Trop J Nat Prod Res, April 2024; 8(4):6940-6947 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Protective Role of Plant Growth Promoting Rhizobacteria Inoculation in the Development of Drought Tolerance in Shallot: Effects on Hydroxygen Peroxide Production, Lipid Peroxidation, and Secondary Metabolite Production

Arum Pratiwi[1,2]*, Mochammad D. Maghfoer[1], Eko Widaryanto[1], Nurul Aini[1]

[1]*Department of Agronomy, Faculty of Agriculture, University of Brawijaya, Veteran Malang 65145, East Java, Indonesia*
[2]*Department of Sustainable Agricultural Extension, Agricultural Development Polytechnic of Malang, Dr. Cipto Bedali-Lawang 65216, East Java, Indonesia*

ARTICLE INFO

Article history:
Received 20 March 2024
Revised 10 April 2024
Accepted 15 April 2024
Published online 01 May 2024

ABSTRACT

Shallot contains secondary metabolites and antioxidants that can be used as raw materials for traditional medicine. However, shallot has the disadvantage of being intolerant to drought. Drought can affect the quality of the compounds in shallot at a certain level. Therefore, this study investigates the protective role of Plant Growth Promoting Rhizobacteria *(PGPR)* inoculation in conferring drought tolerance to shallot during different growth stages. Two factors and three replications were considered in this study. The first factor was the timing of drought stress, comprising four treatments (vegetative phase, bulb initiation, bulb development, and maturation), with one treatment as a control without drought stress. The second factor was the type of bacteria, consisting of two treatments (*Bacillus subtilis* Pb03 *and Pseudomonas fluorescens* Pb04), with one treatment as a control without PGPR inoculation. The research results indicate that oxidative stress triggered by drought stress is evidenced by an increase in hydrogen peroxide production, lipid peroxidation, and secondary metabolites at almost all stages of growth. Treatment *Bacillus subtilis* Pb03 inoculation was more effective than *Pseudomonas fluorescens* Pb04 in mitigating drought stress in shallots. *Bacillus subtilis* Pb03 inoculation inhibited oxidative stress by enhancing the activity of antioxidant enzymes. Additionally, this application suppressed the production of secondary metabolites, thereby maintaining osmotic balance in the plants.

***Keywords*:** Shallot, Rhizobacteria, Growth Stage, Drought Stress, secondary metabolites, Antioxidant, Physiological Characteristics

Introduction

Shallot is a medicinal plant that can potentially be used as a raw material for traditional medicine. Shallot contains essential proteins, dietary fiber, minerals, and vitamins (A, B, C), as well as phenolic compounds and flavonoids such as gallic acid, apigenin, quercetin, and tannic acid, which provide antioxidant benefits for liver and kidney health.[1] The unstable climate conditions and shifting rainfall patterns pose severe challenges to global agriculture. One significant impact is drought increasing frequency and intensity, which can adversely affect crop production values.[2] Shallots, as critical medicine crops, are not exempt from the detrimental effects of drought stress. Drought can induce oxidative stress in shallots, disrupting the photosynthetic processes. Insufficient water availability leads to the formation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), which can damage cellular structures and biological molecules.[3] In response, plants activate their antioxidant defense systems to neutralize ROS and mitigate cell damage. Key antioxidant enzymes involved in ROS elimination include superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and glutathione peroxidase (GPX).[4]

*Corresponding author. E mail: arumpratiwi@polbangtanmalang.ac.id
Tel: +6281331526394

Citation: Pratiwi A, Maghfoer MD, Widaryanto E, Aini N. Protective Role of Plant Growth Promoting Rhizobacteria Inoculation in the Development of Drought Tolerance in Shallot: Effects on Hydroxygen Peroxide Production, Lipid Peroxidation, and Secondary Metabolite Production. Trop J Nat Prod Res. 2024; 8(4):6940-6947. https://doi.org/10.26538/tjnpr/v8i4.27

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

Drought conditions result in a decrease in water potential within plant cells. Consequently, plants regulate secondary metabolites by increasing the accumulation of proline and allicin to preserve cell moisture.[5] Plants also respond to drought by reducing transpiration rates, thereby minimizing water loss through stomata and aiding in maintaining water balance within the plant.[6]

The growth stage can significantly influence how effectively they respond to and cope with drought stress. Different growth stages have varying water requirements, and a plant response to drought can vary depending on the specific growth stage experiencing water stress. Previous studies have indicated that implementing water management strategies and innovative agricultural technologies, such as beneficial Plant Growth Promoting Rhizobacteria (PGPR), can enhance plant tolerance to drought.[7]

Different growth stages affect the production of secondary metabolites in plants. During the vegetative growth stage, the production of secondary metabolites such as chlorophyll and phytohormones is more dominant to accelerate growth. During the bulb initiation stage, the production of secondary metabolites such as flavonoids and carotenoids increases to support the flowering and pollination processes. In the bulb development stage, plants can produce secondary metabolites such as ascorbic acid and lycopene. After reaching peak production (maturation), the production of secondary metabolites decreases as the plant's metabolic activity decreases.[8]

Bacillus and *Pseudomonas*, as plant growth-promoting rhizobacteria (PGPR), can mitigate drought stress effects by regulating stress-responsive genes, producing phytohormones, osmolites, siderophores, volatile organic compounds, and exopolysaccharides, and enhancing 1-aminocyclopropane-1-carboxylate deaminase activity.[9] These PGPR have the potential to enhance drought tolerance in important crops, which could help reduce crop losses under water-limited conditions, ultimately improving photosynthetic characteristics.[10]

PGPR are rhizosphere inhabitants known for their ability to enhance plant growth. Previous studies have shown that inoculating green peas (*Pisum sativum*) with PGPR-containing ACC deaminase improved drought resistance.[11] Additionally, inoculation with *Bacillus tequilensis* U36, which produces IAA, promoted root hair formation and seedling root growth, enhancing water and nutrient absorption and aiding plants in overcoming water scarcity.[12] However, inoculation with Pseudomonas fluorescens not only promoted growth and yield but also mitigated the adverse effects of water deficit stress. These strains exhibit moderate ACC deaminase activity and auxin synthesis, along with high phosphate solubilization and siderophore production abilities. Uzma *et al*.[13] suggested that these five strains are drought-tolerant and capable of producing IAA, ACC deaminase, and siderophores. Inoculation with Pseudomonas has been shown to alleviate drought stress in Vigna radiata, significantly increasing seed yield compared to stressed control plants.

This study aims to understand the impact of drought on various growth stages of shallot and explore the potential mitigation through the inoculation of PGPR, specifically *Bacillus subtilis* Pb03 and *Pseudomonas fluorescens* Pb04. The knowledge gained from this study is expected to provide insights into developing strategies for sustainable drought management, aiming to optimize the physiological metabolism and resilience of shallots amidst the ongoing challenges of climate change.

Materials And Methods

Collection and Identification of Plant Material

The shallot variety employed in this study is known as "Batu Ijo" Shallots (*Allium ascalonicum* L.) were collected on June 2023 in Batu, East Java, Indonesia with GPS -7.9105489, 112.5423891. The botanical specimen with the code SH1624558.08F has undergone taxonomic classification by a *Global Biodiversity Information Facility* (GBIF), and it is currently archived at the Agricultural Instrument Standardization Agency (BSIP) of the Ministry of Agriculture, Indonesia. The taxonomy exhibited a 98% similarity to accession number KU140434 from GenBank.

The Batu Ijo variety was selected as the chosen shallot variety for this research because it is a local variety that is widely cultivated by farmers and is known for its susceptibility to drought stress. This makes it more responsive to the treatments provided in the study.

Collection and Identification of PGPR Material

The PGPR inoculants *Bacillus subtilis* Pb03 and *Pseudomonas fluorescens* Pb04 (Figure 1), were extracted from the roots of shallots in the central production area of Probolinggo, East Java Province, Indonesia on March 2023. Based on 16S rRNA sequencing data, bacterial isolate *Bacillus* PB03 showed a 99% similarity to *Bacillus subtilis* (AB192294.2), while *Pseudomonas* PB04 exhibited a 99% similarity to *Pseudomonas fluorescens* (AB266613.1) from GenBank.

Experimental Design

The experiment was conducted in the greenhouse of the *Agricultural Development Polytechnic of Malang*. The greenhouse maintained a temperature range of 24-29°C, relative humidity of 65-75%, and a 12-16 hour light period. The experiment was conducted in a randomized block factorial design with two factors and three replications. The first factor was the timing of drought stress, comprising four treatments (vegetative phase, bulb initiation, bulb development, and maturation), with one treatment serving as a control without drought stress. The second factor was the type of PGPR, consisting of two treatments (*Bacillus subtilis* Pb03 *and Pseudomonas fluorescens* Pb04), with one treatment as a control without bacterial inoculation.

Gas Exchange Characteristics Parameters

Gas exchange measurements, including stomatal conductance (gs), transpiration rate (Tr), and photosynthetic rate (Pn), were recorded seven days after the application of treatments. A portable Li-COR 6400 photosynthesis system (Li-COR, Lincoln, NE, USA) was utilized for the gas exchange measurements.

Relative Water Content

After seven days of treatment imposition, the relative water content (RWC) of leaves was measured as described previously by Fairoj *et al.*[14] The following formula was used for RWC calculation:

$$RWC = \frac{[\text{ Fresh weight-Dry weight }]}{[\text{ Turgid weight-Dry weight }]} \times 100 \quad (1)$$

Stomatal Characteristics

Obsevations of stomatal characteristics (length and width) were conducted using scanning electron microscopy, and precise measurements were carried out using image-J software developed by the *National Institutes of Health* and *Laboratory for Optical and Computational Instrumentation* (LOCI, University of Wisconsin).

Total Chlorophyll and Carotenoid Content

Total chlorophyll and carotenoid were assessed using Wintermans and De Mots.[15] Total chlorophyll and carotenoid content from fresh leaves were determined on a fresh weight basis and extracted with 80% acetone using a spectrophotometer at specific wavelengths, such as 470 nm, 645 nm, and 663 nm.

Phenol and Flavonoid

Total phenolic extracts were assessed using Folin and Ciocalteu reagents, following the method of Singleton and Rossi.[16] Samples and standard readings were taken using a spectrophotometer at a wavelength of 765 nm. The sample total flavonoid content was determined using the aluminum chloride colorimetric method Chang *et al*.[17] Quercetin was utilized to create a standard calibration curve for this determination. The absorbance of the reaction mixtures was measured at a wavelength of 420 nm using a spectrophotometer.

Proline and Allicin

The proline content was determined following the procedure outlined by Bates *et al*.[18] The allicin content was calculated using the INA 110.001 method from The Institute for Nutraceutical Advancement (National Sanitation Foundation & Internacional, Institute for Nutraceutical Advancement, 2005).[19]

Hydrogen Peroxide and Malondialdehyde

Hydrogen peroxide (H_2O_2) levels were determined following the method of Velikova *et al*.[20], with measurements taken at 390 nm using a spectrophotometer. Malondialdehyde (MDA) levels were estimated according to the procedure outlined by Madhava Rao and Sresty.[21], with the absorbance of the colored supernatant measured at 530 nm and 600 nm using a spectrophotometer.

Antioxidant Enzymatic Activity

Catalase (CAT, EC: 1.11.1.6) activity was assessed by monitoring the reduction using a spectrophotometer, following the method outlined by Islam *et al.* [22] Ascorbate peroxidase (APX, EC: 1.11.1.11) activity was determined according to the procedure established by Islam *et al*.[21] Guaiacol peroxidase (GPX, EC 1.11.1.7) activity was determined using guaiacol as a substrate following the method of Nakano and Asada.[23] Superoxide dismutase (SOD, EC: 1.15.1.1) activity was assayed based on the method by Dhindsa and Matowe.[24]

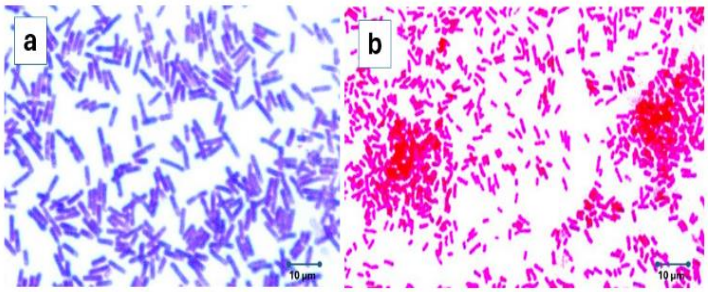

Figure 1: Plant Growth Promoting Rhizobacteria (photo by author collection)
a: *Bacillus subtilis* Pb03; b: *Pseudomonas fluorescens* Pb04

Statistical Analysis
The data were analyzed using DSAASTAT software, and the results were presented as mean ± SD. Subsequently, the Turkey test following ANOVA was employed to compare the outcomes between the treatments and the control treatment. Results were considered statistically significant when P<0.05.

Result and Discussion

Gas Exchange Characteristics
The significant impact ($p < 0.05$) of drought stress is observed in a substantial reduction in the photosynthetic rate (Pn), with 12.08%, 22.78%, and 22.32% during the vegetative, bulb initiation, and bulb development phases, respectively, compared to without stress. However, there was no significant in Pn during the maturation phase compared to without stress (Table 1). The application of PGPR inoculation on drought-stressed shallots significantly enhanced the Pn rate by alleviating the detrimental effects of drought. Notably, the plants treated with *Bacillus subtilis* Pb03 and *Pseudomonas fluorescens* Pb04 inoculation exhibited a 24.79% and 12.40% increase in Pn, respectively, compared to those without PGPR.
Shallot transpiration rate (Tr) varies significantly ($p < 0.05$) due to different drought stress timings. A substantial decrease in transpiration is observed during drought stress at the bulb initiation and development phases, with reductions of 45.69% and 40.72%, respectively, compared to without stress. Conversely, drought stress during the vegetative and maturation phases had insignificance than without stress. PGPR inoculation on drought stress effectively increases the transpiration rate (Table 1). Among the two tested bacteria, *Bacillus subtilis* Pb03 has a significance of 42.12% compared to those without PGPR. On the other hand, inoculation with *Pseudomonas fluorescens* Pb04 is not statistically significant when compared to those without PGPR inoculation.
Drought stress significantly decreases ($p < 0.05$) stomatal conductance (gs), with the most reduction observed in the bulb development phase, at 69.23% compared to without stress. Meanwhile, during the vegetative phase, bulb initiation, and maturation, there are respective reductions of 46.15%, 57.69%, and 30.76% compared to without stress. The amelioration of drought stress through PGPR inoculation increases stomatal conductance. The plants treated with *Bacillus subtilis* Pb03 and *Pseudomonas fluorescens* Pb04 inoculation exhibited a 95.10% and 55.56% increase in stomatal conductance, respectively, compared to those without PGPR.
Drought stress significantly reduced ($p < 0.05$) the photosynthesis rate, stomatal conductance, and transpiration rate of shallot. Drought stress induces stomatal closure, resulting in a reduction in carbon dioxide (CO_2) influx into the leaves, subsequently decreasing photosynthesis.[11] The decrease in turgor pressure enhances stomatal closure, reducing leaf gas exchange and leading to a decline in CO_2 assimilation, ultimately disrupting photosynthesis.[25]

Relative Water Content
Drought stress significantly reduces ($p < 0.05$) the RWC at all growth stages, as outlined in Table 1. The vegetative growth, bulb initiation, bulb development, and maturation with reductions of 24.17%, 35.25%, 33.89%, and 10.83%, respectively, compared to without stress. PGPR inoculation demonstrates enhanced performance in terms of RWC by alleviating the detrimental effects of drought stress. The increase in RWC with the addition of PGPR, compared to without PGPR, is significant, with increments of 23.86% for *Bacillus subtilis* Pb03 and 23.91% for *Pseudomonas fluorescens* Pb04.
The negative impact of drought on RWC is due to reduced ($p < 0.05$) water flow. Decreased water flow increases protoplasm dehydration, causing oxidative damage to chloroplasts, stomatal closure, and reduced CO_2 concentration in mesophyll cells, affecting photosynthesis.[26] However, bacterial inoculation individually increases RWC in shallot plants. These results indicate that, through the activation of the plant defense system, PGPR helps plants adjust to water relations and membrane function under water stress conditions. PGPR has also been reported to contribute to osmotic adjustment and maintain membrane stability and protein and enzyme structure.[27]

Stomatal Characteristics
The length of stomata significantly decreases ($p < 0.05$) during drought stress at the bulb initiation and bulb development phases, 20.92% and 24.29%, respectively, compared to without stress. Conversely, drought stress during the vegetative growth and maturation phases has insignificant differences in stomatal length (Figure 2a). The width of stomata was significantly reduced by 43.09%, 57.81%, and 69.79%, respectively, drought stress at the vegetative growth, bulb initiation, and bulb development phases compared to without stress (Figure 2b). However, the maturation phase is insignificant compared to without stress.
PGPR inoculation under drought stress conditions has been observed to increase stomatal length and width, which is evident in Figure 3. The bacterial inoculation treatments, specifically *Bacillus subtilis* Pb03 and *Pseudomonas fluorescens* Pb04, show respective increases of 30.85% and 26.59% in stomatal length compared to shallot without bacterial inoculation. As for stomatal width, *Bacillus subtilis* Pb03 and *Pseudomonas fluorescens* Pb04 bacterial inoculations are capable of widening stomata to three times the size compared to the treatment without PGPR, as illustrated in Figure 3.

Table 1: Enhanced gas exchange attributes and relative water content of shallot on different timing drought stress and inoculation PGPR

Treatment	Photosynthetic Rate (Pn) (μmol m^{-2} s^{-1})	Transpiration Rate (Tr) (mmol m^{-2} s^{-1})	Stomatal Conductance (gs) (mmol $m^{-2}s^{-1}$)	Relative Water Content (%)
Time Drought Stress				
Without Stress	21.86 ± 0.73 c	6.85 ± 0.46 b	0.026 ± 0.02 c	56.68 ± 2.65 d
Vegetative Growth	19.22 ± 0.50 b	5.62 ± 0.21 b	0.014 ± 0.03 ab	42.98 ± 3.49 b
Bulb Initiation	16.88 ± 1.13 a	3.72 ± 0.52 a	0.011 ± 0.03 a	36.70 ± 2.89 a
Bulb Development	16.98 ± 0.27 a	4.06 ± 0.41 a	0.008 ± 0.05 a	37.47 ± 2.42 a
Maturation	19.46 ± 0.81 bc	6.18 ± 0.50 b	0.018 ± 0.02 b	50.54 ± 1.69 c
Inoculation PGPR				
Without PGPR	16.78 ± 0.32 a	3.68 ± 0.46 a	0.009 ± 0.02 a	38.02 ± 4.43 a
*Bacillus subtilis*Pb03	20.94 ± 1.09 c	5.23 ± 0.19 b	0.019 ± 0.02 c	47.09 ± 3.55 b
*Pseudomonas fluorescens*Pb04	18.86 ± 0.46 b	4.57 ± 0.31 ab	0.014 ± 0.03 b	47.11 ± 1.48 b

Noted: Different letters indicate significant differences among treatments at $p < 0.05$ according to the Turkey test

The physiological metabolism recovery after stress at each growth stage can involve various adaptation mechanisms. After stress, plants will experience tissue damage. In the early stages of recovery, plants will focus on repairing and regenerating damaged or dead tissues. Hormones such as auxin, ethylene, and abscisic acid can play a crucial role in regulating the plant's response to stress and its recovery. Auxin can stimulate growth and regeneration, while ethylene and abscisic acid can regulate defense responses and stress. The production of secondary metabolites such as antioxidants and phenolic compounds may increase during recovery to protect plants from oxidative stress and stimulate the regeneration process. After stress, plants may enhance their ability to absorb water and nutrients from the surrounding environment, aiding in recovery and further growth.[28]

Several factors, including hormones and environmental conditions, control stomatal characteristics. Drought causes changes in osmotic pressure and increased abscisic acid (ABA) levels. It also affects carbohydrate production, ultimately influencing stomatal opening.[29] PGPR can also influence the production of plant hormones, such as ABA, which regulates stomatal opening in response to environmental conditions. Additionally, PGPR can improve plant nutrient availability, affecting plant metabolism and the balance of plant hormones, including those involved in stomatal regulation.

Photosynthetic Pigments

Drought stress significantly reduces ($p < 0.05$) chlorophyll formation in all three phases, with values of 21.56%, 25.46%, and 14.52% at vegetative growth, bulb initiation, and bulb development, respectively, compared to without stress (Table 2). However, chlorophyll is insignificant ($p > 0.05$) during the maturation phase compared to without stress. PGPR inoculation contributes to an increase in the total chlorophyll by mitigating drought stress. The maximum increase in chlorophyll is observed with *Bacillus subtilis* Pb03 and *Pseudomonas fluorescens* Pb04 inoculations, with increments of 34.63% and 25.67%, respectively, compared to shallot experiencing drought stress without PGPR. The carotenoid content is insignificantly between the difference in timing drought stress and the PGPR inoculation treatment.

Chlorophyll and carotenoids are essential components for used physiological indicators as they directly influence photosynthetic efficiency.[30] Drought stress reduces the mesophyll cell potential to utilize CO_2, consequently reducing chlorophyll content. It has been reported that early growth stress maintains high chlorophyll content, exhibiting better performance under drought stress.[31] Consistent with these findings, our results indicate higher chlorophyll content, consequently showing the highest gs, Tr, and Pn during vegetative growth compared to bulb initiation and bulb development.

Table 2: Photosynthetic Pigments of shallot on different timing drought stress and inoculation PGPR

Treatment	Total Chlorophyll (mg g-1)	Carotenoid Content (mg g-1)
Time Drought Stress		
Without Stress	21.01 ± 2.06 c	0.330 ± 0.04
Vegetative Growth	16.48 ± 1.41 ab	0.347 ± 0.02
Bulb Initiation	15.66 ± 1.23 a	0.343 ± 0.03
Bulb Development	17.96 ± 1.44 b	0.353 ± 0.02
Maturation	20.19 ± 1.53 c	0.343 ± 0.02
Inoculation PGPR		
Without PGPR	13.4 ± 1.99 a	0.358 ± 0.02
*Bacillus subtilis*Pb03	18.04 ± 1.78 b	0.348 ± 0.02
*Pseudomonas fluorescens*Pb04	16.84 ± 1.34 b	0.334 ± 0.01

Noted: Different letters indicate significant differences among treatments at $p < 0.05$ according to the Turkey test

The application of *Bacillus subtilis* Pb03 and *Pseudomonas fluorescens* Pb04 bacterial inoculation alleviates the harmful effects of drought and enhances the content of chlorophyll, as well as Pn, gs, and Tr at various growth stages.

Secondary Metabolites

Proline formation during drought stress significantly increases ($p < 0.05$) with values of 38.39% and 31.07% during bulb initiation and bulb development, respectively, compared to the without stress. However, under drought stress during the vegetative growth and maturation phases, there is insignificant e compared to the without stress. Furthermore, PGPR inoculation on drought-stressed significantly reduces the proline content. There is a value of 9.16% and 8.94%, respectively, with *Bacillus subtilis* Pb03 and *Pseudomonas fluorescens* Pb04 compared to without PGPR inoculation.

Drought stress significantly increases ($p < 0.05$) allicin content in all three phases, with values of 26.99%, 86.67%, and 53.09% at vegetative growth, bulb initiation, and bulb development, respectively, compared to without stress. Conversely, allicin content is insignificant in the maturation phase compared to the without stress. PGPR inoculation on shallots experiencing drought stress can reduce the allicin content. Among the two tested bacteria, *Bacillus subtilis* Pb03 and *Pseudomonas fluorescens* Pb04 significantly reduce allicin content by 10.26% and 13.51%, respectively, compared to those without PGPR.

Drought stress significantly increases ($p < 0.05$) total phenol content in the different timing treatments of drought stress. Bulb initiation and bulb development, respective increments of 35.78% and 42.06% compared to without stress.

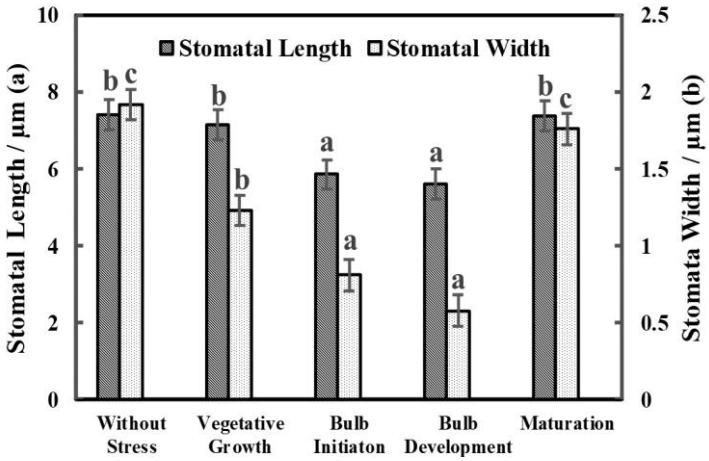

Figure 2: (a) Stomatal length and (b) Stomatal width in shallot leaves mitigate drought stress at different growth stages. Bars are means, and error bars are standard errors (n=3). Different letters indicate significant differences among treatments at $p < 0.05$ by the Turkey test

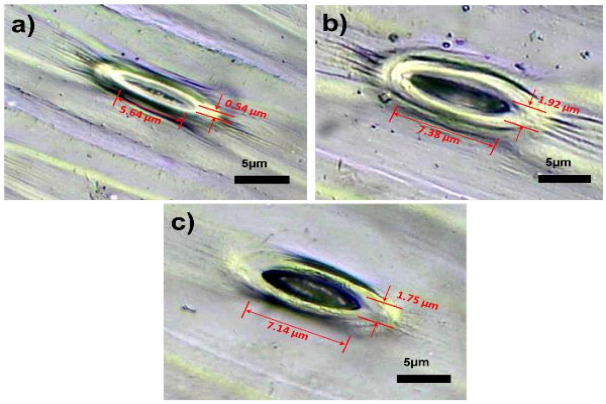

Figure 3: Leaf stomatal morphology of shallot under drought stress (a) without PGPR, (b) *Bacillus subtilis* Pb03, and (c) *Pseudomonas fluorescens* Pb04

On the other hand, drought stress during the vegetative and maturation phases is an insignificant difference in total phenol content compared to without stress. PGPR inoculation on drought-stressed significantly reduces the phenol content. There is a value of 19.65% and 15.80%, respectively, with *Bacillus subtilis* Pb03 and *Pseudomonas fluorescens* Pb04 compared to without PGPR inoculation.

The flavonoid content undergoes a decrease ($p < 0.05$) of 34.69%, 30.73%, and 35.71% in the phases of vegetative growth, bulb initiation, and bulb development, respectively, during drought stress compared to the without stress (Table 3). However, the maturation phase is insignificant compared to without stress. PGPR inoculation on drought-stressed significantly reduces the flavonoid content. There is a value of 18.93% and 17.84%, respectively, with *Pseudomonas fluorescens* Pb04 and *Bacillus subtilis* Pb03 inoculation compared to without PGPR inoculation.

The plant water status may become unbalanced under drought stress, disrupting osmotic adjustment and ultimately causing a higher accumulation of secondary metabolites.[32] Proline has antioxidant activity, reducing lipid peroxidation and promoting cellular homeostasis by protecting redox balance.[33] Several studies have shown that allicin and flavonoid content increases under drought stress. It is reported that plants alleviate drought damage by accumulating higher allicin and flavonoid levels. However, continuous water deficiency due to prolonged drought may damage plant structure and affect protein and sugar synthesis.[34] Phenols are broken down to provide energy and carbon when photosynthesis is limited under prolonged water stress conditions.

Drought stress in the early growth stages can inhibit biomass production, including plant size and weight. Drought stress during the bulb initiation phase can reduce the synthesis of secondary metabolites. This can result in a decrease in the quality of shallots, such as a reduction in nutrient content and bioactive compounds. Drought stress during the bulb development phase can disrupt bulb development. This can harm the quality of shallots, such as smaller bulb size. Drought stress during the maturation phase can disrupt the process of antioxidant accumulation.[35]

During the vegetative growth stage, plants tend to experience mild drought stress because they still have water reserves from the seed. Plants can produce more lateral roots and root hairs to absorb water more efficiently. Another mechanism that occurs is the production of hormones such as IAA (Indole Acetic Acid) to stimulate root growth and increase water absorption. Bulb initiation stage, plants begin to experience more pronounced drought stress as their water needs increase. Plants will close their stomata to reduce water evaporation through transpiration. Additionally, plants may increase the production of osmolytes such as proline to maintain osmotic balance within cells. During the bulb development stage, plants are more vulnerable to drought stress because bulb development requires a lot of water. To cope with this, plants may redirect resources to produce growth hormones such as gibberellins to accelerate bulb development. Additionally, plants may increase the production of antioxidant compounds to protect tissues from damage caused by oxidative stress. And the maturation stage, plants begin to reduce metabolic activity, so drought stress has less impact at this stage.

The mechanism of bacteria and plant secondary metabolites involves a complex interaction between PGPR and plants. Firstly, PGPR can produce antimicrobial compounds that help protect plants from pathogens. Secondly, PGPR can enhance plant nutrient availability by converting complex organic compounds into forms that plants absorb more easily. Thirdly, PGPR can trigger plant immune responses, increasing resistance to stress and diseases. Fourthly, PGPR can produce plant hormones, such as auxin, which stimulate root growth and plant recovery after stress.

Hydrogen Peroxide and Malondialdehyde Content

Drought stress increases ($p < 0.05$) the accumulation of hydrogen peroxide (H_2O_2) in shallots. The interaction resulting from PGPR inoculation affects H_2O_2 levels in all growth phases (Figure 4a). In the vegetative growth phase, *Bacillus subtilis* Pb03 and *Pseudomonas fluorescens* Pb04 reduce H_2O_2 levels by 15.98% and 25.56%, respectively, compared to the without PGPR. Similarly, during the bulb initiation phase, *Bacillus subtilis* Pb03 and *Pseudomonas fluorescens* Pb04 reduce H_2O_2 levels by 38.93% and 25.67%, respectively, compared to the without PGPR. In the bulb development phase, only *Bacillus subtilis* Pb03 can reduce H_2O_2 levels by 34.61% compared to the treatment without PGPR. For the maturation phase, all PGPR inoculation treatments result in H_2O_2 levels that do not significantly compare in plants without PGPR.

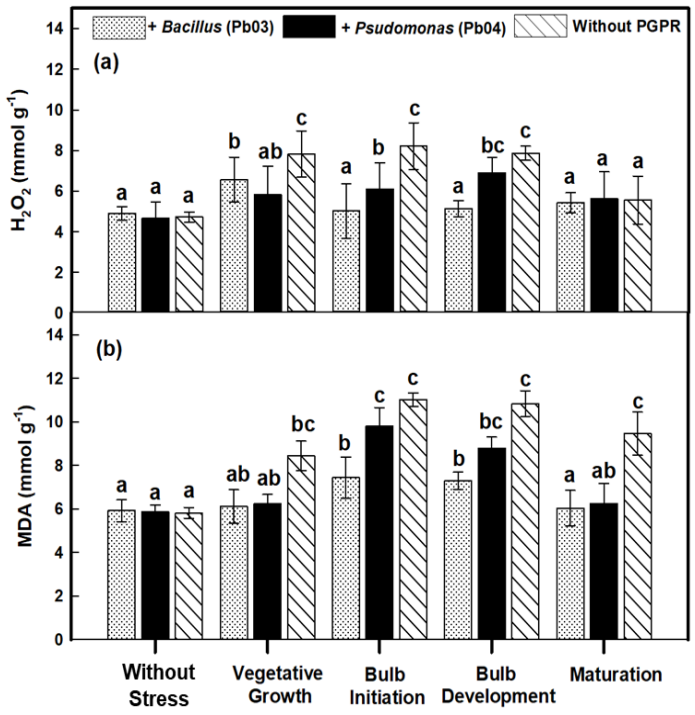

Figure 4: Effect of difference timing drought stress and inoculation of PGPR types on regulating (a) H_2O_2 and (b) MDA content of shallot under drought stress. Mean values with standard error of the mean (n=3). Letters indicate significant differences at $p< 0.05$ according to the Turkey test

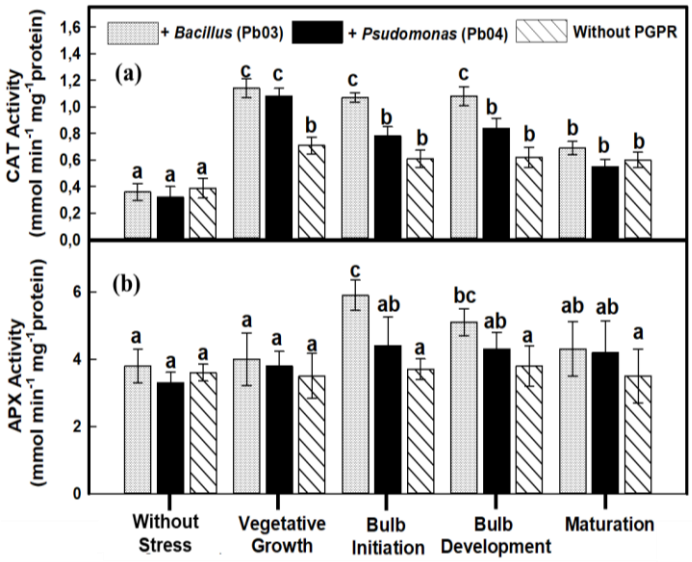

Figure 5: Effect of difference time drought stress and inoculation of PGPR types on (a) Catalase and (b) Ascorbate Peroxidase. Mean values with standard error of the mean (n=3). Letters indicate significant differences at $p< 0.05$ according to the Turkey test

The drought stress influences malondialdehyde (MDA) production. The interaction resulting from PGPR inoculation affects MDA levels in all growth phases (Figure 4b). Adding PGPR inoculation reduces ($p < 0.05$) the MDA production in the same phase compared to plants without PGPR. During the vegetative growth phase, MDA production decreases by 27.61% and 26.07%, respectively, *Bacillus subtilis* Pb03 and *Pseudomonas fluorescens* Pb04. In the bulb initiation phase, MDA production decreases by 32.49% and 10.98%, respectively, in the *Bacillus subtilis* Pb03 and *Pseudomonas fluorescens* Pb04. In the bulb development phase, MDA production decreases by 32.59% and 18.74%, respectively, in the *Bacillus subtilis* Pb03 and *Pseudomonas fluorescens* Pb04, and during the maturation phase, MDA production decreases by 36.15% and 34.04%, respectively, in the *Bacillus subtilis* Pb03 and *Pseudomonas fluorescens* Pb04.

H_2O_2 and MDA production under drought stress are indicators of oxidative stress. Excessive H_2O_2 production occurs due to extreme reduction in the mitochondrial and chloroplast electron transport chain under water pressure, leading to ROS overproduction.[36] MDA is produced through lipid peroxidation under drought stress, while ROS causes lipid peroxidation in plant membranes under drought.[37] It is reported that H_2O_2 is a highly toxic radical that damages various cell components and proteins and increases lipid peroxidation and membrane damage, ultimately causing cell death.[38] It is also reported that excessive production of H_2O_2 and MDA causes interaction with proteins, lipids, and deoxyribonucleic acid (DNA) and causes oxidative damage to plants. Higher H_2O_2 and MDA production increases electrolyte leakage by reducing plant cell membrane integrity under drought stress.

Antioxidant Enzyme Activity

The catalase (CAT) activity in the drought stress treatment increases ($p < 0.05$) in all growth stages (Figure 5a). The interaction resulting in the vegetative growth under drought stress, *Bacillus subtilis* Pb03 and *Pseudomonas fluorescens* Pb04 inoculations increase the CAT activity by 56.34% and 54.22%, respectively, compared to the treatment without PGPR. During bulb initiation and bulb development phases, *Bacillus subtilis* Pb03 and *Pseudomonas fluorescens* Pb04 increase the CAT activity average by 32.72% and 54.8%, respectively, compared to the treatment without PGPR. However, PGPR does not significantly increase CAT activity during maturation.

Drought stress significantly increases ($p < 0.05$) Ascorbate Peroxidase (APX) activity during the bulb initiation and bulb development phases when *Bacillus subtilis* Pb03 inoculation is applied. The activity elevates by 63.89% during bulb initiation and 41.67% during bulb development compared to plants without PGPR (Figure 5b). However, inoculation with *Pseudomonas fluorescens* Pb04 and without PGPR treatment shows no significant difference during the vegetative and maturation phases compared to plants without drought stress.

The interaction between drought stress timing and bacterial inoculation influences the increase in guaiacol peroxidase (GPOX) activity. Inoculation with *Bacillus subtilis* Pb03 during the vegetative, bulb initiation, and bulb development phases increases ($p < 0.05$) GPOX activity by 23.81%, 28.57%, and 26.19%, respectively, compared to the control (Figure 6a). Compared to the treatment without PGPR inoculation, the increase in GPOX with *Bacillus subtilis* Pb03 is 31.71% and 26.19%, respectively, during the bulb initiation and bulb development phases. However, adding *Pseudomonas fluorescens* Pb04 inoculation does not significant difference compared to without PGPR inoculation.

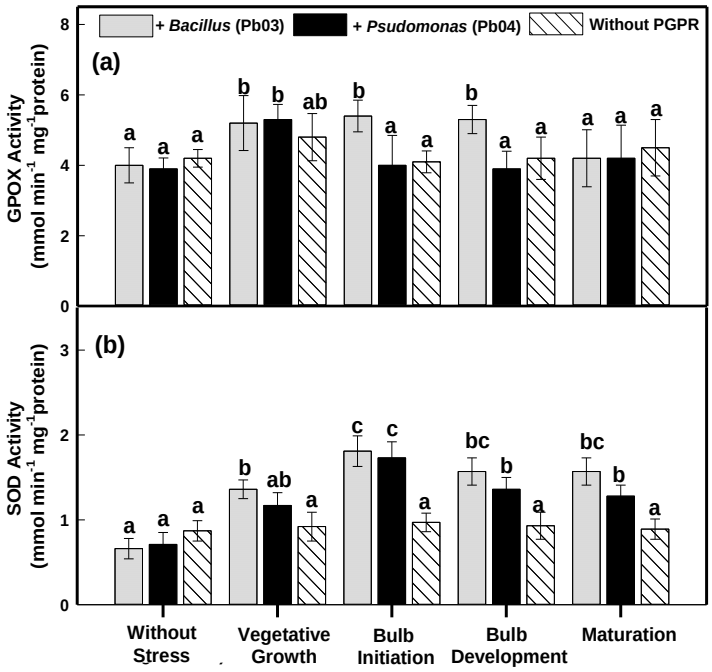

Figure 6: Effect of difference time drought stress and inoculation of PGPR types on (a) Guaiacol Peroxidase and (b) Superoxide Dismutase. Mean values with standard error of the mean (n=3). Letters indicate significant differences at p< 0.05 according to the Turkey test

Table 3: Secondary metabolites of shallot on different timing drought stress and inoculation PGPR

Treatment	Proline content (µg g^{-1})	Allicin content (µg g^{-1})	Total Phenol (mg GAE g^{-1})	Total Flavonoid (mg QE g^{-1})
Time Drought Stress				
Without Stress	37.25 ± 0.12 a	1263 ± 2.25 a	670.7 ± 3.52 a	478.3 ± 2.87 a
Vegetative Growth	42.05 ± 2.97 a	1604 ± 4.56 b	787.5 ± 4.12 a	644.2 ± 2.64 b
Bulb Initiation	60.46 ± 4.41 c	2358 ± 9.60 d	910.7 ± 4.51 b	625.3 ± 3.22 b
Bulb Development	54.04 ± 5.44 b	1933 ± 8.16 c	952.8 ± 2.89 b	649.1 ± 4.85 b
Maturation	39.56 ± 0.22 a	1294 ± 8.33 a	757.8 ± 4.14 a	488.1 ± 4.01 a
Inoculation PGPR				
Without PGPR	49.67 ± 5.59b	1836.5±4.56 b	925.3 ± 3.62b	657.6 ± 3.06b
*Bacillus subtilis*Pb03	45.12 ± 3.81a	1648.0±4.33 a	743.5 ± 3.31a	533.1 ± 3.54a
*Pseudomonas fluorescens*Pb04	45.23 ± 3.94a	1588.3±3.25 a	779.1 ± 1.87a	540.3 ± 4.04a

Noted: Different letters indicate significant differences among treatments at $p < 0.05$ according to the Turkey test

Bacterial inoculation, both with *Bacillus subtilis* Pb03 and *Pseudomonas fluorescens* Pb04, leads to an increase ($p < 0.05$) in SOD activity in all growth stages (Fig. 6b). Compared to the treatment without PGPR inoculation, SOD activity increases by 47.83% and 27.17, respectively, with *Bacillus subtilis* Pb03 and *Pseudomonas fluorescens* Pb04 during the vegetative phase. During the bulb initiation phase, SOD activity increases by 86.6% and 78.35%, respectively, with *Bacillus subtilis* Pb03 and *Pseudomonas fluorescens* Pb04 inoculation. In the bulb development phase, SOD activity increases by 68.82% and 46.24%, respectively, with *Bacillus subtilis* Pb03 and *Pseudomonas fluorescens* Pb04. and in the maturation phase, SOD activity increases by 76.4% and 43.82%, respectively, with *Bacillus subtilis* Pb03 and *Pseudomonas fluorescens* Pb04 inoculation.

CAT is an enzyme that reduces excess H_2O_2 production under oxidative stress by converting H_2O_2 into H_2O and O_2. The ascorbate glutathione pathway is plant cells' primary H_2O_2 detoxification system, where APX reduces H_2O_2, with ascorbate acting as an electron donor. For shallot, under drought stress, the activity of these enzymes is considered one of the most important protective mechanisms against oxidative stress.[39] GPX scavenges ROS and produces related compounds such as lignin, guaiacol, and pyrogallol. These compounds function as electron donors to scavenge H_2O_2 inside and outside the cell. SOD plays a crucial role as the first line of defense against oxidative stress caused by drought.[40] CAT and SOD activities were significantly lower in plants, possibly due to prolonged drought stress. CAT and SOD activities increase under short-term water stress while they decrease under long-term water stress.[41] It is also noted that the effectiveness of antioxidant enzyme activities depends on plant species and the severity and duration of drought stress.[42] Increased APX and GPX were insufficient to neutralize the excess production of H_2O_2 under prolonged drought stress. It has been observed that bacterial inoculation regulates and increases the activity of CAT, APX, GPX, and SOD in various plant species under drought stress. PGPR can enhance plant tolerance to stress, including oxidative stress, which can lead to an increase in antioxidant production as a plant defense response. Research results indicate that PGPR increases antioxidant levels in shallots, suggesting that PGPR has the potential as an additive alternative to naturally increase antioxidant content in plants.

Conclusion

The outcomes of this study underscore the critical role of timing in determining the impact of drought stress. Shallot exhibits a post-drought stress physiological repair metabolism during different growth phases. The maturation growth phase emerges as the most tolerant to drought stress, given that essential metabolic processes have been completed before entering this phase. Following maturation, the vegetative phase provides a controlled environment for stress during early growth stages, allowing a more significant window to stimulate post-stress recovery. However, bulb initiation and development phases become the most vulnerable to drought stress, redirecting photosynthesis intended for bulb growth towards the physiological metabolism repair due to the stress.

Inoculation with *Bacillus subtilis* Pb03 under drought stress has enhanced physiological metabolism and antioxidant enzyme activities. Conversely, the impact of *Pseudomonas fluoresces* Pb04 inoculation on the physiological characteristics of shallot is notably weaker compared to *Bacillus subtilis* Pb03 inoculation, potentially linked to their functional characteristics. These results highlight significant differences in the effects of applying two distinct PGPR to enhance drought tolerance in red onions. This underscores the pivotal role of PGPR selection as a crucial factor influencing the outcomes of their application.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgments

The authors would like to thank the Ministry of Agriculture, Republic of Indonesia, for the grant of research number No.596.1/KPTS/PK.320/A/8/2019.

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