds. The plant growth promotion potential by *Bacillus* spp. could be described through characteristics such as phosphate solubilization,

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nitrogen fixation, as well as biocontrol attributes like the production of hydrogen cyanide (HCN), siderophores, hydrolytic enzymes, and antibiotics (Kumar et al., 2012). The induction of the plant's natural defense system can decrease its sensibility towards diseases caused by a wide range of pathogens and parasites. One of the plants' defense mechanisms that has been described is induced systemic resistance (ISR), which is commonly attributed to plant growth-promoting rhizobacteria (PGPR) (Jaiswal et al., 2020). PGPR, particularly the *Bacillus* species*,* represent a great alternative to chemical fertilizers and pesticides for the promotion of plant growth and ISR (Park et al., 2016). The application of PGPR in the rhizosphere might be used to alleviate stress in plants (Hashem et al., 2019). However, despite numerous reports on PGPR-mediated stress alleviation (Beneduzi et al., 2012), there is a lack of data on their potential role in mitigating oxidative stress specifically caused by herbivorous mites in soybean (Orozco-Mosqueda et al., 2021). This study addresses that gap by evaluating the biochemical responses of soybean plants inoculated with *Bacillus subtilis* under the *Tetranychus urticae* infestation.

The two-spotted spider mite (TSSM), *T. urticae* Koch, is one of the most destructive cosmopolitan agricultural pests, affecting over 1100 plant species worldwide (Suekane et al., 2012). In soybean, the *T. urticae* infestation can result in severe yield losses due to chlorosis, defoliation, and an impaired photosynthetic capacity. In Serbia—where soybean is a strategic crop cultivated on approximately 250,000 ha annually—outbreaks of *T. urticae* have become increasingly common, particularly during hot and dry growing seasons. Yield losses associated with mite infestations have been reported to range from 40% to 60%, depending on the crop's developmental stage and the severity of the infestation (Milovac et al., 2021). Across Europe, rising temperatures and the progressive restriction of synthetic pesticide use under integrated pest management (IPM) and European Green Deal regulations have further contributed to the resurgence of *T. urticae* as a major pest in leguminous crops (Grbić et al., 2011). Although precise global estimates vary, *T. urticae* is widely regarded as one of the most economically damaging mite species worldwide due to its extreme polyphagy, a high reproductive rate, and the ability to rapidly develop resistance to acaricides (Van Leeuwen et al., 2010). These characteristics highlight the urgent need for environmentally sound and sustainable pest management strategies, particularly in key soybean-producing regions like Serbia. These challenges emphasize the need for alternative, sustainable approaches such as PGPR-based strategies. Considering the adverse effects of synthetic growth regulators, herbicides, and chemical fertilizers, Khoso et al. (2024) suggest that these inputs are likely to be increasingly replaced by PGPR in the future. The issue of pesticide use is particularly critical from the perspective of soybean product safety, as the widespread application of pesticides poses potential risks not only to the environment but also to human health (Szarka et al., 2024) and poultry welfare (Mathias et al., 2024). Spider mites feed primarily on leaf surfaces, causing damage to individual cells. Similarly to phloem-feeding insects, TSSM have mouthparts adapted for a sucking mode of feeding (Bensoussan et al., 2016). Under such stress conditions, the metabolism of the plant cell is followed by increased formation of reactive oxygen species (ROS). ROS and reactive nitrogen species (RNS) bursts occur in response to arthropod feeding (Santamaria et al., 2020). ROS include the superoxide anion radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$), and the singlet oxygen ($^1O_2$). ROS are highly reactive and toxic and may cause damage to proteins, lipids, carbohydrates, and DNA molecules (Sachdev et al., 2021). The action of free radicals

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on the cell membrane includes the induction of lipid peroxidation (LP). However, plants have developed a broad range of mechanisms to survive and thrive under stress conditions. The antioxidant defense system of the plants includes a variety of secondary molecules and enzymes such as superoxide dismutase (SOD), peroxidase (POD) with different substrates (e.g., pyrogallol (PPX) and guaiacol (GPX)), glutathion-reductase (GR), glutathione-S-transferase (GST) and catalase (CAT) (Sachdev et al., 2021; Sharma et al., 2012).

In order to evaluate the suppression of *T. urticae* induced stress in soybean using PGPR *B. subtilis*, the aim of this study was to measure the differences in oxidative stress parameters analyzing lipid peroxidation and the activity of antioxidant enzymes (superoxide dismutase, catalase, pyrogallol peroxidase, and guaiacol peroxidase) in soybean leaves and roots.

Materials and methods

Characterization of Bacillus subtilis for plant growth-promoting properties

Two *Bacillus* isolates, B3 and B44, were initially screened in order to determine their suitability for inoculation. The isolates were evaluated for the production of hydrolytic enzymes (amylase, lipase, urease, gelatinase, and cellulase), siderophores, and hydrogen cyanide (HCN), using standard methods and established protocols. Amylase production and lipase production were determined according to Gonzales et al. (1978). The urease activity and gelatinase activity were assessed following Biswas and Paul (2014), while the cellulase activity was tested using 0.5% carboxymethyl cellulose as a substrate, as described by Sreena and Sebastian (2018). Siderophore production on the Chrome Azurol S (CAS) medium was analyzed according to Schwyn and Neilands (1987), and HCN production followed the method of Ahmad et al. (2008).

Experimental design

The experimental trials included 240 soybean plants (cultivar "Maximus") divided in four groups: (1) seeds (S) inoculated with *B. subtilis* (B) and infested with *T. urticae* (T) (SBT, positive control); (2) seeds inoculated with *B. subtilis* without *T. urticae* (SB); (3) seeds without submerging in *B. subtilis* inoculum, but infested with *T. urticae* (ST); and (4) seeds without *B. subtilis* inoculum, and no *T. urticae* infestation (S) (negative control).

Bacillus subtilis strains were isolated from a soil sample and grown on L nutrient agar (Trypton 10.0 g, yeast extract 5.0 g, agar 15.0 g, and 1000 mL distilled H_2O), at 28°C for 24 h and kept in the refrigerator at +4°C. For the purpose of the experiment, in this way preserved *B. subtilis* culture was revived on fresh L agar. The inoculum was prepared by moving a loopful of 24 h old *B. subtilis* culture in 100 mL of inoculum medium (L broth). The inoculated medium was incubated in Environmental shaker – Incubator ES – 20/60 (BiopSAM, Estonia) for 24 h at 150 rpm and 28°C. After OD determination on 600 nm inocula was adjusted at 10^8 cell mL^{-1}. The microbial soil isolates preparation and cultivation was performed in the Laboratory of Microbiology, Faculty of Agriculture, University of Novi Sad. The colony of *T. urticae* was cultivated on the same variety of soybean plants and maintained under controlled laboratory conditions at 25 ± 1°C, 65 ± 5% RH, and a photoperiod of 16:8 h (L:D) in the Laboratory of Zoology, Faculty of Agriculture, University of Novi Sad.

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The seeds used for the cultivation of plants assigned to the first two groups (60 for SBT and 60 for SB) were inoculated before sowing, by submersing in 60 mL of *B. subtilis* inoculum (1 mL of bacterial culture containing approximately 10^8 cells) for 30 min. In order to cultivate plants for the other two groups, 120 seeds (60 for ST and 60 for S) were submerged in the same amount of distilled water (60 mL) for the same period of time (30 min). All seeds (240) were cultivated in clean plastic pots containing sterile soil. Plants were grown for 21 days in a controlled climate chamber at 28°C, with 60% RH, a photoperiod of 18:6 h (L:D), and a light intensity of 10.000 lx. After that period, 120 plants (60 in SBT and 60 in ST) were infested with *T. urticae*. Using a soft thin brush, 10 young *T. urticae* females were evenly distributed per each plant, on leaves, and left for the next 12 days. These plants were kept in transparent plastic compartment boxes in another climate chamber to avoid plant infestation in SB and S, and cross contamination in SBT and ST groups, with the same ambient conditions adjusted (28°C, 60% RH, 18:6 (L:D), and 10.000 lx). On the 33rd day from the beginning of the experiment, plant materials were sampled. Prior to biochemical analyses, fresh mass of stems and roots, as well as length and volume of roots were measured.

Visual symptoms of mite damage were not scored, nor was mite reproduction monitored, as the study focused strictly on biochemical stress responses in the host plant.

Determination of T. urticae induced oxidative stress parameters

The oxidative stress parameters were analyzed using fresh plant material, roots and leaves. The fresh plant material (1 g) was homogenized with a 10 mL phosphate buffer (0.1 M, pH 7.0). After homogenization, extracts were centrifuged at 15.000 × *g* for 10 min at 4°C. Supernatant aliquots were used for a further biochemical analysis, such as membrane lipid peroxidation (LP), and the activity of four enzymes—superoxide dismutase (SOD), catalase (CAT), pyrogallol (PPX) and guaiacol (GPX) peroxidase. LP was measured at 532 nm using the thiobarbituric acid (TBA) test according to Mandal et al. (2008). The total amount of TBA–reactive substances was determined as nM malondialdehyde (MDA) equivalents g^{-1} fresh weight (FW). The slightly modified method from the same reference (Mandal et al., 2008) was used to determine the activity of SOD. The SOD (EC.15.1.1) activity was analyzed by measuring its ability to inhibit the photochemical reduction of nitroblue-tetrazolium (NBT) chloride. The reaction mixture included: 50 mM phosphate buffer (pH 7.8), 13 mM L-methionine, 75 μM NBT, 0.1 mM EDTA, 2 μM riboflavin, and 0.02 ml of enzyme extract (0.02 mL distilled water in control). The mixture was kept under a 15 W fluorescent lamp for 30 min, and the absorbance was read at 560 nm. One unit of the SOD activity was defined as the amount of enzyme required to inhibit the reduction of NBT by 50%. The CAT (EC 1.11.1.6) activity was determined according to Sathya and Bjorn (2010). The Morkunas and Gmerek (2007) method was used to determine the activity of peroxidases (EC 1.11.1.7), where pyrogallol (for PPX) and guaiacol (for GPX) were used as substrates. The PPX activity was based on the purpurogallin (a product of pyrogallol oxidation) content measurement. The enzyme extract (0.02 mL) was added to the assay mixture containing 3 mL of 180 mM pyrogallol and 0.02 mL of 2 mM H_2O_2. The absorbance was recorded at 430 nm. The GPX activity was determined by an assay of tetraguaiacol (a colored product of guaiacol oxidation) in the sample. The enzyme extract (0.02 mL) was added to the assay mixture containing 3 mL of 20 μM guaiacol and 0.02 mL of 3 mM H_2O_2, and the absorbance was recorded at 436 nm. The activities of SOD, CAT and the peroxidases were expressed in U g^{-1} of FW.

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Statistical analyses

All experiments were performed in triplicates. Obtained values of the biochemical parameters were expressed as a standard error of the mean (SE). Differences among samples were tested by ANOVA, and confirmed by Duncan's multiple range test (for $p < 0.05$). Pearson's correlation coefficient (r) was used to determine the correlation between different variables, with $p < 0.05$ significance level. Data were analyzed using Statistica 14.0.0.15 (TIBCO Software Inc., University license).

Results

Characterization of Bacillus subtilis for plant growth-promoting properties

Two *Bacillus* isolates (B3 and B44) were preliminarily screened for key plant growth-promoting (PGP) traits, including the production of extracellular enzymes and secondary metabolites linked to pathogen suppression and plant growth enhancement, in order to identify the most effective strain for subsequent inoculation trials. The results indicated that both isolates produced several hydrolytic enzymes: lipase, urease, gelatinase, and amylase, as well as hydrogen cyanide (HCN). However, only the isolate B3 demonstrated the ability to produce siderophores, forming an orange halo > 10 mm in diameter on Chrome Azurol S (CAS) medium. This siderophore-producing ability was considered a key factor in selecting the isolate B3 for the main experiment, due to its role in iron sequestration and potential induction of systemic resistance in plants. Neither isolate produced cellulase under the tested conditions. These biochemical characteristics are summarized in *Table 1*.

Table 1. *Production of siderophores, hydrogen cyanide (HCN), and enzymatic activity by Bacillus isolates*

Isolate	Siderophores*	HCN**	Lipase	Urease	Gelatinase	Amylase	Cellulase
B3	++	+	+	+	+	+	–
B44	–	+	+	+	+	+	–

*Width of orange zone on CAS medium: + = 0–10 mm; ++ = > 10 mm; – = no halo **HCN: + = produced; – = not produced; enzyme activities: + = hydrolysis observed; – = no hydrolysis observed

Effect of inoculation with B. subtilis in leaves of soybean plants on LP and enzyme activity with and without exposure to mites

In the present study, results in plants treated with mites have shown the highest level of LP intensity (the highest MDA content, an end product of the LP process) in the leaves of the non-inoculated group of soybean plants (*Table 2*). The non-inoculated group of soybean plants treated with mites suffers the greatest oxidative stress due to the most intense LP process which confirms the negative impact of mites on soybean plants. Furthermore, results in plants treated with mites have shown a statistically significant higher LP intensity in non-inoculated plants compared to inoculated ones, which indicates a positive effect of *B. subtilis* inoculation in protecting soybean plants from mite attacks. The lowest MDA content (LP intensity) was observed in the leaves of control plants, followed by MDA content in the leaves inoculated with *B. subtilis*. The statistically significant higher LP intensity in the leaves of soybean plants

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inoculated with *B. subtilis* compared to control plants confirms that *B. subtilis* inoculation causes mild oxidative stress, which prepares plants for the subsequent stress, which in this study was caused by a mite attack. The lower intensity of LP in the plants inoculated only with *B. subtilis* compared to the group of plants exposed to the mite attack indicates that the first stress to which the plant is exposed prepares a plant defense response for the second stress.

Inoculation with *B. subtilis* successfully decreases LP intensity in leaves of soybean plants during the mite attack.

Results in soybean plants treated with mites have shown a lower enzyme activity (SOD, GPX, and PPX) in inoculated plants compared to non-inoculated ones and a higher enzyme activity compared to control soybean plants (*Table 2*).

A statistically significant decrease in the CAT activity in soybean leaves was detected in the group of plants treated with mites compared to the control group, while an increase in the CAT activity was detected in plants inoculated with *B. subtilis* (*Table 2*).

Our results showed an increase in the LP intensity, and the activity of antioxidant enzymes (SOD, GPX, and PPX) in leaves of inoculated plants without exposure to mites, compared to control (*Table 2*). Hence, mild stress was observed.

Table 2. *Lipid peroxidation intensity and enzyme activity in leaves of soybean plants inoculated with Bacillus subtilis and exposed to two-spotted spider mites*

Samples	LP	SOD	GPX	PPX	CAT
Control	84.72 ± 1.019^{a}	189.8 ± 0.11^{a}	25.73 ± 0.49^{a}	9.15 ± 0.14^{a}	0.35 ± 0.002^{a}
B. subtilis	104.42 ± 0.50^{b}	410.1 ± 0.20^{b}	47.58 ± 1.19^{b}	12.37 ± 0.13^{b}	0.40 ± 0.002^{b}
TSSM	146.12 ± 0.60^{c}	701 ± 5.33^{c}	48.55 ± 0.41^{b}	16.53 ± 0.14^{c}	0.25 ± 0.002^{c}
B. subtilis and TSSM	123.46 ± 1.28^{d}	544.4 ± 10.08^{b}	41.6 ± 0.12^{c}	$10.3 \pm 0.21^{a,b}$	0.37 ± 0.013^{b}

Control – non-treated plants; LP – lipid peroxidation (nmol MDA g^{-1} FW), SOD – superoxide-dismutase (U g^{-1} FW), GPX – guaiacol-peroxidase (U g^{-1} FW), PPX – pyrogallol-peroxidase (U g^{-1} FW), CAT – catalase (U g^{-1} FW); the data are mean values ± standard error; a-d – values without the same superscripts within each column differ significantly ($P < 0.05$)

A simple correlation test showed a significantly positive correlation between the MDA content and the SOD activity ($r = 0.99$). In addition, a non-significantly positive correlation was recorded between the MDA content and the peroxidase activity (GPX and PPX). While there was a negative correlation between the MDA content and the CAT activity (*Table 3*).

Table 3. *Correlation of lipid peroxidation intensity (nmol MDA g^{-1} FW) and antioxidant enzyme activity in leaves of soybean plants*

	LP	SOD	GPX	PPX	CAT
LP	1.00	0.99	0.76	0.82	-0.68
SOD	0.99	1.00	0.83	0.81	-0.59
GPx	0.76	0.83	1.00	0.77	-0.20
PPx	0.82	0.81	0.77	1.00	-0.73
CAT	-0.68	-0.59	-0.20	-0.73	1.00

SOD – superoxide-dismutase (U g^{-1} FW), GPX – guaiacol-peroxidase (U g^{-1} FW), PPX pyrogallol-peroxidase (U g^{-1} FW) and CAT – catalase (U g^{-1} FW). Red marked correlations are significant at $p < 0.05$

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Effect of inoculation with B. subtilis in roots of soybean plants on LP and enzyme activity with and without exposure to mites

During the mite attack, inoculation with *B. subtilis* successfully reduced the LP intensity in the roots of soybean plants. The highest level of MDA was measured in the leaves of the non-inoculated group of soybean plants treated with mites, while a similar LP intensity was observed in the inoculated group of soybean plants with *B. subtilis* with and without exposure to mites (*Table 4*).

A significantly higher activity of SOD, CAT and GPX was detected in the roots of non-inoculated infected plants, while the activity of PPX was lower compared to the control group of soybean plants.

Furthermore, inoculation with *B. subtilis* itself significantly increased the activity of antioxidant enzymes SOD, GPX, and CAT in the roots of soybean plants, while there was no significant increase in LP compared to control plants. Despite all this, it was noticed that inoculation significantly decreased the activity of PPX compared to control (*Table 4*).

Table 4. *Lipid peroxidation intensity and enzyme activity in roots of soybean plants inoculated with Bacillus subtilis and exposed to two-spotted spider mites (TSSM)*

Samples	LP	SOD	GPX	PPX	CAT
Control	29.61 ± 0.15^{a}	89.52 ± 3.78^{a}	44.31 ± 0.40^{a}	31.45 ± 0.16^{a}	0.060 ± 0.00^{a}
B. subtilis	32.18 ± 0.38^{a}	113.56 ± 0.57^{b}	70.18 ± 0.32^{b}	23.52 ± 0.16^{b}	0.063 ± 0.19^{a}
TSSM	48.91 ± 0.37^{c}	187.0 ± 4.45^{c}	77.21 ± 0.20^{c}	21.77 ± 0.40^{b}	0.093 ± 0.00^{b}
B. subtilis and TSSM	33.97 ± 0.85^{a}	97.25 ± 0.54^{d}	102.56 ± 0.34^{d}	10.56 ± 0.13^{c}	0.033 ± 0.00^{c}

Control – non-treated plants; the data are mean values ± standard error. a-d – values without the same superscripts within each column differ significantly ($P < 0.05$); LP – lipid peroxidation (nmol MDA g^{-1} FW), SOD – superoxide-dismutase (U g^{-1} FW), GPX – guaiacol-peroxidase (U g^{-1} FW), PPX – pyrogallol-peroxidase (U g^{-1} FW), CAT – catalase (U g^{-1} FW)

A statistically significant positive correlation was noticed between the MDA content and the SOD activity ($r = 0.97$), as well as between the GPX and the PPX activity. A positive correlation was recorded between the MDA content and the GPX activity, but not statistically significant. On the other hand, a negative correlation was recorded between the MDA content and the PPX activity (*Table 5*).

Table 5. *Correlation of lipid peroxidation intensity (nmol MDA g^{-1} FW) and antioxidant enzymes activity in roots of soybean plants*

	LP	SOD	GPX	PPX	CAT
LP	1.00	0.97	0.30	-0.20	0.73
SOD	0.97	1.00	0.15	-0.04	0.85
GPx	0.30	0.15	1.00	-0.99	-0.38
PPx	-0.20	-0.04	-0.99	1.00	0.48
CAT	0.73	0.85	-0.38	0.48	1.00

SOD – superoxide-dismutase (U g^{-1} FW), GPX – guaiacol-peroxidase (U g^{-1} FW), PPX pyrogallol-peroxidase (U g^{-1} FW) and CAT – catalase (U g^{-1} FW). Red marked correlations are significant at $p < 0.05$

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Effect of inoculation with isolate B. subtilis on morphology parameters of soybean plants

Due to plant growth-promoting properties of *B. subtilis*, morphology parameters were measured. Results in plants inoculated with *B. subtilis* have shown a positive impact on the fresh mass of stems and roots, length, and volume of roots (*Table 6*). A statistically significant higher level of fresh mass of stems, fresh mass of roots, and volume of roots was detected in the inoculated soybean plants compared to control, while there were no significant differences in fresh mass of stems, fresh mass of roots and roots length between plants inoculated with *B. subtilis* and exposed to mites after inoculations and the control group of soybean plants. Furthermore, results in plants treated with mites have shown lower volume of roots in non-inoculated and inoculated plants compared to control.

Table 6. *Morphology parameters of soybean plants inoculated with Bacillus subtilis and exposed to two-spotted spider mites (TSSM)*

Sample	Fresh mass of stems [g]	Fresh mass of roots [g]	Length of roots [cm]	Volume of roots [cm^3]
Control	2.61 ± 0.033^{a}	1.27 ± 0.008^{a}	18.3 ± 0.064^{ab}	1.03 ± 0.005^{a}
B. subtilis	3.07 ± 0.021^{b}	1.87 ± 0.017^{b}	19.99 ± 0.283^{a}	1.6 ± 0.021^{b}
TSSM	1.67 ± 0.012^{c}	0.53 ± 0.008^{c}	13.9 ± 1.88^{c}	0.86 ± 0.002^{c}
B. subtilis and TSSM	2.38 ± 0.036^{a}	1.08 ± 0.008^{a}	16.84 ± 0.046^{b}	0.87 ± 0.017^{c}

Control – non-treated plants; the data are mean values ± standard error; a-c – values without the same superscripts within each column differ significantly ($P < 0.05$)

Discussion

Inoculation with PGPR is beneficial to plants and promotes the growth and immunity of plants (Beneduzi et al., 2012). Apart from their influence on plant nutrition and growth, beneficial microbes (such as rhizobacteria and fungi) also contribute to enhanced plant defenses, making the whole plant more resistant to pathogens and pests (Romera et al., 2019).

The increased frequency of pest resistance is a consequence of the excessive use of pesticides. Chemical control of the pest is harmful to humans, animals, and the environment, as well. Therefore, a search for alternative methods has been encouraged (Amemann et al., 2015).

Different environmental conditions (such as drought, quality of soil, temperature variations, anoxia, etc.) may lead to the production of very aggressive ROS, which may attack components of the lipid membrane and another biomolecule (Sharma et al., 2012). Lipid peroxidation of cell membranes is one of the consequences of uncontrolled oxidative stress. Malondialdehyde (MDA) has been widely employed as a biomarker for lipid peroxidation (Ayala et al., 2014).

According to Curá et al. (2017), inoculation with beneficial microorganisms successfully reduces the content of MDA in plants during drought conditions. A higher level of LP in plants under condition stress, such as a spider mite attack, was recorded by Antoniou et al. (2018). The accumulation of ROS and phenolic compounds at the wounding sites caused by the TSSM in barrelclover and thale cress plants has been reported (Santamaria et al., 2020).

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Antioxidant enzymatic and non-enzymatic defense mechanisms control the accumulation of ROS. Plants possess extremely efficient enzymes such as SOD, GPX, PPX, and CAT. Therefore, oxidative stress in plants was monitored by the changes in the activity of antioxidant enzymes (SOD, PX, CAT). SOD acts as a first line of defense, converting O^{2-} into H_2O_2 (Sachdev et al., 2021). Caversan et al. (2016) recorded an increase in the SOD activity under different heat shock treatments. An increase of peroxidase activity in different black gram genotypes upon whitefly feeding was recorded by Taggar et al. (2012).

However, Farouk and Osman (2012) found that mite infestation can cause a significant decrease in the CAT activity. The reduced catalase activity in infested plants could be explained by the Haber-Weiss reaction, which generates hydroxyl radicals from H_2O_2 (Taggar et al., 2012). Also, the biotic stress (in this case – the mite attack) can cause an inhibition of enzyme activity by elevating the level of oxidative stress in plants (Dewanjee et al., 2014).

Secondly, the aim was to assess the beneficial effect of inoculation itself, without the exposure to mites. According to Qiao et al. (2017), these species are well known for their beneficial action on plants stimulating plant growth directly by increasing nutrients or indirectly by inducing plant resistance against pathogens. The stimulation of antioxidant enzymes was observed due to *B. subtilis* inoculation to alleviate the effect of stress (Hashem et al., 2017).

Plants have developed a variety of mechanisms to defend themselves. However, since these mechanisms share key points, facing one type of stress means that plants could cope with other types of stresses more efficiently (Rejeb et al., 2014).

According to Hashem et al. (2017) inoculation of *B. subtilis* to infested plants alleviated the oxidative stress due to the reduction of the MDA content. The increased SOD activity could be considered a proof of a higher level of oxidative stress (Cho and Park, 2000). Studies of Sharma and Dubey (2007) indicate an increased activity of CAT in the roots of rice induced by a toxic concentration of Al^{3+} (abiotic stress). Cho and Park (2000) documented that the activity of GPX was higher in the roots than in the leaves of plants.

PGPR are a diverse and naturally occurring bacteria with the ability to colonize and interact with plant roots (Beneduzi et al., 2012). Those rhizobacteria have a beneficial influence on plant growth, reducing the need for fertilizers and decreasing the pollution of agricultural soils and water, which is essential for agricultural ecosystems (Pineda et al., 2010). In our study, the selected *B. subtilis* isolate B3 demonstrated the ability to produce multiple extracellular enzymes, including lipase, urease, and gelatinase, as well as HCN. Most notably, isolate B3 was capable of siderophore production, a key trait associated with induced systemic resistance (ISR). Siderophores contribute to plant defense by chelating iron in the rhizosphere, thus depriving pathogenic microbes of this essential nutrient and indirectly stimulating plant immune responses (Lin et al., 2023). These biochemical traits may explain the observed enhancement in both plant growth and antioxidant defense mechanisms following inoculation.

Although our study demonstrates the positive effects of *B. subtilis* on oxidative stress alleviation in soybean during the *T. urticae* infestation, it does not provide mechanistic insight into the molecular pathways involved. Specifically, the expression of genes associated with jasmonate/ethylene signaling, PR proteins, or systemic resistance markers have not been investigated, nor have the pest population dynamics or visible symptoms of damage assessed. These limitations restrict the ability to directly link biochemical changes to improved plant tolerance or yield outcomes. Future research should include

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transcriptomic or proteomic analyses to uncover the underlying signaling networks and validate the role of PGPR in induced systemic resistance (ISR).

The application of PGPR for managing *T. urticae*-induced oxidative stress in soybean plants holds not only agronomic and ecological benefits but also significant implications for the nutritional quality of soybean as animal feed. Soybean is a vital component in poultry diets due to its high content of digestible protein, essential amino acids, and bioactive compounds including isoflavones and unsaturated fatty acids. However, biotic stress caused by mite infestation and subsequent oxidative damage can alter the metabolic composition of soybean seeds, potentially affecting feed quality and animal health. Lin et al. (2023) emphasized that plant-based feed should be evaluated not only for processing and nutritional properties but also from a safety perspective, identifying pesticides among the key risk factors. Szarka et al. (2024) further underscored the need to monitor pesticide residues in soybean products as part of a broader food safety strategy, advocating for innovative approaches to pesticide residue control and management.

Ali et al. (2020) reviewed the potentials and limitations of soybean use in animal and human nutrition, highlighting its value as an affordable protein source and a fundamental component of balanced animal feed, particularly in poultry, where its combination with maize provides a complete amino acid profile. However, Mathias et al. (2024) reported adverse effects on poultry health and performance following a dietary exposure to herbicides, underlining the importance of assessing the consequences of herbicide use across the entire food chain.

By enhancing the plant's antioxidant defense system and reducing the impact of oxidative stress, PGPR inoculants help to preserve the integrity of soybean's metabolic profile. Studies have shown that oxidative stress reduces seed protein content, alters lipid composition, and disrupts mineral uptake (Das and Biswas, 2022). This reduction in nutritive value may adversely affect the overall production performance of poultry (Bacou et al., 2021). The study by Tan et al. (2018) confirmed the impact of oxidized soybean oils on the oxidative status of broiler chickens. While the findings indicated that production performance may not always be adversely affected, elevated corticosterone levels were observed, suggesting that broilers still experience physiological stress in response to such dietary components.

The biocontrol action of PGPR not only suppresses pest populations through indirect mechanisms (e.g., induced systemic resistance and the enhancement of antioxidative enzymes like SOD, POD, and CAT), but also improves nitrogen fixation, root development, and nutrient assimilation. Consequently, PGPR-treated soybean plants often yield grains with a higher protein content and improved nutritional indices (Khoso et al., 2024). These quality improvements are directly relevant to poultry producers, as soybean remains a primary protein source in broiler feed formulations. Furthermore, minimizing chemical pesticide use through biological control strategies reduces the risk of pesticide residues in soybean-derived feed, which is a growing concern in poultry production and food safety (Bueno et al., 2023). The integration of PGPR in soybean cultivation thus contributes to a more sustainable livestock production system by ensuring feed quality and safety while reducing the environmental impact.

Conclusions

A significantly higher LP intensity in non-inoculated plants treated with mites compared to the control group was noticed. Also, the control group compared to

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inoculated plants treated with mites had a lower LP intensity. These results show that inoculation with beneficial microorganisms may decrease stress in plants. Following antioxidant enzymes, the SOD activity was in positive correlation with the LP intensity while PPX and GPX were especially active in plant roots following inoculation. The CAT activity was low both in leaves and roots. Notably, inoculation with PGPR itself induced mild biotic stress, which may have primed plants for enhanced antioxidant responses during a subsequent mite infestation. These findings indicate that PGPR inoculation can mitigate oxidative stress in soybean plants exposed to *T. urticae*. However, the study did not assess pest population dynamics, visible damage symptoms, or the expression of defense-related genes, limiting the ability to directly link biochemical responses to whole-plant tolerance or yield outcomes. Future research should address these aspects and explore underlying molecular mechanisms to confirm the role of PGPR in biocontrol strategies. Overall, the integration of PGPR-based inoculants into soybean pest management programs could complement the chemical control methods, reduce pesticide inputs, and enhance crop resilience under field conditions. Beyond agronomic benefits, such practices may also improve the quality and safety of soybean-derived feed, reinforcing their importance across the food and feed production chain.

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