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Biofertilizer application enhance chlorophyll content, membrane stability index, and anatomy of shallot (*Allium cepa* L.) leaves under drought stress

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ABSTRACT. Shallots (*Allium cepa* L.) are plants from the Amaryllidaceae family, Allioieae subfamily, and *Allium* genus. This perennial plant is estimated to have more than 1,000 species. Shallot leaves form a basal sheath. Biofertilizer contains rhizobacteria, which facilitate nutrient availability and uptake by plants. This research was conducted to determine the effect of biofertilizers on the physiological conditions and anatomical structure of shallot leaves (*A. cepa* L.) under drought-stress conditions. The treatments applied included the provision of biofertilizer and different drought stresses; biofertilizer was used with concentrations (10, 15, and 20 L/Ha), and the drought stress applied was 25, 50, and 75%. Data from measurements of chlorophyll a and chlorophyll b levels, Membrane Stability Index (MSI), stomatal density, leaf thickness, and metaxylem diameter were analyzed using SPSS 20, ANOVA test, DMRT with a confidence level of 95%. A biofertilizer concentration of 10 L/Ha is optimal for the Membrane Stability Index (MSI), a concentration of 15 L/Ha is optimal for chlorophyll a and b levels, leaf thickness, and metaxylem diameter, and a concentration of 20 L/Ha is optimal for increasing stomata density shallot leeks under drought stress conditions.

Keywords: biofertilizer; drought stress; growth rate; nitrate reductase; shallots

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INTRODUCTION

Shallots with the scientific name *Allium cepa* L. are one of the agricultural commodities. This plant belongs to the genus *Allium*, which currently counts 1,019 defined species (Royal Botanical Garden Kew, 2023). Shallots are used as a food ingredient that contains many bioactive compounds. Some of the roles of bioactive compounds in shallots include antibacterial, healing cardiovascular disease, healing wounds, antiplatelet, anticancer, antidiabetic, anti hypercholesterolemia, antioxidant, antiobesity, antihypertensive, antiparasitic, gallstone treatment, bone disorder treatment, antidepressant, anti-inflammatory, neuroprotective, insecticide, immunomodulator, lung disturbance, and hepatoprotective (Chakraborty *et al*., 2022).

Shallots possess numerous agronomic and economic benefits. Their cultivation has steadily increased in response to growing demand, establishing them as a principal agricultural commodity. Shallot production in 2021 reached an average of 112,201.3 tons, while in Indonesia, the production of shallots and onions with an agricultural area of 103,024 hg/Ha (10,302.4 kg/Ha) is 2,004,590 tons (FAOSTAT, 2023). The main problems in Indonesia in increasing shallot production include focused production centers in certain areas, conventional cultivation systems, the lack of ability to adopt technology, and the limitation of environmentally friendly controls (Biro Perencanaan, 2018).

Environmentally friendly controls are still limited compared to the size of agricultural land, which could indicate that chemical fertilizers are still used a lot. Chemical fertilizers have a faster effect on plants but have a negative impact, namely environmental damage. Chemical fertilizers initiate severe soil damage. Soil nutritional stability is disturbed, the rhizosphere layer is damaged, and heavy metal activity increases. Organic fertilizer can improve soil's structure and microbial composition so that plant productivity increases (Lin *et al*., 2019). One organic fertilizer that is very easy to apply is biofertilizer. Biofertilizer is a liquid organic fertilizer used to reduce the use of chemical fertilizers and increase plant tolerance capabilities (Wong & Teh, 2021). The advantages of biofertilizers are that they are affordable, reduce the use of chemical fertilizers, increase the use and

availability of soil nutrients that plants can absorb, increase root proliferation by releasing growth hormones, and do not damage the environment (Saif *et al*., 2021).

In addition to fertilizer selection, drought stress is a major challenge in shallot cultivation. Drought stress is different in each region. The impact of drought on plants can be observed through the plant organs. The organs that can be used to observe the effects of drought are leaves. The direct impact of drought on leaves is stomata closure and leaf aging, while the indirect impact is a decrease in chlorophyll levels and cell damage (Ahluwalia *et al*., 2021). Previous research by Sujatha *et al*. (2016) stated that soil with good permeability provides enough water for irrigation and can increase plant productivity. Soil permeability can be increased by giving organic material (Sujatha *et al*., 2016). Based on this description, research was carried out regarding the response of chlorophyll content, membrane stability index, and anatomy in shallot leaves (*A. cepa* L.) to biofertilizer application under drought stress. The results of this study have the potential to support improvements in plant resilience, physiological efficiency, and productivity in marginal lands, as well as to promote environmentally friendly sustainable agricultural practices. Furthermore, the findings may serve as a reference for selecting appropriate biofertilizers to strengthen plant defense systems through anatomical and biochemical approaches.

MATERIALS AND METHODS

Study area. This research was conducted at the Sawitsari Greenhouse, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, from October to February 2023. The planting medium used was a mixture of rice field soil and manure with a ratio of (1:1). Shallots were planted in polybags with a diameter of 20 cm. The shallot plants used in this study were approximately 7 days old at the time of biofertilizer application and drought stress treatment. At this stage, the plants were in the vegetative phase, which is critical for determining the impact of environmental and nutritional factors on bulb formation and final yield. The experiment employed a factorial CRD (Completely Randomized Design) with two factors: biofertilizer dosage (0, 10, 15, and 20 L/Ha) and drought stress levels (0, 25, 50, and 75% of field capacity). Each treatment combination was replicated 3 times.

Treatment. The biofertilizer used is a formula from Siswanti (2015), which contains nine microbes, namely *Azotobacter* sp., *Azospirillum* sp., *Bacillus* sp., *Lactobacillus* sp., *Pseudomonas* sp., *Rhizobium* sp., *Saccharomyces* sp., *Streptomyces* sp., and IAA hormone-producing bacteria. Biofertilizer is applied by diluting it first with a ratio of 1:11. Meanwhile, for drought stress, it is given with different watering levels: 25% stress with 75% field capacity watering, 50% stress with 50% field capacity watering, and 75% stress with 25% field capacity watering.

Measurement of chlorophyll levels. The method used is the Arnon method (1949). A sample of 0.1 grams of fresh leaves was ground and added with 10 mL of 80% acetone. The mixture was filtered, and then the absorbance of the filtrate was measured. The light waves used were 663 nm for chlorophyll a and 645 nm for chlorophyll b (Siswanti & Umah, 2020). The levels were calculated from the absorbance results with the formula from (Siswanti & Umah, 2020).

$$\text{Chlorophyll a} = \frac{(12.7 \times A663 - 2.69 \times A645) \times V}{1000 \times W}$$

$$\text{Chlorophyll b} = \frac{(22.9 \times A645 - 4.68 \times A663) \times V}{1000 \times W}$$

Membrane Stability Index (MSI) measurement. The method used refers to the study (Abid *et al*., 2018). Two sets of samples were prepared, each of which was fresh leaves cut into small pieces weighing 0.1 grams. Each sample was put into a test tube and added with 10 mL of distilled water. The first set was incubated at 40ºC for 30 minutes, while the second set was incubated at 100ºC for 15 minutes. The incubation results were measured for electrical conductivity using a TDS EC meter. The conductivity of the first set was marked as C1 and the second set as C2. The calculation of the

membrane stability index based on the conductivity results was carried out using the formula from the study by Abid *et al*., (2018).

$$MSI\ (\%) = [1 - \left(\frac{C1}{C2}\right) \times 100$$

Stomatal density measurement. Shallot leaf stomata preparations were made by applying nail polish to the leaf surface. The leaves used were the middle part. The removal of the epidermis on the nail polish was assisted by using a solvent, which was then attached to the glass slide. Stomata were observed using a light microscope, and pictures were taken at 100x magnification using Optilab Viewer 2.2. Stomata were counted at a cross-sectional area of 0.94 mm2 with Image Raster 3. Density was calculated using the formula (Humami *et al*., 2020).

$$\text{Stomatal density} = \frac{\text{number of stomata}}{\text{wide field of view}}$$

Making leaf anatomy preparations. Leaf anatomy preparations were made using the embedding method. Fixation was done with the FAA. The following process was staining with 1% safranin in 70% alcohol. Dehydration was done with graded alcohol and continued with dealcoholization because paraffin infiltration and covering would be carried out. Ribbon preparation was done with a rotary microtome and covered with Canadian balsam. The preparations were observed with Optilab viewer 2.2. Leaf thickness, palisade thickness, vascular bundle diameter, and metaxylem diameter were measured with Image Raster 3.

Data analysis. Data were analyzed using ANOVA at a 95% confidence level equipped with the DMRT test. The software used was IBM SPSS version 20.

RESULTS AND DISCUSSION

Chlorophyll a and chlorophyll b levels. Although there are several types of chlorophyll in plants, the most commonly found chlorophyll is chlorophyll a and b. Drought stress can affect the chlorophyll levels of plants, as in the study of Sansan *et al*. (2024), where drought stress reduced the levels of chlorophyll a and b in shallot leaves (*A. cepa* L.). The measured levels of chlorophyll a and b in shallot leaves are in Table 1.

Tabel 1. Chlorophyll a and b levels of shallot (*A. cepa* L.) under drought stress conditions with varying doses of biofertilizer

Chlorophyll a (mg/g)					
Biofertilizer	Drought Stress (%)				
(L/Ha)	0	25	50	75	Average
0	0.627 ± 0.024^{bc}	0.548 ± 0.066^{bc}	0.823 ± 0.050^{de}	0.378 ± 0.042^{a}	0.594 ± 0.172^{y}
10	0.516 ± 0.016^{b}	0.247 ± 0.166^{a}	0.621 ± 0.054^{bc}	0.677 ± 0.082^{cd}	0.515 ± 0.191^{x}
15	0.885 ± 0.202^{e}	0.680 ± 0.016^{cd}	0.689 ± 0.088^{cd}	0.704 ± 0.038^{cd}	0.739 ± 0.130^{z}
20	0.684 ± 0.011^{cd}	0.802 ± 0.072^{de}	0.645 ± 0.017^{bc}	0.594 ± 0.012^{bc}	0.681 ± 0.086^{z}
Average	0.678 ± 0.165^{q}	0.569 ± 0.231^{p}	0.695 ± 0.095^{q}	0.588 ± 0.140^{p}	
Chlorophyll b (mg/g)					
Biofertilizer	Drought Stress (%)				
(L/Ha)	0	25	50	75	Average
0	0.169 ± 0.032^{ab}	0.216 ± 0.059^{ab}	0.454 ± 0.055^{def}	0.295 ± 0.177^{bcde}	0.283 ± 0.141^{x}
10	0.115 ± 0.049^{a}	0.393 ± 0.060^{cdef}	0.216 ± 0.043^{ab}	0.501 ± 0.075^{f}	0.306 ± 0.165^{xy}
15	0.457 ± 0.247^{ef}	0.411 ± 0.001^{cdef}	0.266 ± 0.080^{abc}	0.452 ± 0.034^{def}	0.396 ± 0.138^{z}
20	0.278 ± 0.084^{abcd}	0.529 ± 0.050^{f}	0.273 ± 0.079^{abc}	0.413 ± 0.029^{cdef}	0.373 ± 0.124^{yz}
Average	0.254 ± 0.177^{p}	0.388 ± 0.124^{q}	0.302 ± 0.109^{p}	0.415 ± 0.116^{q}	

Notes: Numbers in rows and columns followed by the same letter are not significantly different at the level ($p<0.05$)

ANOVA analysis showed that the average levels of chlorophyll a and chlorophyll b increased with the administration of biofertilizer, especially at a dose of 15 L/Ha, significantly different from the control. The administration of biofertilizer had a significant effect ($p < 0.05$) on the levels of chlorophyll a and chlorophyll b in dealing with drought stress. The application of biofertilizer under 50% field capacity (equivalent to 50% drought stress) resulted in relatively high levels of chlorophyll a and b. Biofertilizer contains nine microbes that can enrich the content of soil elements that are ready for plants' use. The N element in the soil is crucial for plant synthesis, including chlorophyll synthesis (Li *et al*., 2018). As much as 70% of the N element in leaves accumulates in chloroplasts. The availability of sufficient N elements can increase the levels of chlorophyll a and b (Fathi, 2022). Drought stress treatment without biofertilizer administration affects the decrease in chlorophyll levels (Kalaji *et al*., 2015). Biofertilizer helps maintain chlorophyll levels in shallot leaves under drought stress.

Membrane stability index. The cell membrane regulates and selects ion traffic needed by plant cells. When the distribution of ion charges passing through is uneven, membrane potential will be created (Bhatla & Lal, 2018). Membrane stability is influenced by the presence of ions and the presence of oxidative stress (Zayed *et al*., 2023). The results of measuring the membrane stability index of shallot leaves are in Table 2.

Tabel 2. Stability index of shallot leaf membrane (*A. cepa* L.) under drought stress conditions with varying doses of biofertilizer

Biofertilizer (L/Ha)	Drought Stress (%)				
	0	25	50	75	Average
0	52.700 ± 4.411^{efgh}	42.463 ± 5.416^{cde}	39.427 ± 1.190^{bcd}	$15.713 \pm 6.452a^{a}$	37.576 ± 14.727^{x}
10	61.430 ± 3.638^{ghi}	54.767 ± 5.391^{fgh}	50.887 ± 1.657^{efg}	63.137 ± 4.134^{hi}	57.555 ± 6.175^{z}
15	66.603 ± 2.571^{i}	48.050 ± 9.728^{def}	35.497 ± 5.088^{bc}	47.343 ± 1.120^{def}	49.373 ± 12.587^{y}
20	31.197 ± 14.283^{b}	56.997 ± 0.971^{fghi}	57.637 ± 7.138^{fghi}	51.180 ± 5.765^{efg}	49.253 ± 13.342^{y}
Average	52.983 ± 15.613^{r}	50.569 ± 7.982^{qr}	45.862 ± 10.001^{pq}	44.343 ± 18.762^{p}	

Notes: Numbers in rows and columns followed by the same letter are not significantly different at the level ($p<0.05$)

Based on the results in (Table 2.) show that the provision of biofertilizer has a significant effect on the membrane stability index of shallot leaves under drought stress conditions. The membrane stability index increased the most at a biofertilizer dose of 10 L/Ha (57.55%). Biofertilizers can increase the membrane stability index, while drought stress decreases the membrane stability index. Biofertilizers can increase soil nutrition and improve soil conditions. Sufficient nutrition for leaves in protein and enzyme synthesis will effectively reduce cell damage and increase membrane stability (Akhtar *et al*., 2022). Drought stress can disrupt the membrane structure by forming Reactive Oxygen Species (ROS) so that membrane stability decreases (Rachmawati *et al*., 2018). Increasing the N element in the soil by adding biofertilizers helps plants produce enzymes that can suppress ROS. Adding N elements to the soil can increase stomatal activity and membrane stability (Abid *et al*., 2016). The provision of biofertilizers can help plants overcome membrane instability caused by drought stress.

Stomatal density. Stomata are gaps on the surface of the leaf epidermis that play a role in the exchange of gases and water vapor (Humami *et al*., 2020). Stomatal density was calculated to observe how leaves respond to biofertilizer application under drought stress. The stomatal density of *A. cepa* L. leaves was measured and presented in Table 3.

Tabel 3. Stomata density of shallot leaves (*A. cepa* L.) under drought stress conditions with varying doses of biofertilizer

Biofertilizer	Drought Stress (%)				
(L/Ha)	0	25	50	75	Average
0	59.577 ± 8.033^{bc}	43.617 ± 6.471^{a}	60.283 ± 11.275^{bc}	61.347 ± 13.475^{bc}	56.206 ± 11.556^{xy}
10	81.560 ± 2.212^{de}	45.033 ± 0.612^{a}	41.490 ± 4.638^{a}	60.637 ± 12.265^{bc}	57.180 ± 17.463^{xy}
15	45.390 ± 1.299^{a}	49.643 ± 5.030^{ab}	52.837 ± 2.677^{ab}	71.983 ± 6.048^{cd}	54.963 ± 11.215^{x}
20	44.680 ± 3.832^{a}	84.753 ± 1.625^{e}	67.730 ± 2.678^{c}	49.290 ± 6.922^{ab}	61.613 ± 17.001^{y}
Average	57.802 ± 16.104^{p}	55.762 ± 17.995^{p}	55.585 ± 11.494^{p}	60.814 ± 12.085^{p}	

Notes: Numbers in rows and columns followed by the same letter are not significantly different at the level ($p<0.05$)

ANOVA analysis showed that the administration of biofertilizer at a dose of 20 L/Ha had an average stomatal density value (61.61%) significantly different from the control (Table 3). The highest stomatal density was in the biofertilizer dose of 20 L/Ha with 25% drought stress (84.75%). The number of stomata on the leaves of plants given biofertilizer in the planting medium was higher than in the planting medium that was not given biofertilizer (Madusari, 2016). Plants reduce water loss and maintain stability in the tissue by reducing the size and number of stomata (Seleiman *et al*., 2021). When water is limited, signals reduce hydraulic conductivity and increase abscisic acid production, causing the turgor pressure of guard cells to decrease and the stomata to close. Reducing the number and size of stomata may be effective in increasing efficient water use and dealing with drought stress without reducing production yields (Bertolino *et al*., 2019). The provision of biofertilizers helps plants use K elements and growth hormones that play a role in the opening and closing of stomata. Indirectly, plants will be more effective in using water and adjusting the number and opening of their stomata (Wong & Teh, 2021).

Leaf thickness. Drought stress causes dehydration, to which plants respond by changing leaf thickness (Khan *et al*., 2023). The leaf thickness of *A. cepa* L. was measured to see the effect of biofertilizer given under drought-stress conditions. The results of leaf thickness measurements are in Table 4.

Tabel 4. Leaf thickness (µm) of shallot (*A. cepa* L.) under drought stress conditions with varying doses of biofertilizer

Biofertilizer	Drought Stress (%)				
(L/Ha)	0	25	50	75	Average
0	198.157 ± 12.490^{fg}	180.250 ± 2.547^{ef}	146.757 ± 5.548^{ab}	184.153 ± 12.910^{ef}	177.329 ± 21.300^{x}
10	155.567 ± 7.416^{bc}	187.927 ± 8.302^{ef}	170.983 ± 7.263^{cde}	210.650 ± 4.921^{gh}	181.282 ± 22.205^{x}
15	162.257 ± 8.936^{bcd}	249.223 ± 4.461^{i}	268.643 ± 16.346^{j}	222.360 ± 3.855^{h}	225.621 ± 42.709^{y}
20	178.863 ± 16.835^{de}	184.057 ± 16.679^{ef}	222.653 ± 7.977^{h}	133.290 ± 2.279^{a}	179.716 ± 34.793^{x}
Average	173.711 ± 20.005^{p}	200.364 ± 30.725^{r}	202.259 ± 49.976^{r}	187.613 ± 36.338^{q}	

Notes: Numbers in rows and columns followed by the same letter are not significantly different at the level ($p<0.05$)

Based on the results shown (Table 4.), the administration of biofertilizer at a dose of 15 L/Ha had the highest average leaf thickness value (225,621 µm) and was significantly different from the control. The leaves with the highest thickness were in the 15 L/Ha biofertilizer treatment and 50% drought stress (268,643 µm). The availability of essential soil elements, particularly nitrogen, influences leaf thickness regulation (Tian *et al*., 2016). These elements are related to synthesizing structural proteins that play a role in cell formation. The larger the size of the cells that make up the tissue, the thicker the leaves will be (Khan *et al*., 2023). Leaf thickness also increases due to the role of nitrogen in the widening of epidermal cells (Onyango, 2015). Leaves can decrease or increase thickness as a mechanism when facing environmental stress. An increase in the thickness of the adaxial epidermis causes leaf thickness to rise as an adaptation to reduce transpiration (Yavas *et al*., 2024).

Metaxylem diameter. The xylem, tissue that transports water and nutrients, is divided into two types, namely protoxylem and metaxylem. The transport capacity differs, so they have various sizes (Scuezt *et al*., 2013). The results of measuring the diameter of the metaxylem of *A. cepa* L. leaves are presented in Table 5.

Tabel 5. Metaxylem diameter (µm) of shallot leaves (*A. cepa* L.) under drought stress conditions with varying doses of biofertilizer

Biofertilizer	Drought Stress (%)				
(L/Ha)	0	25	50	75	Average
0	18.637 ± 5.392^{abcde}	23.693 ± 3.193^{cdefg}	23.400 ± 1.480^{cdefg}	12.523 ± 2.546^{a}	19.563 ± 5.234^{x}
10	26.963 ± 4.931^{fg}	15.417 ± 2.252^{ab}	$19.380 \pm 7.398^{abcdef}$	$21.823 \pm 5.267^{bcdefg}$	20.896 ± 4.830^{xy}
15	$21.763 \pm 2.275^{bcdefg}$	24.567 ± 2.768^{cdefg}	20.517 ± 4.294^{bcdef}	29.257 ± 3.752^{g}	24.026 ± 3.877^{y}
20	26.060 ± 4.477^{efg}	16.853 ± 4.644^{abc}	17.460 ± 4.194^{abcd}	24.877 ± 1.658^{defg}	21.313 ± 4.829^{xy}
Average	23.356 ± 3.879^{p}	20.133 ± 4.667^{p}	20.189 ± 2.484^{p}	22.120 ± 7.088^{p}	

Notes: Numbers in rows and columns followed by the same letter are not significantly different at the level ($p<0.05$)

Based on the ANOVA analysis (Table 5.) shows that the highest average metaxylem diameter was found in the treatment of biofertilizer administration at a dose of 15 L/Ha (24.03 µm), which was significantly different from the control. The administration of biofertilizer increased the size of the metaxylem diameter, while drought stress decreased the size of the metaxylem diameter. The size of the metaxylem is influenced by the efficiency of water use (Prince *et al*., 2017). The larger the diameter of the metaxylem, the more efficient the plant is in using water and nutrients. The diameter will affect the area of the tissue, which will impact the transpiration rate. A small diameter will accelerate the transpiration rate so that the diameter is expanded to reduce the transpiration rate. As a form of adaptation, plants increase the size of the metaxylem diameter in drought stress by adding soil nutrients (Cary *et al*., 2019). The increase in metaxylem diameter can be supported by the provision of biofertilizers that enrich the elements in the soil that are ready to be transported by plants.

CONCLUSION

Biofertilizer application has significantly different effects on chlorophyll a and chlorophyll b levels, membrane stability index, stomatal density, leaf thickness, and metaxylem diameter of shallot (*Allium cepa* L.) leaves under drought stress conditions. A biofertilizer concentration of 10 L/Ha is optimal for the Membrane Stability Index (MSI), a concentration of 15 L/Ha is optimal for chlorophyll a and b levels, leaf thickness, and metaxylem diameter, and a concentration of 20 L/Ha is optimal for increasing stomata density of shallot under drought stress conditions. This research provides insights into the effects of biofertilizer application on the physiological and anatomical conditions of shallot leaves and determines the optimal dosage for each parameter. Further research can be conducted on other stress conditions, both in shallots and other food crops, to broaden our understanding of how to address various environmental constraints that hinder plant productivity.

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