HAYATI
Journal of Biosciences

Vol. 32 No. 3, May 2025 700-711
DOI:10.4308/hjb.32.3.700-711
ISSN: 1978-3019
EISSN: 2086-4094

Research Article

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Soil Symbiosis Reimagined: *Rhizobium* and Mycorrhiza Influence on Soybean Performance in Early Oil Palm Ecosystems

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ARTICLE INFO

Article history:
Received October 29, 2024
Received in revised form January 9, 2025
Accepted January 15, 2025

KEYWORDS:
Mycorrhiza,
palm oil,
rhizobium,
soybean

ABSTRACT

The high demand for soybeans in Indonesia is not in line with national soybean production due to the harvest area decreasing every year. To increase national soybean production, the area of IOP (Immature Oil Plantation) one year has potential for soybean development. This study aimed to investigate the effect of *Rhizobium* spp. and arbuscular mycorrhizal fungi (AMF) inoculation on the physiological capacity, growth, and yield of the soybean plant planted between rows of IOP one-year-old. This research was carried out at PT Perkebunan Nusantara IV (PTPN IV), Adolina Afdeling 1, Serdang Bedagai Regency, North Sumatra, from March 2023 to June 2023. A two-factor Randomized Complete Block Design (RCBD) with four replications for all treatments was conducted in a field experiment. Experimental treatments include: 1) non-inoculated *Rhizobium* spp. + non-inoculated AMF (R0M0) (Control), 2) non-inoculated *Rhizobium* spp. + inoculation AMF (R0M1), 3) inoculation *Rhizobium* spp. + non-inoculated AMF (R1M0) and 4) inoculation *Rhizobium* spp. + inoculation AMF (R1M1). The results showed that the single arbuscular mycorrhizal fungi (AMF) inoculation treatment significantly enhanced soybean physiology, growth, and yield including relative water content (85.87%), nitrate reductase activity (8.98 µmol NO_2-g BS^{-1} $hour^{-1}$), stomatal density (775.16 mm), proline (17.76 µmol proline g BS^{-1}), and ascorbate peroxidase (0.0068 U/min/mg).

1. Introduction

Soybeans are regarded as one of the most important food crops, following rice and corn. As the population grows, the need for soybeans in Indonesia increases every year. Hence, it is necessary to balance this demand by increasing soybean production and productivity. However, the high demand for soybeans to meet people's food needs is not in line with the increase in domestic production. The Ministry of Agriculture estimates that Indonesia's soybean production will continue to decline from 2021 to 2024. In 2021, the projected domestic soybeans production will reach 613.3 thousand tons, down 3.01% from 2020, which reached 632.3 thousand tons. Meanwhile, in 2024, soybeans originating from Indonesia will decrease by 3.12% to 558.3 thousand tons (Kementerian Pertanian 2021).

To increase national soybean production while supporting the government's efforts to increase food crop production and maintain national food security, it is possible to increase the area of soybean harvest by optimizing and utilizing the spaces between rows of IOP (Immature Oil Palm) in the first year (Effendy *et al.* 2020). The extent of IOP plantation in Indonesia spans 2,037,401 hectares (Ditjenbun 2020). In IOP plantation one year old, the potential land area for soybean cultivation is determined by the aisles with width of 7 meters and approximately 5% shading conditions (Marwoto *et al.* 2012).

In addition, the development of the canopy and growth of oil palm plants has not yet reached the optimal phase in the IOP one-year-old. Therefore, the large distance between oil palm and soybeans will continue

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to support the environmental conditions for growing soybeans with adequate sunlight interception and minimal nutrient competition. The selection of soybeans as an intercropping crop in oil palm plantations is based on the fact that soybean plants are included in the legume category can also improve soil fertility, and are still in the same family as LCC (Legume Cover Crop), which has been planted previously on the sidelines of oil palm land.

Efforts to increase soybean production can also be made through optimal fertilization by adding biological fertilizers to agricultural land, such as *Rhizobium* and mycorrhiza. In order to increase the efficiency and uptake of nutrients in soil, biological fertilizer can be added (Herman *et al.* 2012). Mycorrhiza and *Rhizobium* increase plant growth by regulating the nutrient and hormone balance, functioning as growth regulators, dissolving nutrients, and inducing plant resistance to pathogens (Singh *et al.* 2019).

Mycorrhiza is defined as a symbiotic association between the roots of plants and fungi (Dowarah *et al.* 2022). This association involves the infection of the root system of the host plant by the production of a network of external hyphae that grow quickly and penetrate the underground layers, thereby increasing the capacity of the roots to absorb nutrients and water (Nadeem *et al.* 2014). Concurrently, *Rhizobium*, a category of soil microbes, has the capacity to bind free nitrogen from the atmosphere into ammonia (NH_3). This ammonia is then converted into amino acids and subsequently into nitrogen compounds that are indispensable for the growth and development of plants (Igiehon & Babalola 2017).

The capacity of mycorrhiza to discharge exudates into the mycorrhizosphere can serve as a stimulus for soil organisms such as *Rhizobium*. These microbial organisms then utilize the exudate as a substrate, facilitating the conversion of soil organic matter and minerals into available nutrients that plants can absorb. This finding suggests that the incorporation of mycorrhiza in legumes may yield favorable outcomes due to the concurrent tripartite symbiotic association with *Rhizobium*. This association is believed to enhance land fertility and plant productivity (Olivera *et al.* 2004).

Several research findings show that the application of arbuscular mycorrhiza inoculation and *Bradyrhizobium japonicum* provides an increase in shoot growth, dry biomass, AMF root colonization, chlorophyll content, nitrogen uptake, and phosphorus uptake in NRC 37 soybean varieties (Prasad 2021). Additionally, there is a significant increase in the concentration of various macronutrients and micronutrients (Mitra *et al.* 2021), a 169% rise in the number of root nodules, and the amount of fixed N (Yakubu *et al.* 2010). Therefore, this research was directed to examine the physiological capacity, metabolism, growth, and yield of soybeans inoculated with Arbuscular Mycorrhizal Fungi (AMF) - *Rhizobium* spp when they were planted between rows of IOP one-year-old.

2. Materials and Methods

2.1. Plant Materials and Condition

The research utilized one-year-old IOP (Immature Oil Palm) plantations area, Anjasmoro soybean variety, Arbuscular Mycorrhizal Fungi (AMF) inoculum from Sikendang brand, *Rhizobium* spp. inoculum from Legin brand, and Mutiara NPK fertilizer 16:16:16. The oil palm land is located at PT Perkebunan Nusantara IV (PTPN IV), Kebun Adolina Afdeling 1, Serdang Bedagai Regency, North Sumatra, at coordinates of NL: 0478633° and SL: 0370526° with an altitude of 15-130 meters above sea level.

The land class is classified as S3 and falls under the classification of rain-fed land. Throughout the soybean planting period, the total rainfall ranged from 88-175 mm/month (Figure 1), which is classified as too low for soybean cultivation (350-1,100 mm) according to the Ministry of Agriculture No. 79 of 2013. In addition, the air temperature was in the range of 29.3°C-38.9°C (Figure 2), and the average air temperature at the research location during the soybean planting period was 34.8°C, slightly exceeding the optimal soybean growth temperature of 34°C (Sumarno 2016). Average soil moisture in the pod-filling phase ranged from 12.4-26% (Figure 3). Based on the environmental conditions in this research location, it is suspected that drought stress

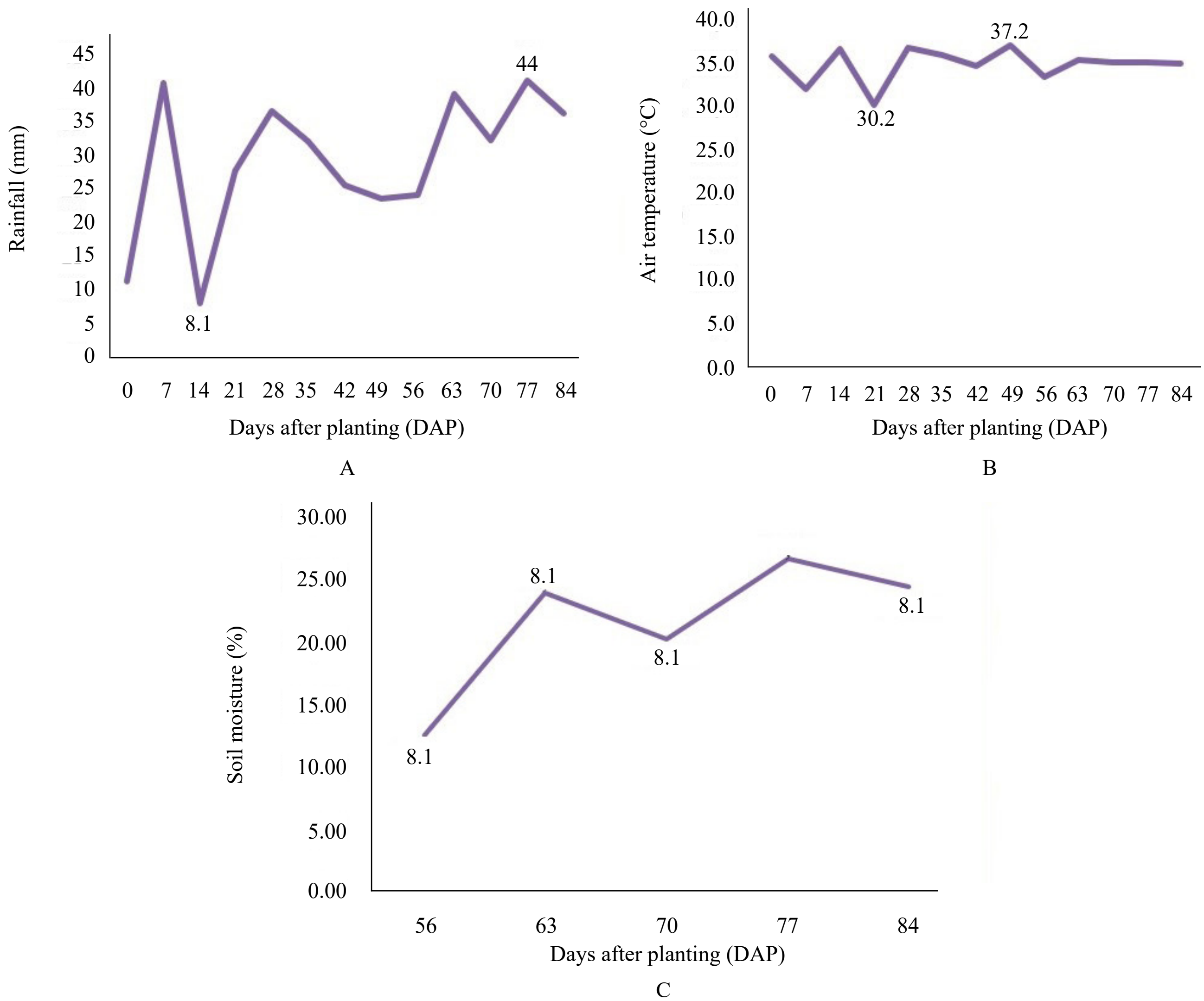

Figure 1. (A) Average rainfall during planting period, (B) average air temperature during planting period, (C) average soil moisture in the pod filling phase

occurred during the soybean planting period. Planting was carried out from March 8th, 2023 to June 8th, 2023.

2.2. Experimental Design

The land area utilized was measured at 45 m × 12 m (540 m^2), maintaining a distance of 2.4 m between soybeans and oil palm plants. Each treatment plot covered an area of 10.5 m × 3 m, with soybean planting spacing set at 30 cm × 20 cm. This research was conducted using a two-factor Randomized Complete Block Design (RCBD) with four replications for all treatments. The first factor is *Rhizobium* isolates, consisting of two levels: without inoculation (R0) and *Rhizobium* inoculation (R1). Similarly, the second factor is mycorrhiza isolates, which consists of two levels: without inoculation (M0) and mycorrhiza inoculation (M1). Overall, there were four treatment combinations: 1) non-inoculated *Rhizobium* spp. + non-inoculated AMF (R0M0) (Control), 2) non-inoculated *Rhizobium* spp. + inoculation AMF (R0M1), 3) inoculation *Rhizobium*

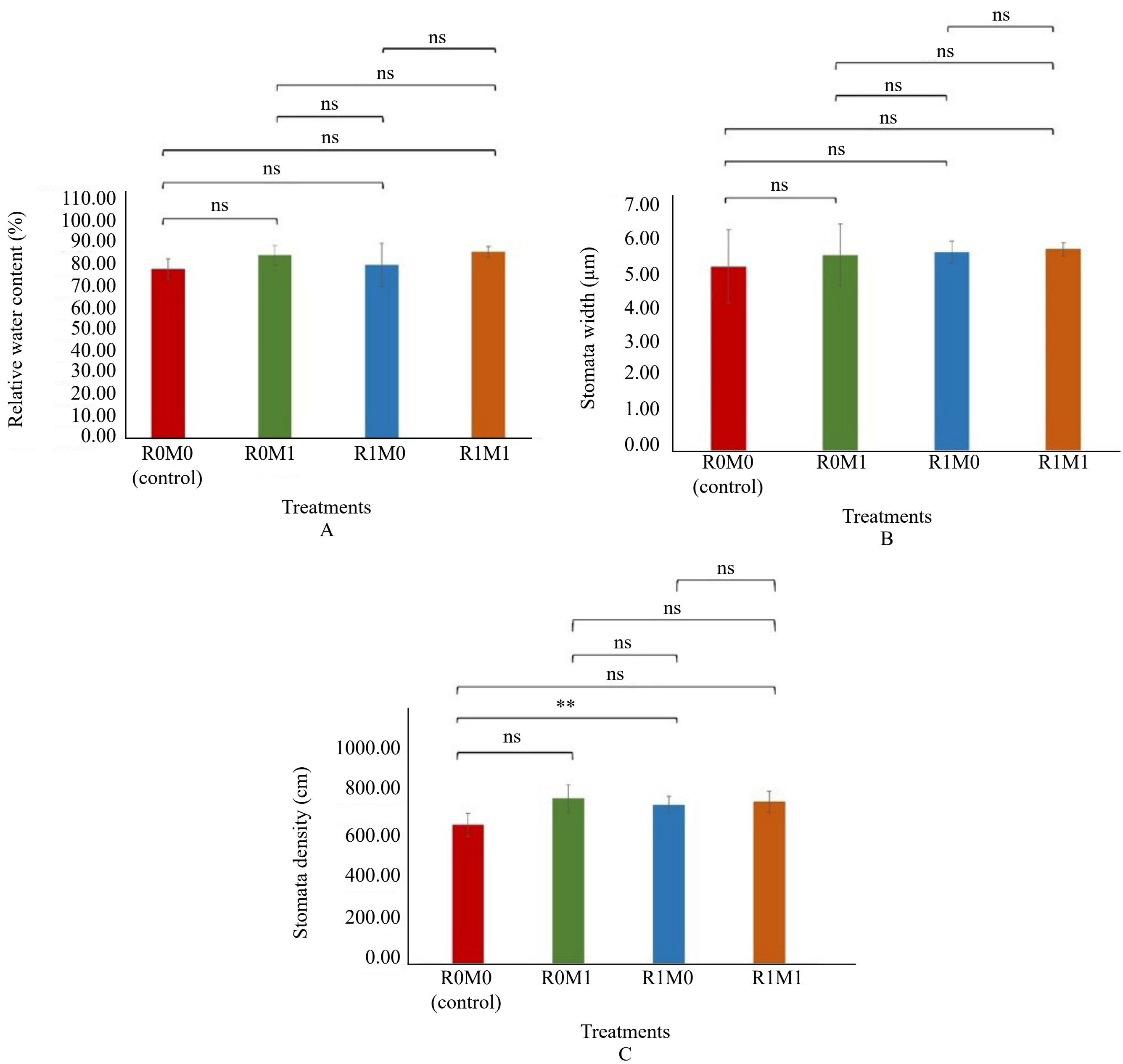

Figure 2. (A) Relative water content, (B) stomata width, (C) stomata density of four treatments. Values are the means of four replicates ±S.E. Bars with the same line are not significantly different (P>0.05) according to LSD's test

spp. + non-inoculated AMF (R1M0) and 4) inoculation *Rhizobium* spp. + AMF (R1M1).

2.3. Soil Fertility Analysis

Soil analysis assessed physical properties (texture and moisture using the YY-1000 N tool) and chemical properties, including total nitrogen (Kjeldahl method), available phosphorus (Bray I method), potassium (HCl 25% extract), organic carbon (Walkley-Black method), Cation Exchange Capacity (CEC), and soil pH (pH meter).

2.4. Plant Physiology Analysis of Soybean

The physiological parameters included stomatal behavior and relative water content (RWC). Stomatal width and density were observed on fully grown, dark green fourth leaves between 7-9 AM (UTC+7) using a microscope at 100x and 40x magnifications, respectively. RWC was calculated using fresh, saturated, and dry leaf weights:

$$RWC = \frac{fw - dw}{sw - dw} \times 100\%$$

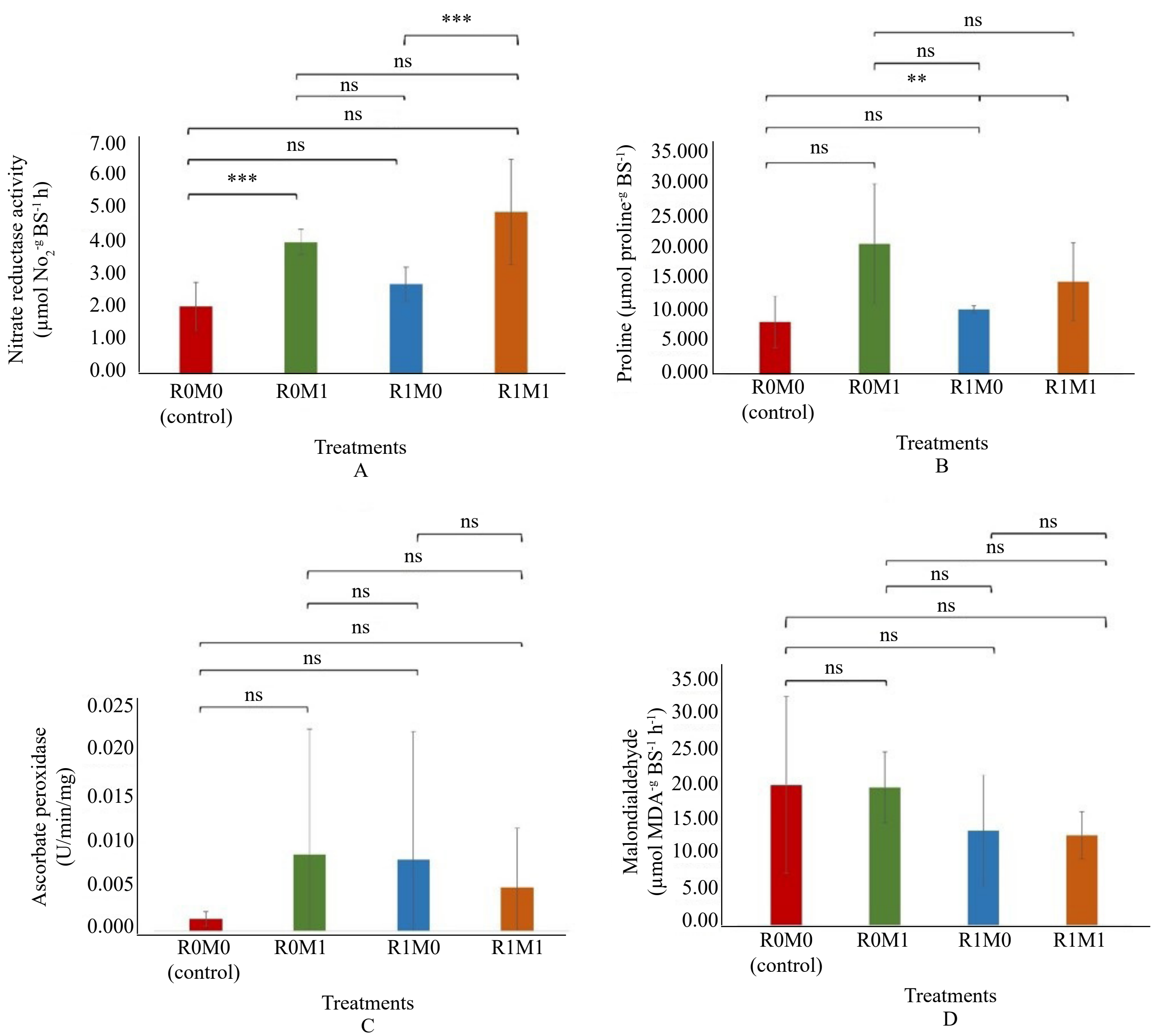

Figure 3. (A) Nitrate reductase activity content, (B) proline content, (C) ascorbate peroxidase content, (D) malondialdehyde content of four treatments. Values are the means of four replicates ±S.E. Bars, of which ns are not significantly different (P>0.05), ** are significantly different (P≤0.01), *** are significantly different (P≤0.001) according to LSD's test

Where:
fw : fresh weight
dw: dry weight
sw : saturated weight

2.5. Biochemical Analysis of Soybean Leaf

The biochemical parameters of soybean plants consist of Malondialdehyde (MDA), Ascorbate Peroxidase (APX), Nitrate Reductase Activity (NRA), and proline.

MDA levels were measured using the thiobarbituric acid (TBA) method. One gram of soybean leaves was ground and mixed with 2 ml of 0.1% trichloroacetic acid (TCA). After centrifuging 1.5 ml of the mixture at 15,000 g for 10 minutes, 0.5 ml of the supernatant was combined with 1.5 ml of 0.5% TBA in 20% TCA. The mixture was incubated at 90°C for 20 minutes, cooled on ice, and centrifuged again at 10,000 g for 5 minutes. The absorbance of the supernatant was measured at 532 nm and 600 nm using a spectrophotometer, with MDA calculated using an absorption coefficient of 155 mM^{-1} cm^{-1}. A 0.1% TCA solution served as the blank.

APX activity was determined using the Nakano and Asada (1981) method. Fresh leaves were ground in a chilled mortar with 50 mM potassium phosphate buffer (PPB). The extract was centrifuged at 4°C for 15 minutes. The reaction mixture, containing PPB, ascorbic acid, H_2O_2, and EDTA, was mixed with the enzyme extract to initiate the reaction. Absorbance at 290 nm was recorded at 15-second intervals for 60 seconds using a UV spectrophotometer.

For NRA measurement, 200 mg of leaf slices (1 mm thick) were incubated in 5 ml of 0.1 M phosphate buffer (pH 7.5) for 25 hours in the dark. After incubation, 0.1 ml of the solution was mixed with sulfanyl amide and N-naphthyl ethylene diamide solutions, turning pink. The total volume was adjusted to 3 ml with distilled water, and absorbance was recorded at 540 nm with a spectrophotometer.

Proline content was measured using the method of Bates *et al.* (1973). Half a gram of leaves was ground in 10 ml of 3% sulfosalicylic acid, filtered, and mixed with a ninhydrin solution containing phosphoric acid and distilled water. The mixture was heated at 100°C for an hour and then extracted with toluene. The upper layer containing proline was collected, and its absorbance was measured at 520 nm using a spectrophotometer.

2.6. Nutrient Uptake Analysis in Soybean Leaf Tissue

The nutrients analyzed consist of macronutrients, including N (Nitrogen), P (Phosphorus), and K (Potassium). Analysis of N in plant tissue was carried out using the Kjeldahl method with H_2SO_4 and H_2O_2, which were then distilled (Sulaeman *et al.* 2005). Meanwhile, the P plant tissue analysis was carried out using the Kjeldahl method, which is like N analysis. Still, it was then extracted employing the Bray method and measured with a spectrophotometer at a wavelength of 693 nm. For K plant tissue analysis, the destruction stage was identical to that of P plant tissue analysis. Subsequently, the diluted destruction solution is read with a flamephotometer.

2.7. Hormone Analysis in Soybean Leaf Tissue

The plant hormones analyzed were the auxin (IAA) and gibberellin hormones. Analysis of the IAA (auxin) hormone involved a combination of methods, including extraction method (Fu *et al.* 2011) and spectrophotometric methods with Salkowski reagent (Patten and Glick 2022) for measuring hormone levels. Meanwhile, gibberellin analysis was conducted following the method outlined by Barendse *et al.* (1987), with gibberellin compounds being read using a UV-VIS detector at a wavelength of 254 nm.

2.8. Statistical Analysis

The data obtained were analyzed for variance (ANOVA) and continued with the LSD test at a 5% level if the ANOVA results showed significant differences between treatments. The closeness of the relationship between observation variables was determined through correlation analysis. Both analyses were carried out through Rstudio 4.3.0. The observation parameters with the greatest contribution to the results were determined through path analysis using Jamovi 2.3.21.

3. Results

3.1. Soil Fertility Characteristics

The results of soil analysis after the research showed an improvement in several soil chemical properties such as C-organic, total N, P available, K available, and soil CEC, even though it was still in the low category (Table 1).

3.2. Effect of *Rhizobium* and Mycorrhiza on Soybean Physiology

Figure 2 shows the influence of *Rhizobium* spp. and Arbuscular Mycorrhizal Fungi (AMF) on the physiological components of soybean plants. *Rhizobium* spp. and AMF treatments did not show significantly different ($P>0.05$) in the relative water content and stomata width of soybean plants. Meanwhile, the *Rhizobium* spp. inoculation + non-inoculated AMF (R1M0) treatment showed significant results on the stomata density of soybean plants and was significantly different from the control (R0M0). This indicates that the higher stomata density, which is influenced by *Rhizobium* spp., can increase the water absorption process in soybean plants.

3.3. Effect of *Rhizobium* and Mycorrhiza on Soybean Biochemistry

The prediction of the occurrence of drought stress at the research location is strengthened by several enzyme activities in plants that are directly related to when plants are under drought stress conditions. The result shows that *Rhizobium* spp. and AMF did not show significantly different ($P>0.05$) in Ascorbate Peroxidase (APX) and Malondialdehyde (MDA). While Nitrate Reductase Activity shows significantly different ($P\leq0.001$) between non-inoculated *Rhizobium* spp. + AMF inoculation (R0M1) treatment with control (R0M0), and inoculation *Rhizobium* spp. + AMF (R1M1) with inoculation *Rhizobium* spp. + non-inoculated AMF (R1M0). In addition, *Rhizobium* spp.

inoculation + AMF inoculation (R1M1) significantly different ($P \leq 0.01$) with R0M0, and R1M0 treatment on proline.

3.4. Nutrient Uptake of N, P, and K in Soybean Leaf Tissue

Nutrient analysis in plant tissue aims to determine the status of nutrients absorbed by plants. Although the results of soil analysis show an increase in the availability of total N (low), P (very high), and K (very high) in the soil after the research (Table 1), the nutrient content contained in the tissue still has not reached the level sufficient to support maximum growth. The absorption of N, P, and K nutrients in plant tissue was hampered due to the rainfall and very low soil moisture at the research site (Figure 4).

3.5. Auxin and Gibberellin Content in Soybean Leaves

Stimulation of plant growth and acquisition of nutrients by beneficial microbes correlates with the biosynthesis of growth regulators that greatly influence plant metabolism. The result showed that *Rhizobium* spp. inoculation and AMF inoculation (R1M1) treatment increased levels of the hormone auxin (13.24%) and gibberellin (5.48%) in soybean plant tissue compared to other treatments (Figure 5). This indicates that the *Rhizobium* spp. and AMF

Table 1. The analysis results of soil nutrient content before and after research

Variables	0 WAP	12 WAP			
		R0M0 (control)	R0M1	R1M0	R1M1
Sand (%)	51	53	57	51	49
Dust (%)	33	32	32	36	40
Clay (%)	16	15	11	13	11
H2O pH	5.5 S	4.8 S	6.2 SS	5.4 S	5.5 S
KCl pH	4.1 N	4.1 N	5.6 N	4.2 N	4.5 N
C-Organic (%)	0.95 VL	1.64 L	1.85 L	1.04 L	1.29 L
C/N	10 M	9 M	10 M	7 L	8 M
N total (%)	0.10 L	0.19 L	0.19 L	0.14 L	0.16 L
P total (%)	0.060 M	0.040 M	0.040 M	0.050 M	0.040 M
P available (ppm)	36.58 VH	58.52 VH	40.48 VH	62.93 VH	71.32 VH
K available (m.e/100 g)	0.06 L	0.99 VH	3.46 VH	0.93 VH	0.91 VH
Cation exchange capacity (CEC) (m.e/100 g)	8.36 L	13.65 L	3.15 VL	9.55 L	10.72 L

The value is based on the soil chemical properties assessment criteria (Hardjowigeno 1995), which includes L: low, VL: very low, M: medium, H: high, VH: very high, VS: very sour, S: sour, SS: slightly sour, N: neutral, SA: slightly alkaline, A: alkaline. (WAP: weeks after planting). R0M0: non-inoculated *Rhizobium* spp. + non-inoculated AMF, R0M1: non-inoculated *Rhizobium* spp. + AMF inoculation, R1M0: inoculation *Rhizobium* spp. + non-inoculated AMF, R1M1: inoculation *Rhizobium* spp. + AMF

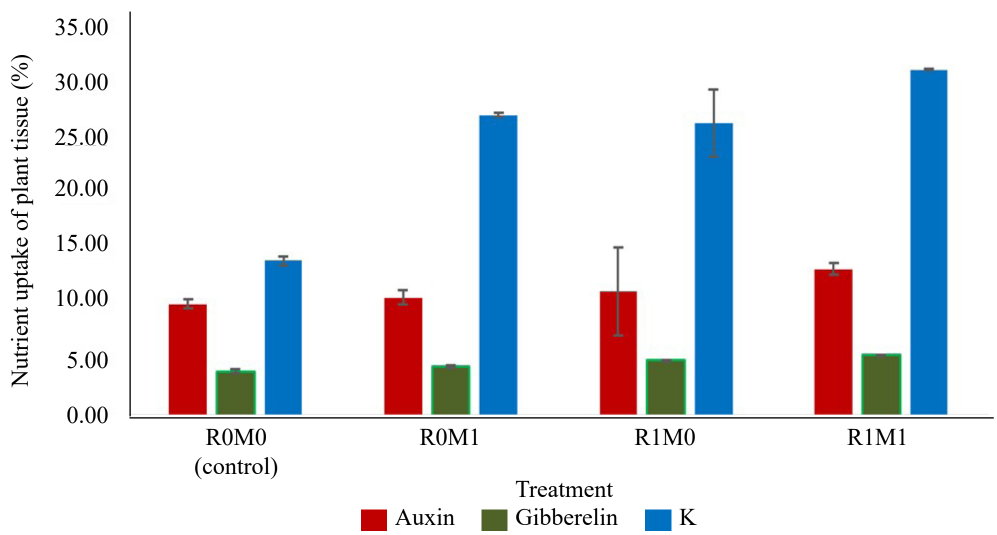

Figure 4. Nutrient of N, P, and K uptake of four treatments. Values are the means of four replicates ±S.E. Bars

support plant growth through the production of the phytohormones auxin and gibberellin.

3.6. Correlation Analysis

The results of correlation analysis revealed that there were positive and negative correlations between components of growth, physiology, biochemistry, and yield of soybean plants. Analysis shows that plant height is positively correlated to the number of leaves (0.70**), NRA and proline are positively correlated to seed weight per plant (0.59*, 0.69**), and leaf area is positively correlated to the number of pods per plant (0.74***) and APX (0.59*) (Figure 6). This suggests that an increase in plant height will affect the number of leaves, followed by an increase in plant leaf area, and will affect soybean yields.

3.7. Path Analysis

Based on the results of the analysis, it is known that not all parameters have significant correlation values that provide a high direct effect. Path analysis revealed that leaf area and nitrate reductase activity contributed positively to production. Leaf area was the parameter that had the highest direct effect (0.59), followed by nitrate reductase activity (NRA) (0.40). Meanwhile, proline (-0.08) and weight per 100 seeds (-0.05) had a direct negative effect and were regarded as low (Figure 7).

4. Discussion

Soybean growth and development are significantly influenced by environmental factors. In the research area, insufficient water availability in the soil due to low rainfall, high light intensity, and high air temperatures results in dehydration of the plants during the growing period. This condition leads to soybean plants experiencing drought-stressed conditions. Numerous studies have demonstrated that the inoculation of Rhizobium enhances nodulation, nitrogen assimilation, and the yield of legume plants (Htwe *et al.* 2016). However, in this study, it is hypothesized that the presence of drought stress and an acidic soil pH hinders the active role of *Rhizobium* spp., thereby impeding the formation of root nodules. Drought stress has been shown to reduce nodule growth and function (Kung 1971), consequently inhibiting N fixation (Wilmowicz *et al.* 2020). Other studies have demonstrated that the formation of root nodules is subject to change, with a 55% decrease observed in drought conditions (Prudent *et al.* 2015).

A study by Yu et al (2015) demonstrated that soil moisture levels have a direct impact on evapotranspiration and stomatal conductance, with a concomitant increase in canopy temperature. However, the present study did not yield significant findings regarding stomata width, relative water content (Figure 2), and nutrient uptake in

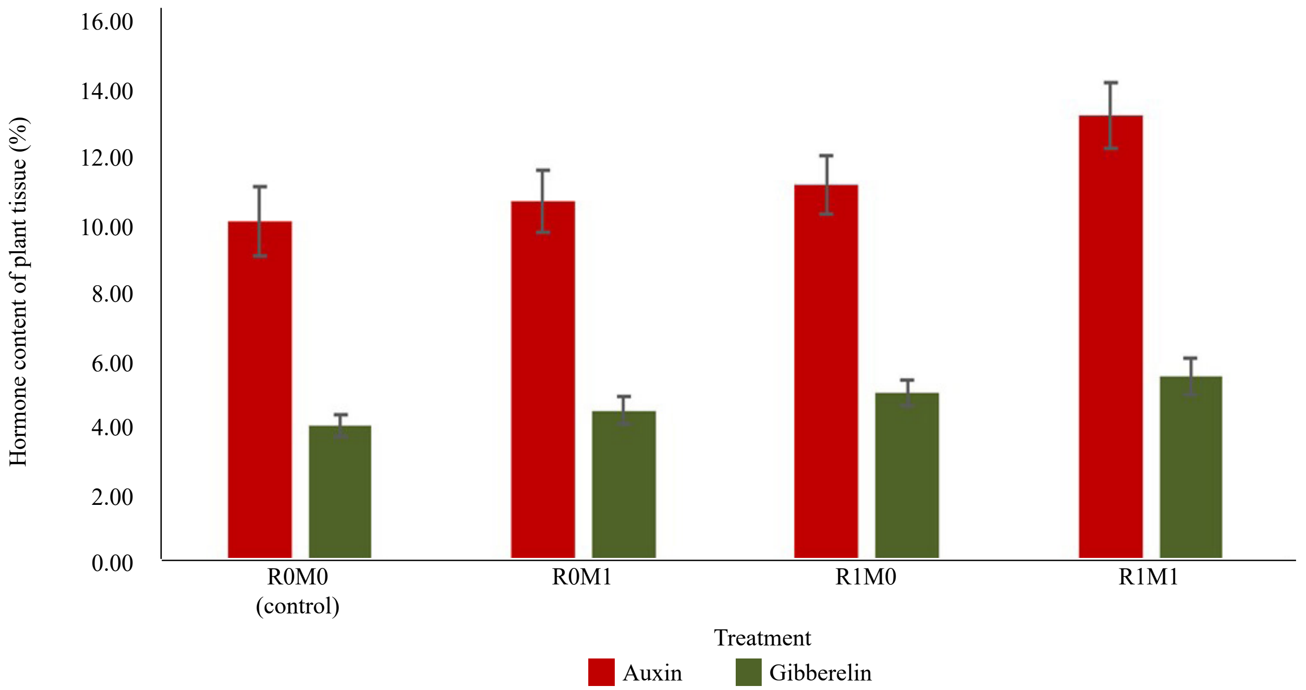

Figure 5. Auxin and gibberellin content of four treatments. Values are the means of four replicates ± S.E. Bars

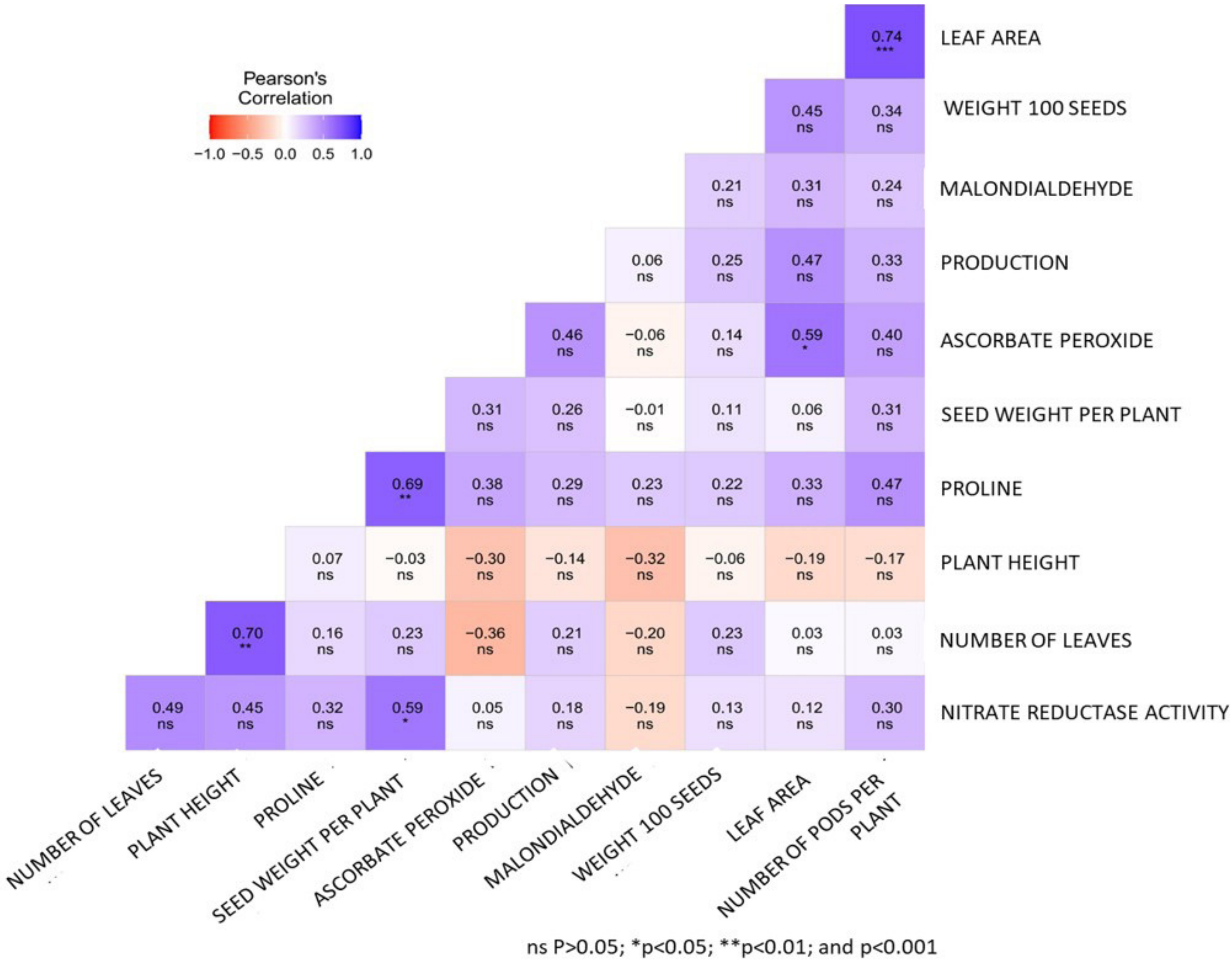

Figure 6. Correlation analysis between growth, physiology, biochemistry, and yield of soybean plants

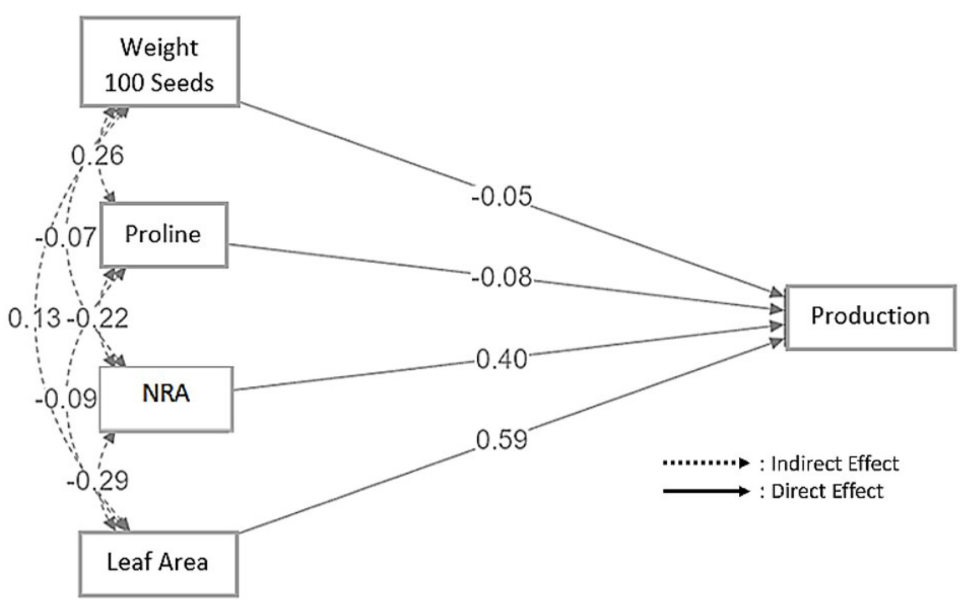

Figure 7. Path analysis diagram of the relationship and effect between physiology and growth on soybean yield (NRA: nitrate reductase activity)

soybean plant tissue (Figure 4). This finding suggests that *Rhizobium* spp. and Arbuscular Mycorrhizal Fungi (AMF) have not been effective in enhancing stomatal performance or nutrient uptake under drought conditions in this study. However, in terms of enhancing soil fertility, *Rhizobium* spp. and AMF have demonstrated the capacity to elevate soil chemical properties, including N-total, P-available, and K-available, although these increases have not been statistically significant (Table 1). The enhancement of mineral elements in the soil is attributable to the secretion of phosphate or organic acids by AMF and *Rhizobium* spp., which can dissolve or chelate primary mineral ions, thereby releasing elements such as K and P into the soil thus increasing the availability of K and P (Priyadharsini & Mut Hukmar 2016). The increase in N-total is influenced by *Rhizobium* (Meitasari & Wicaksono 2017).

Moreover, soybean-inoculated *Rhizobium* spp. and AMF increased the concentration of IAA and gibberellin in plant tissue (Figure 5). This indicates that *Rhizobium* spp. and AMF help soybean plants grow in drought-stress conditions through the production of phytohormones. (Figure 5). IAA concentrations also increased in research by Da *et al.* (2019). The secretion of Indole Acetic Acid (IAA) helps plants to increase cell elongation, thus increasing root growth and encouraging the development of lateral roots, so it has a positive effect in increasing water and nutrient absorption (Inbaraj 2021), while gibberellins play a role in determining plant source-sink relationships, expansion leaves and promotes root hair growth (Mishra *et al.* 2019).

Proline is an amino acid produced by plants as an osmotic protector and prevents water loss in plants under drought conditions. Accumulation of proline in plants subjected to microbially-induced stress has been shown to decrease the osmotic potential of plant cells, thereby increasing water absorption and, consequently, maintaining osmotic balance (Ortiz *et al.* 2015). AMF inoculation can induce proline accumulation so it can support the growth of soybean plants experiencing drought stress in this research (Figure 3). Mycorrhiza upregulates biosynthetic enzymes involved in proline synthesis. Proline accumulation protects plant proteins and membranes from damage due to excess levels of ROS. Osmolytes contribute to maintaining tissue water content in conditions of water deficit (Abeer and Salwa 2016).

Proline influences nitrogen metabolism, which is necessary for stress recovery. Increasing proline in soybeans was followed by increases in nitrate reductase activity (Figure 3). These results show that the ability of AMF facilitated plant nitrate absorption. Other research revealed that plants inoculated with AMF showed better activity of nitrogen metabolism enzymes, resulting in increased amino acid synthesis (Ahanger *et al.* 2014).

In addition, the accumulation of MDA (Malondialdehyde) in plant tissue reflects the level of oxidative stress damage to cell membranes. Although there were no significant results, plants inoculated with microbes reduced the MDA in soybeans. Other research also shows that plants inoculated with mycorrhiza under drought stress conditions reduce MDA values in soybean plants (Bressano *et al.* 2010). This indicates that AMF can reduce cell damage on soybean plants due to drought stress.

Plants have a defense system that produces antioxidant enzymes such as Ascorbate Peroxide (APX) to reduce oxidative damage caused by drought stress. Inoculated soybeans with AMF showed a higher APX activity, although it was not significantly different from other treatments in this research. Mycorrhiza can increase enzymatic antioxidant activity, thereby reducing free radicals and increasing plant resistance to drought stress in Sesamum indicum plants (Aalipourm *et al.* 2020). Chandra *et al.* (2019) also showed that the application of PGPR has a higher activity of antioxidant enzymes (GPX, CAT, SOD, and APX) in wheat plants under drought-stress conditions.

Based on the results, it can be implied that the combined inoculation of AMF and *Rhizobium* spp. has the potential to increase the physiological capacity of soybean plants, such as proline content, nitrate reductase activity, IAA, and gibberellin hormones that support plant growth. However, the effect on stomatal growth, relative water content, and antioxidant enzyme activity has not been significant. Nevertheless, the use of AMF and *Rhizobium* spp. can help soybean plants overcome drought stress and increase the efficiency of nitrogen metabolism, thus potentially supporting soybean productivity in suboptimal lands such as immature oil palm plantations.

Acknowledgements

The author expresses deep gratitude to PT Perkebunan Nusantara IV for the opportunity to use plantation land and plants as a research site, and also thank you to Mr. Dennis Nichova, S.P., for giving me valuable suggestions and contributions during the planning and development of this research work.

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