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Research Article

Salt-Tolerant Bacteria (*Brachybacterium sp.* and *Bacillus spizizenii*) Enhance Growth, Photosynthetic Pigments, and Antioxidant Defense of *Vicia faba* under Drought Stress

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Abstract:

This study evaluates the mitigating effects of five salt-tolerant bacterial strains on drought-induced growth suppression in bean plants. An experiment was conducted with *V. faba* subjected to drought and treated with five salt-tolerant bacterial strains. Growth traits were measured at 7 days, 15 days, and during a recovery stage. All bacterial treatments enhanced growth traits relative to the control across time points. At 7 days, Bact2 (*Brachybacterium sp.*) and Bact4 *(Bacillus spizizenii*) showed the strongest responses, with increases of 275%,339% in shoot fresh weight, 209%, 373% in shoot dry weight, 153%, 170% in shoot length, and 205%, 222% in leaf number, respectively. By 15 days, Bact2 and Bact4 again yielded the highest values across traits, with substantial but slightly reduced relative gains. During recovery, Bact2 and Bact4 markedly continued to boost growth metrics over the control. Fresh and dry weights rose steadily from day 7 to recovery, Bact4 leading, followed by Bact 2; plant length and leaf number also increased over time, with Bact2 and Bact4 driving the largest gains. Pigment content rose significantly in all bacterial treatments, with Bact2 and Bact4 achieving the greatest enhancements. Salt-tolerant bacteria enhanced pigment content and antioxidant enzymes in *Vicia faba* under drought. By day 15, Bact2 and Bact4 most strongly boosted chlorophylls, carotenoids, APX, and PPO versus control, with recovery further elevating these markers. These strains show promise for sustainable strategies to bolster crop productivity in arid environments. These findings highlight potential microbial inoculants for sustainable faba bean cultivation under water scarcity.

1. Introduction

Faba bean *(Vicia faba L.)* is one of the main legumes grown in Egypt (Singh et al., 2013). Faba beans are a key protein source for both people and animals, play an important role in crop rotation, and their seeds feed over a billion people globally but climate change is putting legume production at risk (Mansour et al., 2021)

Faba bean cultivation particularly in arid and semi-arid regions is unsuitable because this crop is not sufficiently drought and heat tolerant as it is susceptible to moisture and high temperature stresses (Loss and Siddique, 1997).

Long-term droughts are detrimental to the land and reduce farming productivity. They also threaten food security, the energy supply, and public health. Therefore, effective management of land and water is essential for long-term growth. Extended drought leads to stunted plant growth and lower respiration, disrupts ATP production, boosts the creation of harmful reactive oxygen species (ROS), and ramps up both enzymatic and non-enzymatic antioxidants. It also triggers more osmoregulatory compounds like glycine betaine and proline, raises levels of soluble sugars and proteins, increases lipid peroxidation, and can ultimately cause plant death (Cvikrová et al., 2013; Seleiman et al., 2021a; Bondok et al., 2022b). Various methods and technologies have been created to counteract drought's harmful effects on plant growth, including plant growth regulators, genetic engineering, biochar, fertilizers, beneficial microbes, seaweed extracts, and seed priming (Ali et al., 2017; Hussain et al., 2018).

Using rhizobia as biofertilizers has been demonstrated to enhance nitrogen fixation and improve soil health, particularly under stressful conditions. New research also indicates that combining native rhizobia with plant growth-promoting rhizobacteria (PGPR) can significantly enhance drought resistance in legumes (Mansour et al., 2021).

The usage of bacillus species as PGPR has been shown to be an ecofriendly method of enhancing agricultural yields by encouraging plant development via a directly and indirectly mechanism. Plant growth promoting bacillus strains regulates physiologic and metabolic balance, induces resistance against drought stress, and solubilizes minerals for efficient plant uptake(Azeem et al., 2023). Inoculation of *Brachybacterium sp.* strains improved the germination of a drought-sensitive variety of maize landraces(Arellano-Wattenbarger et al., 2024).

In this study we hypothesized that salt-tolerant bacteria would enhance growth, pigment accumulation, and antioxidant defense in drought-stressed faba bean, so the aim of this study was to evaluate salt-tolerant bacteria isolated from wastewater/saline soils for their ability to alleviate drought stress in faba bean.

2. Materials and Methods

2.1. Experiment location

This study was conducted at the Agricultural Experiment Farm, Botany Department, Faculty of Agriculture, Tanta University, Egypt, during the 2022 agricultural season. The study focused on the faba bean (*Vicia faba*) cultivar 'Sakha 1'. Seeds were obtained from Sakha Agricultural Research Station (SARS), Food Legumes Research Section, Kafr El-Sheikh, Egypt.

2.2. Microorganism Isolation and Treatments

Samples were collected from agricultural wastewater and saline soil, then serially diluted. Dilutions of 10^5 and 10^6 were plated on Nutrient Agar containing different salt concentrations (0.25, 0.5, and 1 µM NaCl).

2.3. Molecular identification of isolated strains

2.3.1. DNA Extraction and PCR reaction

DNA was extracted from the bacteria according to (Abed, 2013). A total of 50 µl of 1X reaction buffer,1.5 mM MgCl2, 1U Taq DNA polymerase (Promega), 2.5 mM dNTPs, 30 pmol of forward and reverse primers of 16SrRNA gene (F:5'-, AGAGTTTGATCCTGGCTAG -3′ and R:5'- GGTTACCTTGTTACGACTT -3′), and 30 ng of genomic DNA were used as a template. An initial denaturation cycle lasting 5 minutes at 94°C was followed by 40 cycles of PCR amplification using a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems) protocol. Each cycle included a denaturation phase lasting 30 seconds at 94 °C, an annealing step lasting 30 seconds at 45 °C, and an elongation stage lasting 1 minute at 72 °C. In the last cycle, the primer extension phase was prolonged to 7 min at 72°C. The PCR products were detected by electrophoresis on a 1.5% agarose gel. Amplified products were purified using an EZ-10 spin column according to user manual(Sharaf-Eldin et al., 2023).

2.3.2. 16S-rRNA amplified product sequencing analysis

Using Big Dye TM Terminator Cycle Sequencing Kits according to user manual, the resultant PCR was sequenced in an automated sequencer ABI PRISM 3730XL Analyzer before being exposed to electrophoresis in an ABI 3730xl sequencer (Microgen Company), Korea. The sequences were analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). Sequences were aligned using Align Sequences Nucleotide BLAST. The phylogenetic tree was constructed by data matrix following the neighbor-joining method using MEGA 6.1 software.

2.3. Experimental design

Plastic bags (38×34 cm) were planted with Completely Randomized Design. Three replicates per treatment and 15 beans in each pot. After 21 days, the first five treatments were inoculated with bacteria. The first treatment acted as the drought control. Measurements were taken 7 and 15 days after treatment application. Plants were irrigated on the 16th day after treatment, and samples were collected three days after irrigation.

2.4. Sampling

Five random samples were collected from each treatment at the following times: 7 days after treatment application, 15 days after treatment application, and 3 days after the first recovery irrigation.

2.5. Growth measurements

Five seedlings from each replicate were used to measure the following traits: shoot length (cm), shoot fresh weight (g), shoot dry weight (g), and leaf area (LA) (cm^2).

2.6. Physiological traits

Physiological traits, including chlorophyll content, antioxidant enzyme activities, malondialdehyde (MDA) content, and proline content, were measured.

2.7. Photosynthetic pigments

Chlorophyll levels were estimated using the spectrophotometric method (UV1901PC), as described by (Lichtenthaler and Wellburn, 1983). Chlorophyll was expressed as µg/ml methanol by this equation:

Chlorophyll a (mg g^{-1} FW) = 15.65 A666 - 7.340 A653

Chlorophyll b (mg g^{-1} FW) = 27.05 A653 - 11.21 A666

Carotenoids (mg g^{-1} FW) = (1000*A470) - (2.86 – Ch a) – (129.2*Ch b /245)

Where,

A 666, A470 and A653 are absorbance's at A653, A470 and A666 nm.

2.8. Antioxidant enzyme activity assay

2.8.1. Preparation of buffer solution

Monosodium phosphate and disodium phosphate are used. The molecular weight of each is known either from within the container or by calculating it to prepare a solution with a concentration of 0.1 mol/L in distilled water. The concentration is then adjusted according to the pH (Gomez et al., 2001). Tissue preparation for the determination of antioxidant enzymes followed this procedure: samples of fresh leaf (0.5 g) were pulverized in liquid nitrogen and homogenized in 4 mL of a solution comprising sodium phosphate buffer (50 mM). The mix homogenate was centrifuged for 20 minutes at 15,000 rpm under 4°C; the supernatant was utilized for the enzyme activity assay.

2.8.2. Polyphenol oxidase activity (PPO)

The activity of PPO was assessed using the method established by (Mayer and Harel, 1979) at a wavelength of 490 nm. 200 µl of enzyme solution mixed with 0.01 mM catechol solution, which was made in 0.1 M sodium phosphate buffer at pH 6.5. The blank was made using an equivalent quantity of catechol and 0.03 mL of 50 mM sodium phosphate buffer, excluding the enzyme extract.

2.8.3. Ascorbate peroxidase enzyme activity

The following reaction mixture was used (Yoshimura et al., 2000): phosphate buffer (25 mM, pH 7), enzymatic extract (0.2 ml) hydrogen peroxide (1 mM), ascorbic acid (0.25 mM), and EDTA (0.1 mM). Hydrogen peroxide was incorporated into the mixture to initiate the enzymatic reaction. The light absorption was measured for one minute at 290 nm using a spectrophotometer following the addition of the enzyme extract.

2.9. Biochemical contents

2.9.1. Proline content

The following proline concentration was measured spectrophotometrically at 520 nm using the ninhydrine methodology (Bates et al., 1973). The homogenate was filtered through filter paper after roughly 0.5 g of plant material was homogenized in 10 mL of 3% aqueous sulfosalicylic acid (SSA). In a test, 2 milliliters of the filtrate were combined with 2 milliliters of glacial acetic acid and 2 milliliters of ninhydrin acid. The tubes were heated to 100 degrees Celsius for one hour in a water bath, and then they were placed in an ice bath to complete the reaction. Finally, four milliliters of toluene were added to the reaction mixture. For twenty to thirty seconds, it was vigorously mixed. After heating it to room temperature, toluene was used as the blank, and the absorbance was measured at 520 nm. Using a standard curve as a guide, the proline concentration was computed using fresh weight as follows:

(mg proline x ml toluene) / 115.5]/[(g sample)/5] = moles of proline/g of fresh weight material. 115.5 is the molecular weight of proline

2.9.2. Malondialdehyde (MDA)

MDA content was measured according to (Heath and Packer, 1968). Samples weighing approximately 0.5 g were dissolved in 10 mL of trichloroacetic acid (0.1%) (w/v). After centrifuging the homogenate at 1500 × g for 10 minutes, the liquid was filtered and used for the analysis. Two milliliters of the diluted extract and two milliliters of TBA (0.67%) made with TCA (20%) were mixed. It should be incubated in water (95–100°C) for 30 minutes. After five minutes at room temperature, it should be put in an ice bath. In that order, the absorbance of the aqueous phase was measured at 450, 532, and 600 nm.

The following formula was used to determine the MDA concentration in the aqueous phase: Concentration μmol/L) = 6.45 × (A532 − A600) − 0.56 × A450.

2.10. Statistical analysis

The data were given as means ± standard deviation (SD). Statistical significance was determined using two-way ANOVA via SPSS 20 software, with individual treatment comparisons assessed by the Tukey multiple range test at $P < 0.05$.

3. Results

3.1. Salt tolerance range for bacterial isolates

Several bacteria were initially isolated at a concentration of 1 μM NaCl. However, bacterial growth was limited at two μM, so further selection was done on media containing 1.5 μM NaCl. This process resulted in five bacterial isolates.

3.2. Molecular identification of bacteria

A phylogenetic tree in Figure (1) that shows how a group of nucleotide sequences (shown by their GenBank accession codes) are related to each other. The numbers at the nodes are bootstrap values, which show how sure each branch is (the closer the value is to 100, the greater the support). The tree is divided into two major clades (branches). The upper clade (bootstrap values mostly 58–60) contains strains such as EU873259.1, DQ312470.1, and KP027802.1, among others. The lower clade (bootstrap support 52–96) comprises strains such as JX949864.1, OR131162.1, and JX454452.1, among others. The phylogenetic analysis indicates that the query sequence (Bact (2), (lcl|Query 6070701) is most closely associated with JX454476.1 (*Brachybacterium sp.* 11394, Gram-positive bacteria) and JX454452.1 (*Brachybacterium sp.* 11427 Gram-positive bacteria), as evidenced by a high bootstrap value (96). This suggests that the query strain probably belongs to the same lineage or species group as the two reference sequences.

On the other hand Figure (2) showed the the classification of Bact (4). At the very bottom, there is lcl|Query 2181043, representing the query sequence used in the analysis. The numbers at the nodes (e.g., 41, 23, 64, 59) are likely bootstrap values from resampling tests. These indicate the confidence level (%) of the branching pattern at that node. The query sequence (lcl|Query 2181043) is most closely related to the sequence gb|OR187152.1|:5-874 (*Bacillus spizizenii*. They form a clade with high bootstrap support (63). That cluster is nested with sequences gb|OL708413.1| *Bacillus subtilis* and gb|OR591420.1|*Bacillus rugosus,* suggesting close evolutionary relationships.

3.3. The effect of bacteria on the growth traits of bean plants (*Vicia Faba* L.) under the influence of drought (7 and 15 days after applying the treatments) and the recovery stage (3 days after irrigation of the treatments)

An agricultural experiment was conducted to investigate the mitigating effect of bacteria on drought stress in bean plants (*Vicia faba*) using five strains of salt-tolerant bacteria. The results indicated that all bacteria resulted in an increase in growth traits after 7 days, including shoot fresh and dry weight, shoot length, leaf area, and leaf number (Fig. 3), compared to the control group. Notably, Bact 2 and Bact 4 exhibited the highest values across all parameters compared to the negative control, with increments of 275% and 339%, 209% and 373%, 153% and 170%, and 205% and 222% respectively.

The results in Figures (3) indicate that all bacteria resulted in an increase in growth traits after 15 days, including shoot fresh and dry weights, shoot length, leaf area, and leaf number compared to the control group. Notably, Bact 2 and Bact 4 exhibited the highest values

across all parameters compared to the negative control, with increments of 156%, 177%; 129%, 194%; 132%, 158% and 116%, 163% respectively.

The results during the recovery stage showed that bacteria (Bact 2 (*Brachybacterium sp.*) and Bact 4 *(Bacillus spizizenii*) significantly increased growth traits, including shoot fresh and dry weight, shoot length, leaf area, and leaf number, compared to the control group. The increases were as follows: 164 %, 185 %, 139 % and 145 %, 223 %, 138 % and 136 %, 155 %, 121 % and 112 %, 159 %, 122% respectively (Figure 3).

Fresh and dry weights rise steadily from 7 days (1) to recovery period (3) across all treatments, with Bact 4 leading to the highest gains by day 3 closely followed by Bact 2. The control group stays lowest throughout, highlighting how the bacterial treatments (especially Bact 4) powerfully promote both water content and biomass accumulation over time. Both plant length and number of leaves increase from day 1 to day 3 across all treatments, with the biggest jumps clearly seen for Bact 2 and Bact4 in length and leaf number, especially by recovery. The control always lags behind, highlighting how certain bacterial treatments really boost growth and leaf development over time (Fig. 3).

3.4. The effect of bacteria on the pigment content under the influence of drought (7 and 15 days after treatment application) and the recovery stage (3 days after irrigation of the treatments)

In the context of bacteria, a significant rise in pigment content was observed in the treatments with bacteria compared to the negative control. Specifically, Bact2 and Bact4, in comparison to other strains, exhibited increases of (239%, 299%), (144%, 252%), (234%, 280%), and (298%, 424%), respectively, when contrasted with the negative control (Figure 4)

Figures (8) show the effect of bacteria on pigment content in *Vicia faba* under drought stress after 15 days of treatment. There was a noticeable increase in pigment content in the treatments with bact4 compared to the other bacteria and the control group, with increases of 176% and 118 % and 155 %, 259 % compared to the control.

Figures (4) illustrate the effect of bacteria and fungi on the pigment content during the recovery stage. Regarding bacteria, there was a significant increase in the pigment content (chlorophyll a, b, total chlorophyll and carotenoids) compared to the negative control. Bact2 and bact4 showed the highest values compared with the other treatments. In comparison to the negative control, bact2 and bact4 gave an increase by 142 % , 148 % , 128 % and 204% , 245% , 120% and 162% , 179% , 126% and 174% , 197% , 139% in chlorophyll a, b, total chlorophyll and carotenoids respectively.

Chlorophyll a levels rise with all treatments over time, peaking on period 3 especially with Bact 2, and 4 showing enhanced photosynthetic activity compared to the control. Treatments Bact 2 (*Brachybacterium sp.*) and Bact 4 *(Bacillus spizizenii*) are clear winners for boosting plant health, while the control lag behind throughout. Chlorophyll b levels increase over time for most bacterial treatments, with the most dramatic peak on day 3 (blue line), especially with Bact 2 and Bact 4, these stand out above others. The control remain lower overall, suggesting Bact 2 and Bact 4 really boost photosynthetic pigment content by day 3. Total chlorophyll content rises over the three times for all treatments, with Bact 2, and Bact 4 peaking highest on day 3. In contrast, the control stay consistently lower, suggesting these particular bacterial treatments really boost chlorophyll production and likely support stronger plant growth. Carotenoid levels increase after bacterial treatments, with the biggest jumps on period 2 and especially period 3 for Bact 2 and Bact 4. The control stay much lower, suggesting these specific treatments are especially good at boosting carotenoid production, which could mean better plant health or stress protection (Fig.4)

3.5. The Effect of Bacteria on Antioxidant Enzymes under the influence of drought (7 and 15 days after applying the treatments) and the recovery stage (3 days after irrigation of the treatments).

As for bacteria, there was a significant increase in antioxidant enzymes compared to the control group. The most notable increases were observed in bacterial strains (bact2, bact4, bact5) for the two enzymes, Ascorbate Peroxidase (APX) and Polyphenol Oxidase (PPO). The values for these enzymes were markedly higher across all parameters when compared to the negative control, showing increases of 403%, 852%, and 637% for APX, and 250%, 343%, and 248% for PPO (Figure 5).

Figures (5) show the effect of bacteria on antioxidant enzymes in *Vicia faba* under drought stress after 15 days of treatment. There was a noticeable increase in antioxidant enzymes in the treatments with bactria compared to the control group, the most significant increase was bacterial (bact2, bact4) for APX and PPO with increase 127 %, 189 % and 131% , 127% for bact2 and bact4 respectively.

The effect of bacteria on antioxidant enzymes during the recovery stage (3 days after irrigation of the treatments). As for bacteria, it led to a significant increase in antioxidant enzymes compared to the control group. The most notable increases were observed with bacterial treatments (bact2, bact4) for the two enzymes Ascorbate Peroxidase (APX) and Polyphenol Oxidase (PPO) with increase of 118 % , 131% , 94 % and 501% , 515% , 334 % (Fig.5).

The PPO enzyme levels increase over time across all treatments, with the highest activity on period 2 (15 days), especially in Bact 4, which stands out from the rest. Day 1 and 2 (7 days and recovery period) remain pretty stable and much lower, showing little change between treatments. APx enzyme activity rises sharply on 15 days (2) and recovery period (3) for all bacterial treatments, peaking at Bact 2 and Bact 4, while the control and day 1 levels stay low and steady. This suggests bacterial treatments especially Bact 2 and Bact 4 are boosting the plant's antioxidant defense over time.

3.6. The effect of bacteria on proline levels in plants under drought stress (7 and 15 days after treatment application) and during the recovery stage (3 days after irrigation)

In the case of 7 days, the concentration of proline significantly increased compared to the control group, with the highest increases observed in bact2 and bact4, showing values of 254% and 704% respectively (Figure 6).

Fifteen days after applying the treatments, the content of proline decreased in the bacteria-treated group compared to the control group, with the most significant decrease observed in bact4, which recorded a 37% decrease compared to the control.

Figure (6) shows the effect of bacteria on proline content in plants during the recovery stage. As for bacteria, the proline content increased in bact 3 (181%) and bact 4 (123%), compared to the control group

The marked increase in proline on periods 2 and 3, especially with Bact 3, points to heightened stress adaptation or osmoprotective effects triggered by this treatment. Notably, although the control also rises on day 2, Bact 3 consistently drives higher proline levels by periods 3, indicating sustained protection or metabolic activity compared to other treatments (Fig. 6).

3.7. The effect of bacteria on MDA in plants under the influence of drought (7 and 15 days after treatment application) and the recovery stage (3 days after irrigation of the treatments)

Regarding bacteria, the concentration of Malondialdehyde (MDA) was lower in treated plants compared to the control group, with the lowest values observed in bact2 and bact4, which showed reductions of 19% and 14% respectively, after 7 days (Figure 7).

The effect of bacteria on MDA in plants under drought conditions (15 days after applying the treatments). Regarding bacteria, they led to a decrease in MDA in all treatments, especially in bact4 and bact5. They record decrease by 23% and respectively compare with the control (Figure 7).

Figures (7) show the effect of bacteria on Malondialdehyde (MDA) content in plants during the recovery stage. Regarding bacteria, the concentration of MDA was lower in treated plants compared to the control group, with the lowest levels observed in bact2 and bact4 at 43 % and 61 % respectively.

Across the three periods, MDA levels drop steadily in the bacterial treatments, with the lowest values by the recovery period (3) especially for Bact 2 and Bact 4 highlighting their strong protective effects as time goes on. In contrast, the control stays high throughout, showing that without bacterial help, plants experience more sustained oxidative stress (Fig.7).

4. Discussion

The application of salt-tolerant bacteria significantly reduces drought stress in bean plants (*Vicia faba*), according to the findings of the experiment. The utilization of salt-tolerant bacteria seems to be an effective approach for promoting the growth of drought-stressed bean plants. The pronounced benefits of Bact 2 (*Brachybacterium sp.*) and Bact 4 (*Bacillus spizizenii*) underscore the potential for specific bacterial strains to be utilised in agricultural techniques to enhance crop resilience against climatic unpredictability. Further exploration of these strains could lead to innovative solutions for sustainable farming in challenging environments.

Between the drought-tolerant microbes belonging to different genera have been sorted out and characterized for plant growth-promoting microbes under abiotic stress of drought is *Brachybacterium* (Kour et al., 2019). *Brachybacterium* enhanced plant tolerance against the osmotic and toxic effects of salinity(Barnawal et al., 2016). Also, *Bacillus spizizenii* is as a plant growth salt-tolerant bacterium able to alleviate salt stress in crop plants by improving physiological parameters and antioxidant defense mechanisms (Masmoudi et al., 2021)

During the vegetative phase, the observable indications of water deficiency encompass reduced plant height, leaf wilting, and alterations in leaf quantity and surface area (Husen et al., 2017). Plant height, significantly impacted by drought, is intricately associated with cell expansion and leaf aging. The reduction in plant height is mostly due to diminished cell expansion, heightened leaf abscission, and compromised mitosis under drought conditions (Yang et al., 2021). Grain legumes display various morphological, physiological, morpho-physiological, physio-biochemical, and molecular effects under drought stress. The growth process involves cell division, cell expansion, and differentiation, encompassing genetic, physiological, ecological, and morphological events along with their complex interrelations. These events, influenced by water scarcity, dictate the quality and quantity of plant growth.

Cell growth is highly susceptible to drought due of the decline in turgor pressure. Cell elongation in higher plants can be inhibited by disrupting water transport from the xylem to the adjacent elongating cells during periods of significant water scarcity (Farooq et al., 2008). Impairments in mitosis, cell elongation, and expansion result in diminished leaf area, plant height, and crop growth. Drought stress impairs root and shoot growth, leading to diminished overall plant growth and development (Fathi and Tari, 2016). Drought-tolerant varieties of grain legumes significantly increase their rooting depth compared to sensitive varieties (Ye et al., 2018).

Drought causes the mesophyll cells in the leaves to become dehydrated; a severe water shortage causes the roots to constrict and causes induced deposition in the leaves; at the beginning of water stress, cell proliferation is stopped, which results in a decrease in leaf development. (Khatun et al., 2021) explained how drought stress inhibits turgor pressure, which in turn inhibits cell development. Since turgor pressure nor-

mally controls the expansion of leaf area, drought stress also results in a decrease in both the number of leaves and leaf area. Additionally, it was said that the fresh/dry weight ratio is always constrained because of the scarcity of water supplies (Iqbal et al., 2020)

The observed favourable effects of bacteria may be ascribed to multiple mechanisms, such as improved soil moisture retention, augmented nutrient availability, or the synthesis of growth-promoting chemicals (e.g., auxins, gibberellins). Microbial communities associated with plants, including nitrogen-fixing bacteria, and plant growth-promoting rhizobacteria (PGPR), enhance agricultural productivity and confer stress resistance. PGPR encompasses a diverse array of root-colonizing bacteria that exhibit superior root colonisation capabilities and the ability to synthesise various enzymes and metabolites, aiding plants in withstanding both biotic and abiotic stressors (Ngumbi and Kloepper, 2016)

Microorganisms protect plants from drought by making phytohormones, antioxidants, and xeroprotectants. These are the biochemical processes that are talked about most often (Vílchez et al., 2016). Because it works as a xeroprotectant, trehalose can help plants fight off the damage that drought does. Microbial species that can survive in dry conditions have been shown to protect some plants from drought. It depends on the microorganism's ability to control the amount of trehalose in the plant as a sign of damage from drying out (Hanaka et al., 2021).

Photosynthesis is significantly affected by water stress, as the products generated through leaf photosynthesis provide the essential materials for plant growth. The photosynthetic rate serves as a reliable indicator of material productivity per leaf area, thus measuring the biological production level of plants. Research shows that the photosynthetic rate declines with decreasing soil relative water content, primarily due to stomatal and non-stomatal limitations. Stomatal limitations are the main factor affecting photosynthesis during mild drought conditions, while under severe drought, non-stomatal factors become predominant.

Water deficiency reduces photosynthesis by limiting CO_2 availability, leading to diffusion constraints in the stomata and mesophyll (Flexas et al., 2004). Stomatal closure limits CO_2 absorption and reduces transpiration to maintain turgor pressure and water potential. For instance, drought conditions in wheat have been shown to decrease stomatal conductance and increase stomatal resistance, resulting in lower photosynthetic and transpiration rates (Ahmed and Stockle, 2016). As water stress intensifies, non-stomatal factors increasingly contribute to reduced potential rates of CO_2 assimilation, a decrease that cannot be remedied by raising external CO_2 levels. This decline in photosynthesis, influenced by non-stomatal factors, is associated with reduced activity or content of several key elements involved in photosynthesis (Yang et al., 2021).

Moreover, photosynthetic pigments are important indicators for plant health with environmental stress (Mansour et al., 2020). Our findings indicate that drought stress results in a significant decline in chlorophyll a and b content, as well as carotenoids, across all periods. This reduction in photosynthetic attributes represents one of the first responses of plants to water deficits, which adversely affects metabolite accumulation and overall productivity. Studies have established a positive correlation between the content of pigments and yield traits in faba beans (Janusauskaite and Razbadauskiene, 2021; Desoky et al., 2021b). Therefore, enhancing the photosynthetic pigments expression is crucial for improving yield traits of faba beans under drought conditions.

This study investigated the influence of inoculating faba beans with salt-tolerant bacteria on their growth under drought stress. Drought stress substantially reduced the photosynthetic pigments (Mansour et al., 2021). The findings showed that adding bacteria that can handle salt improved the production of photosynthetic pigments in plants that were stressed by drought compared to plants that weren't given the bacteria. This might have happened because the plants were better able to absorb water and nutrients, which are needed for the production of photosynthetic pigments (El-Nahrawy and Omara, 2017; Rabhi et al., 2018).

The results agree with (Enebe and Babalola, 2018; Fasciglione et al., 2015), who reported that, Using PGPR can change the way plants work and the structure of their cell walls, which might help the production of proteins and enzymes that are needed for color biosynthesis. Also agree with (Syamsia et al., 2018) that reported in dry paddy conditions, the amount of chlorophyll in the leaves was smaller than when it was wet.

When there isn't enough water, ROS like O2−, OH−, H2O2, and O2 are made. They damage cells, lower lipid peroxidation, and make it harder for cells to do their jobs. (Banik et al., 2016; Vardharajula et al., 2011). Plants create both enzymatic and non-enzymatic antioxidant defense systems to reduce oxidative damage and stop ROS buildup. (Bharti et al., 2016; Elrys et al., 2020).

One important strategy for reducing the detrimental effects of drought is to increase the production of ROS scavengers in stressed plants. (Habib et al., 2016; Saravanakumar et al., 2011). CAT lowers H_2O_2 levels and prevents OH-radicals from forming. (Elkelish et al., 2019; Semida and Rady, 2014). POX is essential for removing H_2O_2 and lowering oxidative damage. (Rady et al., 2013; Desoky et al., 2021a). In the current study, the activities of PPO, APX substantially increased with increasing water stress times and this agree with (Mansour et al., 2021).

Crop output can be significantly impacted by abiotic stressors such as drought, excessive salt, alkalinity, and high temperatures. Microbes, both naturally occurring and injected, have been shown to be effective in reducing abiotic stress in plants, including crops. In particular, by reducing ROS production, enhancing leaf hydration status, promoting water uptake, and preserv-

ing ionic balance, Rhizobium inoculants increase plant tolerance to abiotic stress (Thrall et al., 2008). Native rhizobia gathered from coexisting leguminous plants and targeted host plants can be used as resources to create novel and enhanced crop inocula (Li et al., 2023).

One interesting technique that can be used as a substitute source of phosphorus and nitrogen in legume crops is the application of enhanced biofertilizers, such as rhizobia. The low effectiveness of native rhizobial strains is a typical issue when cultivating leguminous plants, including faba beans (*Vicia faba* L.). Finding effective nitrogen-fixing inoculant strains that can boost crop output is therefore necessary. The purpose of this study was to evaluate the effects of both single and dual inoculation with AMF and Rhizobium laguerreae on faba bean plant growth and yield. In addition to gas-exchange characteristics, a variety of parameters were assessed during the flowering stage (number of flowers, stems, and leaves, shoot and root biomass, leaf area, leaf mass per area, and leaf area ratio) and harvesting stage (number and weight of pods and seeds) (Pereira et al., 2019).

Important plant cellular osmolytes produced under stress include proteins, soluble carbohydrates, and proline. They improve plant adaptability, preserve the stability of cell membranes, remove reactive oxygen species (ROS), and preserve normal cellular shape. These metabolites build up to lower osmotic pressure in plants during drought stress. (Ghosh et al., 2021; Jing et al., 2023).

In plants grown under drought conditions, the proline content rises proportionately more quickly than that of other amino acids; for this reason, proline content is used to evaluate stressed plants (Essa et al., 2023).

Reduced plant growth and respiration, altered ATP synthesis, increased production of reactive oxygen species (ROS), increased biosynthesis of enzymatic and non-enzymatic antioxidants, increased biosynthesis of osmoregulatory compounds such as proline and glycine betaine, increased content of soluble sugars and proteins, increased lipid peroxidation, and potential plant death are all consequences of prolonged drought (Seleiman et al., 2021b; Cvikrová et al., 2013; Bondok et al., 2022a).

Drought resistance is mostly influenced by factors such as improved root system architecture (RSA), fewer and smaller leaves, stress-induced phytohormones, stomatal closure, antioxidant defense mechanisms, solute buildup (such as proline), and changed gene expression. To create a drought-tolerant legume without compromising crop output, a number of agronomic, breeding, and biotechnology techniques are employed as management techniques. Legumes are more drought-tolerant when plant-growth regulators (PGRs), osmoprotectants, and rhizobacteria and arbuscular mycorrhizal fungi are applied exogenously (Khatun et al., 2021).

In adverse conditions, the use of highly effective and stress-tolerant rhizobial inocula can further increase the production of legume crops (Peoples et al., 2009; Denton et al., 2017; Kanonge-Mafaune et al., 2018). Through a number of mechanisms, including promoting the activity of both enzymatic and non-enzymatic antioxidants, increasing photosynthetic activity and biomass accumulation, and inducing the biosynthesis of osmolytes like ectoines, polyamines, proline, and glycine betaine to protect cells from osmotic stress- and desiccation-related damage, rhizobia appear to improve the stress resilience and growth of host plants (Reina-Bueno et al.; Wdowiak-Wróbel et al., 2013; Lunn et al., 2014; Ding, 2022)). All things considered, it is evident that rhizobia can promote plant health under adverse circumstances. This capacity might be essential for improving the production of legumes in semi-arid and dry areas as well as on marginal land.

MDA is the primary byproduct of membrane lipid peroxidation, and its composition may indicate the extent of reactive oxygen species-induced cell membrane damage (Benmoussa et al., 2022)

ROS-induced oxidative damage commonly takes place under water stress, leading to membrane lipid peroxidation at the cellular and subcellular levels. This further reduces growth, biomass production, and seed yield (Habib et al., 2020). The current study found that other plant species, such as wheat and mung beans, have higher MDA and H_2O_2 concentrations in faba beans under water stress (Ali et al., 2018) and sugar beet (Ghaffari et al., 2019). Therefore, increased oxidative stress resulting from the disruption of the enzymatic response in growing plants under water stress causes raised lipid peroxidation and H_2O_2 levels.

Reducing the irrigation level from 100% to 60% ETc hindered the growth and productivity of faba beans, reduced the effectiveness of the machinery involved in photosynthesis, and affected the stability of the leaf tissue. The excessive production of oxidative stress markers (H_2O_2 and O_2•–) linked to an increase in osmoprotectant chemicals and an elevation of non-enzymatic and enzymatic antioxidants, which combat oxidative damage under drought stress, therefore led to an increase in lipid oxidation (MDA) (Hasanuzzaman et al., 2019). Osmotic stress may be the cause of adverse effects made worse by water deprivation, which include loss of cell turgor and/or increased ROS generation during drought stress (Upadhyaya et al., 2013; Osakabe et al., 2014).

When bacteria were introduced into plants experiencing water deprivation, the amount of MDA and H_2O_2 in their tissues dramatically dropped. Compared to plants that were not under stress, the concentration of these chemicals was even lower (Barquero et al., 2022). When rhizobia strains were introduced into water-stressed plants, the amount of H_2O_2 and MDA in the plant tissues dramatically dropped. Numerous works have reported similar findings (Wang et al., 2012; Gontia-Mishra et al., 2016; Curá et al., 2017; Kumar et al., 2019). Plants under drought and salinity stress create H2O2, a ROS that results in oxidative damage (Gill and Tuteja, 2010). Fatty acid peroxidation produces MDA, which is thought to be a sign of the extent of damage in stressed plants (Morales and Munné-Bosch, 2019). High levels of MDA indicate a serious stress

condition because ROS generated under biotic or abiotic stress damage organic molecules, such as lipids, which raise the MDA content and the permeability of the plasma membrane (Shi et al., 2018; Sun et al., 2020; Zhang et al., 2021). As a result, MDA and H_2O_2 content serve as markers of oxidative stress in plants, and lowering both entails lowering the stress levels.

The current study belong to *Bacillus spizizenii* and *Brachybacterium* in enhancing the plant growth under stress agree with the other studies. (Masmoudi et al., 2021) reported that *Bacillus spizizenii* decreased malondialdehyde and H_2O_2 levels under saline circumstances and enhanced membrane integrity, phenol peroxidase (POX) concentrations, and chlorophyll content. Additionally, *Bacillus spizizenii* inoculation enhanced K+/Na+ and Ca2+/Na+ ratios by increasing K+ and Ca2+ absorption and considerably reducing endogenous Na+ accumulation. IAA production was maximum in *Brachybacterium sp*. AB440 (101.4 ± 0.5 µg/ml). *Brachybacterium sp.* AB439 biopriming of maize seeds produced the longest shoots and roots. Additionally, biopriming has reduced reactive oxygen species and induced strong enzymatic and non-enzymatic antioxidative defensive responses in maize seedlings (Mondal et al., 2024). Additionally, *Brachybacterium sp*. contributed to the modification of maize plant defense, indicating the beneficial effectiveness of bio-priming in helping plants manage biotic stress (Mondal et al., 2024). In conclusion, the two bacterial strains, Bact 2 (*Brachybacterium sp.)* and Bact 4 (*Bacillus spizizenii*), significantly improved the development and physiological characteristics of bean plants subjected to drought stress, enhancing biomass, leaf count, pigment concentration, and antioxidant enzyme activities. These findings indicate that these salt-tolerant bacteria may be cultivated as efficient bio-inoculants to enhance bean crop resilience and productivity in dry and drought-prone agricultural settings.

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Conflicts of Interest: The authors declare no conflict of interest

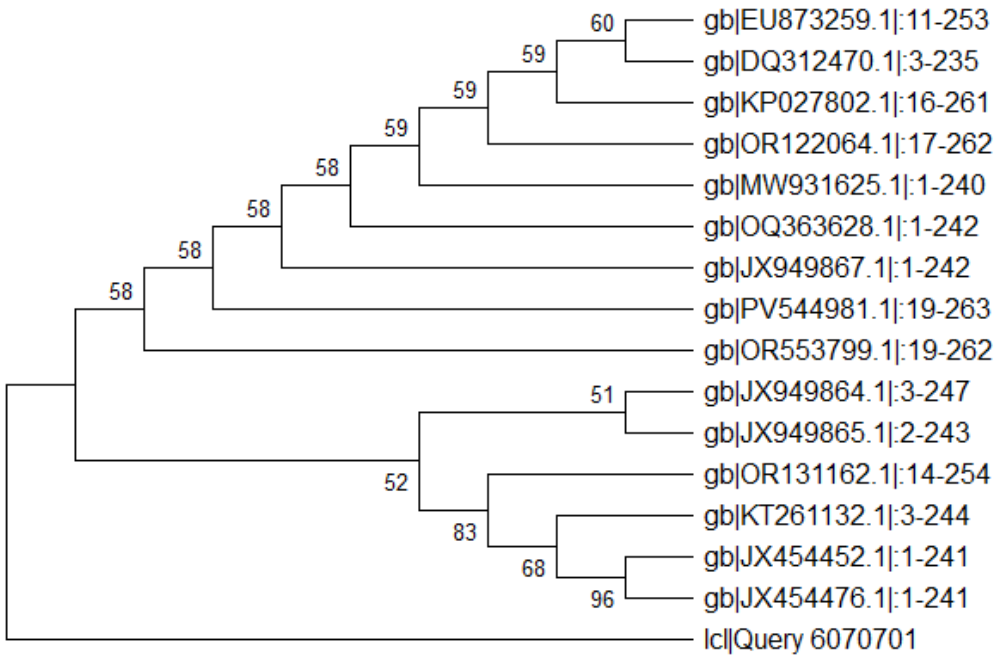

Figure 1. Phylogenetic tree using MEGA-5 of bacterial lcl|Query 6070701 using 16S rRNA, with accession numbers, showing phylogenetic relatedness of lcl|Query 6070701 along with closely related species (JX454476.1 (*Brachybacterium sp.* 11394, and JX454452.1 (*Brachybacterium sp.* 11427) from NCBI database

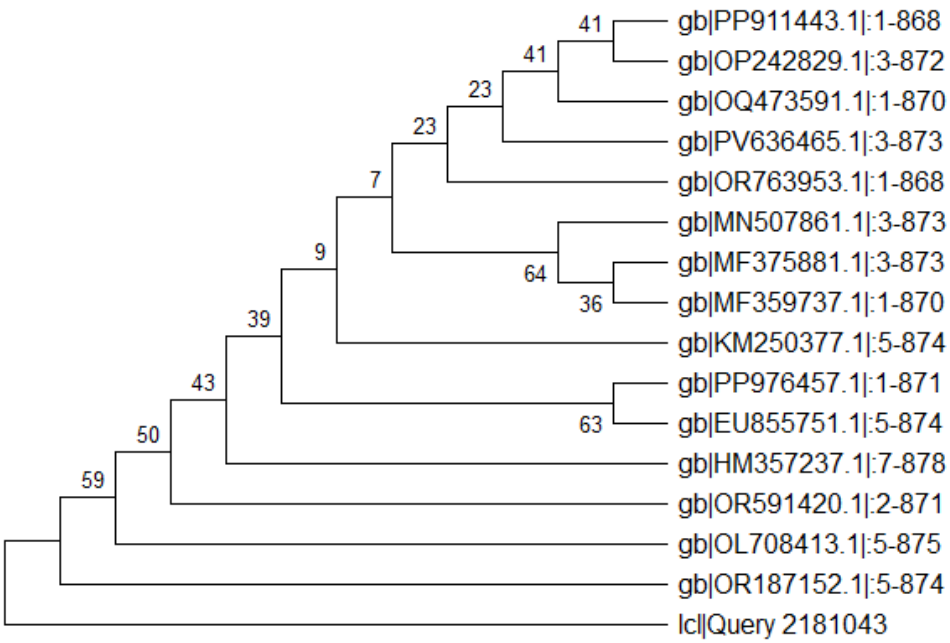

Figure 2. Phylogenetic tree using MEGA-5 of bacterial lcl|Query 2181043 using 16S rRNA, with accession numbers, showing phylogenetic relatedness of lcl|Query 2181043 along with closely related species (gb|OR187152.1|:5-874 (*Bacillus spizizenii*.) from NCBI database.

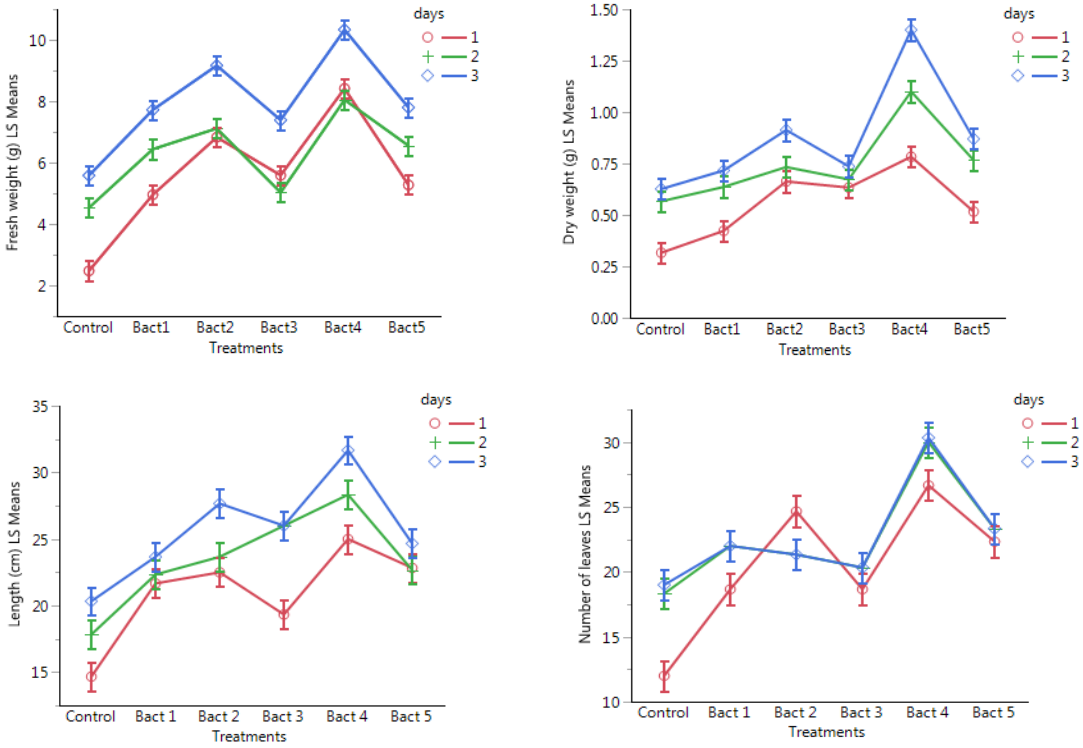

Figure 3. Changes in shoot fresh and dry weights, shoot length, and number of leaves, in *Vicia faba* treated with bacteria under the influence of drought stress, after three periods. Lines designated at significant $P < 0.05$.

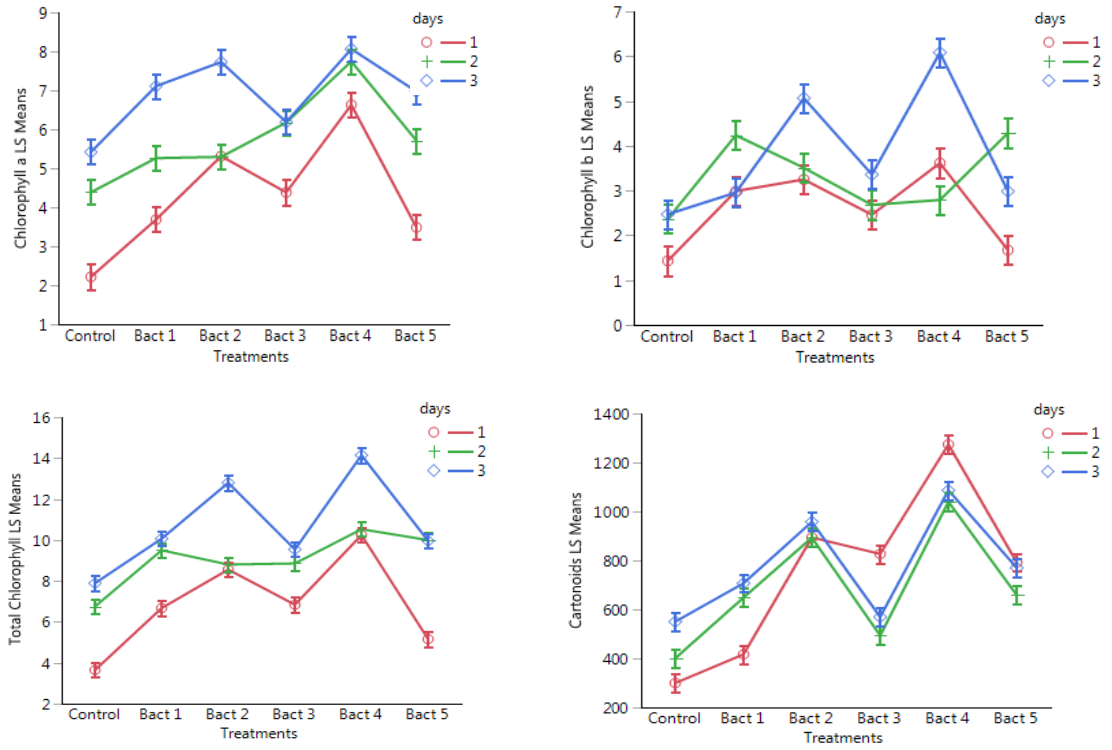

Figure 4. Changes in chlorophyll a, b, and total chlorophyll a and b from *Vicia fab* plant growth after three periods after placing the bacterial treatments under the influence of drought . Lines designated at significant P < 0.05.

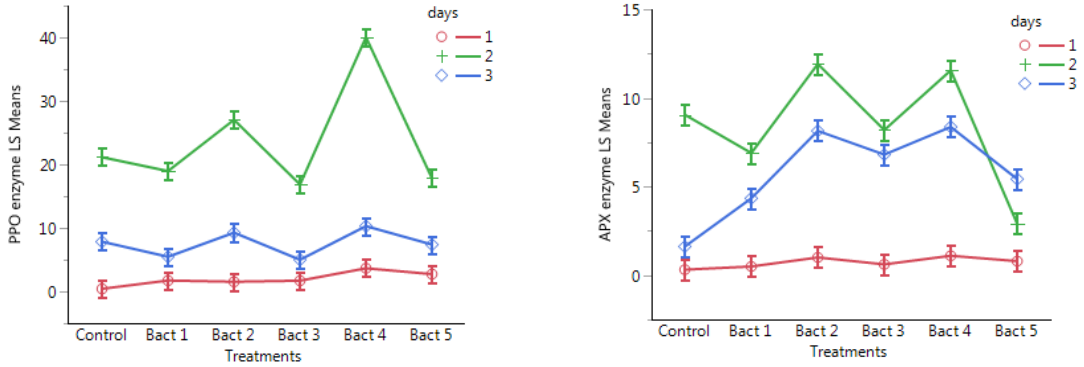

Figure 5. Changes in PPO and APX levels in *Vicia faba* under drought conditions after 7, 15 days of treatment with bacteria and during the recovery stage (3 days after irrigation of the treatments). Lines designated at significant P < 0.05.

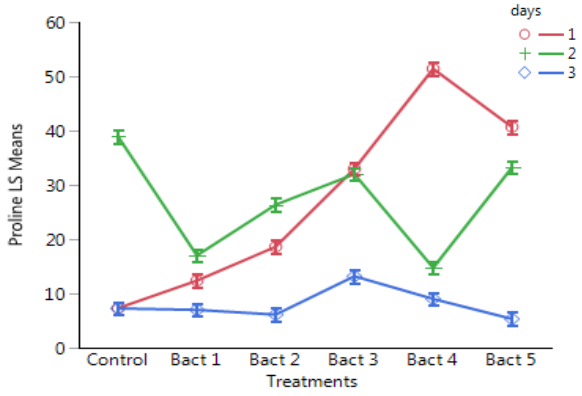

Figure 6. Changes in proline levels in *Vicia faba* under drought conditions after 7, 15 days of treatment with bacteria and during the recovery stage (3 days after irrigation of the treatments. Lines designated at significant P < 0.05.

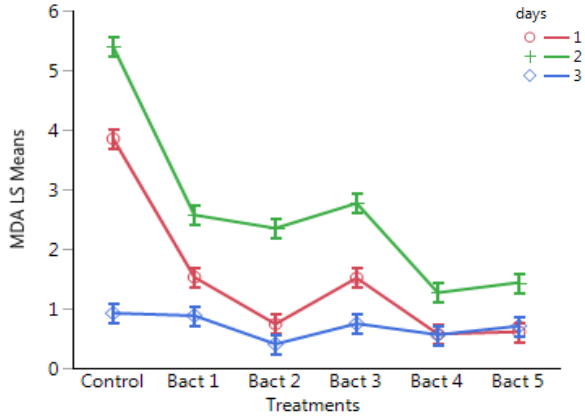

Figure 7. Changes in MDA production in *Vicia fab* under drought stress and treated with salt-tolerant bacteria after 7 and 15 days and during the recovery stage. Lines designated at significant $P < 0.05$.

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