



Effect of *Glomus manihotis* inoculation and salt stress on antioxidant and biochemical properties of Chia (*Salvia hispanica* L.)

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ABSTRACT

Plant productivity is often constrained by abiotic stress in the form of high salt levels. However, a symbiosis between plant and arbuscular mycorrhizal fungi can reduce the severity of the effect of salt stress on cultivated plants. The aim of this study was to determine the impact of salt stress on the antioxidant substances and biochemical parameters of chia (*Salvia hispanica* L.) plants that had been inoculated with the fungus *Glomus manihotis*. A factorial completely randomized design with seven replicates was used with status of inoculation by the fungus *G. manihotis* (inoculated vs. not inoculated) as one of factors and the concentration of sodium chloride (NaCl) (0, 50, 100, and 200 mM) as the other status factor. Several parameters in the chia plants were measured including: root infection, phosphorus content, chlorophyll and carotenoid contents, antioxidant enzyme activities (superoxide dismutase and catalase), and malondialdehyde content. The results showed that chia plants inoculated with *G. manihotis* (mycorrhizal plants), even under salt stress conditions, had higher phosphorus content than non-mycorrhizal plants. High salt levels reduced the percentage of root infection by the mycorrhizal fungus of *G. manihotis*. Under salt stress conditions, chlorophyll and carotenoid contents of chia leaves were higher in mycorrhizal plants than in non-mycorrhizal plants. The activities of superoxide dismutase and catalase of mycorrhizal chia plants were higher than those of non-mycorrhizal plants, even though they were grown under conditions of high salt levels. The malondialdehyde content of chia plants increased with salt concentration, but decreased in chia plants inoculated with *G. manihotis*. The findings of this study indicate that *G. manihotis* inoculation is effective in reducing the effect of salt stress on chia plants.

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1. INTRODUCTION

Cultivation on high salinity soil disrupts agricultural production (Rivero et al., 2018). The occurrence of high salinity land is worsened by population growth and global warming (Okur & Örcen, 2020). Ability of plants to survive under high salinity conditions is due to mechanisms that maintain ion homeostasis and chloroplast function (Li et al., 2020). Specifically, plants tolerate high salt levels by producing antioxidant enzymes against free radicals attack (Cen et al., 2020).

Currently, research is focuses on biological methods aimed at reducing stress related to high salt levels, which can affect agricultural production (Noreen et al., 2021). More than 90% of the plants living on the earth's surface have a symbiotic relationship with fungi, either wholly (endophytes

or partially (mycorrhizae), in plant cells or tissues (Heydari & Pirzad, 2020). Symbiosis allows plants to adapt well to biotic and abiotic factors such as high salinity stress. Additionally, symbiosis is beneficial for the survival of the plant itself and its fungi in the face of stressful habitat conditions (Diao et al., 2021).

Glomus manihotis is a mycorrhizal fungus of the order Glomales and belongs to the arbuscular mycorrhizal type. In this type of mycorrhiza, plants exchange photosynthate with minerals and water taken up by mycorrhizal fungi from the soil (Borde et al., 2017). However, it is not clear whether the ability of plants to tolerate high salt levels is directly by the plant itself or mediated by the presence of mycorrhizal symbiosis.

A plant's response to various environmental factors such as high salt level is thought to be influenced by these fungal symbionts. Inoculation with arbuscular mycorrhizal fungi can enhance plant growth and nutrient absorption in high-salt soil conditions and reduce crop loss (Ait-El-Mokhtar et al., 2020). Additionally, the ability of arbuscular mycorrhizae to destroy reactive oxygen species is believed to be the mechanism responsible for increasing the effectiveness of host plant defenses (Abdelaziz et al., 2019). However, the mechanism by which arbuscular mycorrhiza increases the resistance of host plants to high salt stress is not yet understood.

Understanding the nature of the symbiosis between mycorrhizal fungi and their host plants will enhance strategies to deal with disorders caused by abiotic environmental factors such as high salinity stress (Kong et al., 2020). The symbiosis between mycorrhizal fungal and host plants is believed to be an inexpensive and viable way to reduce the effects of global warming on plants and plant communities. Owing to global warming, the proportion of land affected by high salinity is increasing, making it necessary to understand the mechanism by which plants withstand high-salt stress, which is necessary for maintaining high agricultural productivity (Malhi et al., 2021).

Chia (*S. hispanica* L.) plants are horticultural crops that are sensitive to high salinity levels (Aguilar-Toalá et al., 2020). The role of mycorrhiza in the mechanism of resistance of chia plants to high salt stress is not yet well understood (Mlinarić et al., 2020). This study tested the hypothesis that *G. manihotis* can increase the tolerance of chia plants grown under high salt conditions. Furthermore, the effects of mycorrhiza on antioxidant enzyme activity and lipid peroxidation during salt stress are not yet known. The aim of this study was to determine the impact of salt stress on the antioxidant enzyme activity and biochemical parameters of chia plants inoculated with the fungus *G. manihotis*. To measure antioxidant activities, of superoxide dismutase and catalase activities and the content of malondialdehyde as the end product in lipid peroxidation were measured. The biochemical parameters measured included chlorophyll and carotenoids contents, percentage of root infection, and concentration of phosphorus in chia plants. Moreover, the pairwise correlations among all parameters were calculated.

2. MATERIAL AND METHODS

2.1. Plant material and mycorrhizal fungi preparation

Chia seeds were purchased from an agricultural shop in Tanjungsari sub-District, Sumedang District, West Java. The mycorrhizal culture of *G. manihotis* was propagated in the Integrated Garden of Saintek UIN Bandung. A *G. manihotis* inoculum of 100 g (containing more than 1000 spores) was applied to a depth of 3 cm in a 2 kg polybag already containing sterile zeolite medium. Seedlings of 7-day-old chia plants were planted in the zeolite medium (pH 7) inoculated with mycorrhiza. Chia plants not inoculated with mycorrhiza were prepared as a control. All chia plants were watered with aquaDM every 3 days and with a half-concentrated nutrient solution of the Hoagland formula every 7 days.

2.2. Experimental design

Factorial patterns in a randomized block design were prepared for this experiment. One factor was inoculation status with two factor levels, inoculated with *G. manihotis*, (+Gm) and non-inoculated (-Gm). The other factor was sodium chloride with four factor levels (S0 = 0 mM, S1 = 50 mM, S2 = 100 mM, and S3 = 200 mM). Experiments were conducted in a greenhouse at an integrated garden in Saintek UIN Bandung, for three months from September to November 2021. The main experiments were conducted for 45 days with an ambient temperature coefficient of 27/21°C (day/night), at a relative humidity of 70%, and a light intensity of 6500 lux.

2.3. Root infection percentage

Root samples from 45-day-old chia plants (at the end of the experiment) were cleaned in running water to remove the medium used during the study. The roots were stored in 5% KOH solution but for observations they were soaked in trypan blue for 6 h. Calculation of root infection by mycorrhizal fungi was done using the grid-line intersection method under a surgical microscope (Parvin et al., 2020). The percentage of infected roots was calculated using the following formula:

$$\% \text{ root infection} = 100 \times (\text{number of mycorrhizal roots} / \text{total observed roots}) \dots\dots\dots [1]$$

2.4. Phosphorus estimation

The third leaf from the 45-day-old chia plant shoot tip was dried at 70°C for 48 h. After drying, is the leaf was incinerated in a furnace at 550°C for 24 h. Phosphorus concentration was measured using a spectrophotometer at a wavelength of 882 nm. Phosphorus concentration was expressed in units of mg g⁻¹ DW (Qiu et al., 2020).

2.5. Chlorophyll & Carotenoid estimation

A 1 g-leaf sample of chia plants was weighed for maceration and extracted with 10 ml of 80% acetone. The supernatant was filtered using Whatman filter paper no. 1 and the filtrate was made up to 50 ml in a volumetric flask by adding solvent. The absorbance of the solution was measured by a spectrophotometer at wavelengths of 440.5, 646, and 663 nm. To obtain the concentrations of chlorophyll and carotenoids, the following formulas were used:

$$\text{Chl a} = 12,21(A_{663}) - 2,81(A_{646}) \dots\dots\dots [2]$$

$$\text{Chl b} = 20,13(A_{646}) - 5,03(A_{663}) \dots\dots\dots [3]$$

$$\text{Car} = 4,69(A_{440,5}) - 0,268((\text{Chl a} + \text{Chl b})) \dots\dots\dots [4]$$

Chlorophyll content was expressed as mg g⁻¹ FW and carotenoid concentration was expressed as µg g⁻¹ FW (Kazemi et al., 2019).

2.6. Estimation of the superoxide dismutase antioxidant activity

Superoxide dismutase was measured according to the method described by Santander et al. (2020). The ready-to-use enzyme sample was mixed with 2.9 ml of 30% hydrogen peroxide. The absorbance of the prepared mixture was measured at a wavelength of 240 nm using a UV-Vis spectrophotometer. The level of this enzyme indicates its activity expressed in mmol min⁻¹ mg⁻¹ protein.

Table 1. Phosphorus concentration, root infection, Superoxide dismutase and catalase activity, malondialdehyde content in chia plants with and without *G. manihotis* inoculation grown in various NaCl concentration.

| | PHO ($\times 10^{-3}$ mg g ⁻¹ DW) | INF (%) | SOD mmol min ⁻¹ mg ⁻¹ protein | CAT mmol min ⁻¹ mg ⁻¹ protein | MDA μ mol min ⁻¹ mg ⁻¹ protein |
|-------|--|------------------|---|---|--|
| S0+Gm | 157 \pm 7.6 (a) | 75 \pm 2.7 (a) | 8.7 \pm 0.45 (a) | 8.3 \pm 0.09 (a) | 1.4 \pm 0.05 (d) |
| S1+Gm | 135 \pm 5.5 (b) | 68 \pm 4.3 (a) | 8.5 \pm 0.37 (a) | 8.1 \pm 0.11 (a) | 1.6 \pm 0.08 (d) |
| S2+Gm | 136 \pm 6.8 (b) | 49 \pm 5.2 (b) | 8.3 \pm 0.55 (b) | 6.7 \pm 0.15 (b) | 1.8 \pm 0.09 (d) |
| S3+Gm | 122 \pm 4.9 (b) | 48 \pm 3.9 (b) | 7.6 \pm 0.49 (b) | 4.7 \pm 0.10 (c) | 2.2 \pm 0.06 (d) |
| S0-Gm | 85 \pm 4.5 (c) | | 6.8 \pm 0.36 (b) | 4.2 \pm 0.12 (c) | 2.2 \pm 0.09 (c) |
| S1-Gm | 50 \pm 7.8 (d) | | 5.5 \pm 0.57 (b) | 5.9 \pm 0.14 (b) | 2.3 \pm 0.10 (c) |
| S2-Gm | 50 \pm 9.9 (d) | | 5.1 \pm 0.65 (c) | 4.7 \pm 0.09 (c) | 2.9 \pm 0.07 (b) |
| S3-Gm | 43 \pm 8.8 (d) | | 4.3 \pm 0.77 (c) | 4.3 \pm 0.12 (c) | 3.5 \pm 0.08 (a) |

Notes: Data were expressed as the mean \pm standard error of seven repetitions. ANOVA and Duncan's test were used in data analysis. Different letters in the column to indicate statistical significance ($p \leq 0.05$). PHO=phosphorus concentration; INF=root infection; SOD=superoxide dismutase; CAT=catalase; MDA=malondialdehyde; S0=0 mM NaCl (control); S1=50 mM NaCl; S2=100 mM NaCl; S3=200 mM NaCl; +Gm=with *G. manihotis*; -Gm=without *G. manihotis*

2.7. Estimation of catalase antioxidant activity

The catalase enzyme was identified using the method described by Wang et al. (2020). The ready-to-use enzyme sample was mixed with 2.9 ml of 30% hydrogen peroxide. The absorbance of the prepared mixture was measured at a wavelength of 240 nm using a UV-Vis spectrophotometer. The concentration of this enzyme indicates its activity expressed as mmol min⁻¹ mg⁻¹ protein.

2.8. Estimation of Malondialdehyde content

Lipid peroxidation was tested from the formation of malondialdehyde using the thiobarbituric acid method described by Kabir et al. (2020). A mass of 0.2 g of the third leaf sample from the shoot tip was homogenized by adding 1 mL of 5% trichloroacetic acid solution. The homogenate was centrifuged at 12,000 rpm for 12 min at room temperature. The absorbance of the supernatant was measured on a spectrophotometer with a wavelength of 532 nm. The malondialdehyde content was expressed as μ mol min⁻¹ mg⁻¹ protein.

2.9. Statistical analysis

Data were reported as mean \pm standard error of seven replications. Two-way analysis of variance was used to test for significant differences among treatment groups. Comparison among groups were calculated using Duncan's multiple range test. Pearson's rank correlation coefficient among parameters was estimated. A $p \leq 0.05$ was considered statistically significant. All statistical analyses were performed in SPSS version 25.0 (IBM, Chicago, IL, USA).

3. RESULTS

Chia plants inoculated with the mycorrhizal fungus *G. manihotis* had a higher Phosphorus concentration than those that were not inoculated. The highest concentration of Phosphorus (0.157 mg g⁻¹ DW) was recorded in mycorrhizal chia plants not exposed to a high salt content (control). High salt treatment reduced the phosphorus concentration of both mycorrhizal and non-mycorrhizal chia plants (Table 1).

Chia plants that were not inoculated with *G. manihotis* did not show any root infection. Root infection was observed in plants inoculated with *G. manihotis* in which the percentage of root infection decreased as the salt levels increased. The root infection percentage of chia plants at 50 mM NaCl concentration was not significantly different from that of the control ($p \leq 0.05$) (Table 1).

The activity of the antioxidant enzyme superoxide dismutase in mycorrhizal chia plants was higher than in chia plants without mycorrhizae. Superoxide dismutase activity reached its peak in mycorrhizal chia plants treated with 50, 100 mM, and 0 mM NaCl (control). The activity of superoxide dismutase in chia plants without mycorrhizae and treated with 50 mM NaCl concentration was not significantly different from that of chia plants exposed to 100 and 200 mM NaCl treatment (Table 1).

The activity of the catalase enzyme in chia plants with mycorrhizae was higher than in chia plants without mycorrhizae. Increasing NaCl concentration resulted in a decrease in catalase activity. Peak catalase activity was recorded in mycorrhizal chia plants treated with 50 mM NaCl and those in the control (without NaCl treatment) (Table 1). Malondialdehyde content in mycorrhizal chia plants was lower than in non-mycorrhizal chia plants (Table 1). The highest level of malondialdehyde (3.5 μ mol min⁻¹ mg⁻¹ protein) was obtained in non-mycorrhizal chia plants treated with 200 mM NaCl (Table 1).

Pearson's correlation coefficients among all measured parameters (i.e. biochemical and antioxidant properties) of chia plants treated with NaCl and *G. manihotis* inoculation are shown in Figure 1. There was a significant negative correlation among malondialdehyde and phosphorus concentration, percentage root infection, superoxide dismutase antioxidant activity, catalase antioxidant activity, chlorophyll content, and carotenoids content. Furthermore, the results showed that there was a significant positive correlation between percentage of root infection and activities of all antioxidant enzymes and content of all photosynthetic pigment. Carotenoid content showed a weak positive correlation with phosphorus concentration, superoxide dismutase and catalase activities (Figure 1).

| | PHO | INF | SOD | CAT | MDA | CHL | CAR |
|-----|--------|--------|--------|--------|--------|-------|-----|
| PHO | 1 | | | | | | |
| INF | 0,80* | 1 | | | | | |
| SOD | 0,87* | 0,82* | 1 | | | | |
| CAT | 0,81* | 0,87* | 0,98* | 1 | | | |
| MDA | -0,91* | -0,89* | -0,99* | -0,98* | 1 | | |
| CHL | 0,97* | 0,93* | 0,90* | 0,89* | -0,95* | 1 | |
| CAR | 0,75 | 0,97* | 0,68 | 0,74 | -0,78 | 0,88* | 1 |

Figure 1. Correlation among all measured parameters of chia under NaCl treatment and *G. manihotis* inoculation. A correlation of 1 (solid blue) indicates a perfect positive correlation, while -1 (solid red) depicts a perfect negative correlation. PHO=phosphorus concentration; INF=root infection; SOD=superoxide dismutase; CAT=catalase; MDA=malondialdehyde; CHL=chlorophyll; CAR=carotenoid; *significant at $p \leq 0.05$.

Table 2. Total chlorophyll and carotenoid content in chia plant leaves with and without mycorrhizal inoculation grown in various NaCl concentration.

| Treatment | Salt (mM NaCl) | Total Chlorophyll (mg g ⁻¹ FW) | Total Carotenoids (µg g ⁻¹ FW) |
|--------------------|----------------|---|---|
| Without Mycorrhiza | 0 | 3,55(c) | 7,32(c) |
| | 50 | 3,49(c) | 7,28(c) |
| | 100 | 3,22(d) | 7,07(d) |
| | 200 | 3,18(d) | 7,03(d) |
| With Mycorrhiza | 0 | 4,60(a) | 8,35(a) |
| | 50 | 4,46(b) | 8,27(a) |
| | 100 | 4,39(b) | 8,00(b) |
| | 200 | 4,31(b) | 8,05(b) |

Notes: Data were expressed as the mean \pm standard error of seven repetitions. ANOVA and Duncan's test were used in data analysis. Different letters in the same column indicate significantly different ($p \leq 0.05$)

Leaf chlorophyll content of chia plants inoculated with *G. manihotis* was higher than that of non-mycorrhizal chia plants (Table 2). The chlorophyll content decreased as salt levels increased in both mycorrhizal and non-mycorrhizal chia plants. Mycorrhizal chia plants that did not receive salt treatment had the highest chlorophyll content (4.60 mg g⁻¹ FW). The content of carotenoids in mycorrhizal chia plants was higher than that in non-mycorrhizal chia plants. Exposure to high salt levels reduced levels of carotenoids in chia plant leaves. Mycorrhizal chia plants that were treated with 50 mM and 0 mM NaCl (control) had the highest carotenoid content of 8.27 and 8.35 µg g⁻¹ FW, respectively (Table 2).

4. DISCUSSION

The presence of mycorrhizal fungi on plant roots helps the plants to get more Phosphorus nutrients (Diagne et al., 2020). In this study, chia plants inoculated with the arbuscular mycorrhizal fungus, *G. manihotis*, had a higher Phosphorus content than non-mycorrhizal plants (Table 1). The high Phosphorus content in mycorrhizal chia plants enhances the growth rate of the chia plants and their production of antioxidant enzymes to minimize the impact of salt stress in the environment. Phosphorus concentration had a significant

positive correlation with all measured parameters, except malondialdehyde and carotenoid content (Figure 1). This is in line with Wang et al. (2021)'s observation that available P in the soil under salt stress is an important factor determining the effectiveness of arbuscular mycorrhizae in providing an adequate plant resistance.

Salt stress conditions reduced the percentage of root infection of chia plants by the arbuscular mycorrhizal fungus *G. manihotis* (Table 1). The roots of chia plants are usually able to form a symbiotic relationship with arbuscular mycorrhizal fungi, which will greatly influence the physiological and phytochemical attributes of these plants (Younis et al., 2021). The ability of mycorrhizal fungal infections can be influenced by environmental stresses such as high salt levels (Ebrahim & Saleem, 2017). The germination of arbuscular mycorrhizal fungus spores followed by the formation of hyphae decrease salt levels increase in the surrounding environment (Garg & Bhandari, 2016). However, percentage of root infection showed significantly correlated with all measured parameters. Chlorophyll and carotenoids contents decreased levels of salt in the environment increased. However, *G. manihotis* inoculation significantly increased the chlorophyll and carotenoid content of chia plants under salt stress conditions (Table 2). The beneficial effect of mycorrhizae on plant roots is the increased chlorophyll content and high photosynthetic activity (Hashem et al., 2015). Ouzounidou et al. (2015) reported that *Glomus* sp inoculation increased the chlorophyll content of chia plants. Inhibition of photosynthetic reactions occurs in conditions of high salt level due to inhibition of diffusion of chemicals that support photosynthesis through stomata and leaf mesophyll cells (Shin et al., 2020). Likewise, high salt levels in cultivated land results in the accumulation of minerals in the soil resulting in low water potential. The absorption of water carrying minerals will be hampered resulting in a lack of water supply to the leaf mesophyll tissue. Furthermore, such conditions will affect the closing and opening of stomata, which affects the process of plant photosynthesis (Lotfi et al., 2020).

In this study, the effect of salt stress on superoxide dismutase activity in non-mycorrhizal chia plants was lower than that in mycorrhizal plants (Table 1). Shahvali et al. (2020) reported that the application of arbuscular mycorrhizal fungi enhances the antioxidant system rendering them more

tolerant of salt stress originating from the soil where they grow. Sadak et al. (2019) found a correlation between environmental stress and antioxidant enzymes such as superoxide dismutase in cucumber plants, which function to prevent more severe cell damage. According to Porcel et al. (2016) antioxidant compounds such as superoxide dismutase play an important role in reducing free radicals attack.

Catalase activity occurred at the beginning of the symbiosis between *G. mossae* and asparagus plant roots (He et al., 2020). Soybean plants inoculated with *G. intraradices* in sufficient water or dry growing media showed high catalase activity (Etesami & Shafiei, 2020). Similarly, in this study, catalase activity of mycorrhizal chia plants was higher than that of non-mycorrhizal chia plants (Table 1). According to Haque and Matsubara (2018) the response of antioxidant enzymes varies with mycorrhizae, plant species, and stress conditions. In principle, the response of chia plants varies to interaction between the presence of the mycorrhizal fungus *G. manihotis* and salt stress conditions.

Malondialdehyde is a compound produced in lipid peroxidation reactions (Álvarez-Robles et al., 2020). So its presence indicates to what extent the oxidation reaction damages lipids occurring in plant tissues such as those of chia plants. Hu et al. (2020) reported that mycorrhizal plants had lower levels of malondialdehyde than non-mycorrhizal plants. The presence of mycorrhizae reduces oxidative damage to lipids during environmental stress conditions such as high salt levels. This is in line with the results of this study in that the level of malondialdehyde was low because there were mycorrhizae in the chia root system that reduced high salt stress effect (Table 1). The application of mycorrhizae on chia plants is not only at the acclimatization stage but is continued as long as the chia plants grow until harvest time (Sabouri et al., 2021). Thus, inoculation with mycorrhizae can be used as a strategy to reduce the damaging effects of high salt stress. The right combination of arbuscular mycorrhizal fungi species and their host plants can overcome the effects of high salt stress.

5. CONCLUSION

Chia plants showed good performance when inoculated with the arbuscular mycorrhizal fungus *G. manihotis* even under salt stress conditions. The high concentrations of Phosphorus, chlorophyll and carotenoids in chia plants grown under salt stress conditions was due to inoculation with *G. manihotis*. Likewise, under salt stress conditions, the activity of antioxidant enzymes such as superoxide dismutase and catalase were higher in mycorrhizal plants than in non-mycorrhizal plants. Lipid peroxidation indicated by malondialdehyde content in chia plants remained high in the salt treatment and decreased when the plants were inoculated with *G. manihotis*. Inoculation with the fungus *G. manihotis* can increase the tolerance of chia plants because it produces antioxidant enzymes that will be able to resist salt stress.

Declaration of Competing Interest

The authors declare that no competing financial or personal interests that may appear and influence the work reported in this paper.

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