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# Growth and edamame soybean (*Glycine max* L. *Merr*.) production inoculated with mycorrhiza and nano-inoculant indigenous *Rhizobium* sp. at different watering frequency

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**Abstract** The present work of inoculation of edamame soybean using the nano formula of mycorrhizae-*Rhizobium* sp at different watering frequency demonstrated the interaction between inoculum (mycorrhiza-*Rhizobium* sp) and the watering frequency at once-in-6 day-period. The interaction between the inoculum and watering frequency was observed on the mycorrhiza colonization, root volume, fresh root weight, and shoot dry weight. It was also observed, however, that the nano formula of mycorrhiza-*Rhizobium* sp. resulted in the higher soybean yield compared to other treatments, i.e. crude mycorrhiza, and the mixture of crude mycorrhiza-*Rhizobium* sp. and nano formula of *Rhizobium* indigenous. In addition, the watering frequency at once-in-6 day-period did not result in significant soybean yield compared to once-in-4-day period but it showed significant difference with the once-in-2-day period.

**Keywords:** Nano formula, Watering frequency, Edamame soybean, Mycorrhiza, *Rhizobium* sp.

## Introduction

The main market for Indonesian edamame soybeans export is still held by Japan, with a high demand of 75,000 tons per year. However, Indonesia can only send 5,000 tons of frozen edamame beans in 2019, which equals 6.67% of total edamame soybeans demand (Indonesian Ministry of Agriculture, 2020). According to (Sudiarti, 2017), the productivity of edamame soybeans can reach 10-12 tons/ha, but Indonesia can only produce 8.8 tons/ha. The vast gap indicates that optimization to increase the yield of edamame soybeans in Indonesia is crucial. The average productivity of edamame soybeans was 3.5 tons/ha higher than local soybeans (1.7-3.2 tons/ha) (Sudiarti and Hasbiyati, 2018). Nevertheless, Indonesia still had lower edamame soybeans productivity than the target.

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Originating from Japan, the edamame soybean is a variety of soybeans with large pods and high protein content harvested early (Santi *et al.*, 2019; Setiawati *et al.*, 2018). Due to the high protein content (Samruan *et al.*, 2012), edamame soybeans require more nutrient absorption, up to three to four times more than local soybeans. The required fertilizer by local soybeans is 50 kg/ha of Urea, 75 kg/ha of triple superphosphate, and 100 kg/ha of KCI. Meanwhile, edamame needs 200 kg/ha of Urea, 200 kg/h of SP-36, and 150 kg/ha of KCl fertilizers (Setiawati *et al.*, 2018). Excessive application of chemical fertilizers can lead to various issues, including reduced land productivity and damage to soil ecosystems. Therefore, it is possible to enhance the productivity of edamame soybeans by implementing innovative cultivation techniques on existing agricultural land. One of the techniques that has a high potential is utilizing inoculation of mycorrhiza and *Rhizobium* sp. bacteria as biofertilizers. Nusantara *et al.* (2019) stated that various biofertilizers used in dry land in Indonesia lead to higher nutrient availability and land productivity of different crops to 20 – 100%.

Vesicular-arbuscular mycorrhiza (VAM) is a fungus that can interact symbiotically with the root of a plant and increase nutrient absorption (especially phosphate), improve plant durability from drought, produce growth hormone, protect the plants from pathogens and toxic compounds in the root, enhance the surface area for water and nutrient intake from soils as well as distributing them to all parts of a plant (Campbell *et al*., 1999). VAM can interact with *Rhizobium* sp. synergically to produce root nodules, enhance nutrient absorption, improve growth, and produce a better yield (Diagne *et al*., 2020). A study by Astuti *et al.* (2016) found that inoculation of *Rhizobium* sp. and mycorrhiza in soybeans can improve nodule weight and enhance the growth of root, leaves area, and pod weight per plant in a drought-stressed land. According to research by (Sarawa *et al*., 2014), watering twice a day can result in better growth of soybeans compared to watering frequency of 4, 6, and 8 times per day.

The *Rhizobium* sp lives inside the soil and interacts with the roots of legumes. The bacteria play a crucial function in promoting plant growth, particularly in addressing the issue of nitrogen availability for the host plant. It can provide a substantial amount of nitrogen, ranging from 100-300 kg N/ha, which can fulfil around 75-80% of the Nitrogen requirements in plants (Masson-Boivin and Sachs, 2018). According to (Sari *et al*., 2015), the application of *Rhizobium* sp. inoculum at a rate of 5 g/kg of soybean seeds yielded the most favourable outcomes in terms of dry weight of root nodules, number of root nodules, plant height, number of leaves, and leaf area. A similar study by (Pattipeilohy and Sopacua, 2014) reported that the administration of 7 g/kg seeds of *Rhizobium* sp. inoculum on soybeans significantly impacts plant height, leaf and root count.

The effectivity of *Rhizobium* sp. in maintaining nitrogen is controlled by various aspects, including compatibility and the choice of the carrier material (Wang *et al*., 2018). Previous research by Astuti *et al*. (2021) found indigenous *Rhizobium* sp. isolates compatible with edamame soybeans. However, after practical application in the field, the effectiveness was lacking. The carrier formula of biofertilizer is a significant factor in determining the success of its use, as it plays a vital role in preserving the viability and efficacy of the bacteria it contains. The carrier material must have a small particle size to bind to soybean seeds when inoculated effectively and completely to cover the seeds, as seen in nanoparticle materials. The nanoparticles offer the benefits of being able to permeate intercellular regions penetrated by colloidal particles. Furthermore, flexible nanoparticles can be integrated with a variety of technologies (Buzea *et al*., 2007).

A study by Astuti and Matara (2022) discussed the inoculation of nano-formula *Rhizobium* sp. to edamame soybeans, which can hasten flowering time and improve yields. When the carrier formula of 90% Nano peat + 5% Nano Biochar + 5% Nano bone was used, the highest yield was obtained (10.28 tons/ha) with a 2.3% increase as compared to the control. Nevertheless, the proportion of empty pods remains higher, demanding the addition of a biological fertilizer that enhances phosphate's solubility and water availability during pod filling. It is hypothesized that an interaction between mycorrhiza and nano-formula indigenous *Rhizobium* sp., as well as the watering frequency, can influence the efficacy, growth, and yield of edamame soybean plants.

This study aimed to investigate the interaction impact of watering frequency on the efficacy of Mycorrhiza and *Rhizobium* sp inoculation, to develop an indigenous nano formula for edamame soybeans, and determine the optimal watering frequency and most efficient inoculum for enhancing the growth and productivity of edamame soybeans.

## Materials and methods

### *Experimental design*

The research outline was a 3x3 factorial experiment in completely randomized design. The first factor was inoculum types i.e.: (1) mycorrhiza crude obtained by trapping using corn plant from Gunung Kidul, (2) nano-formula of *Rhizobium* sp and, (3) combination of crude mycorrhiza and nano-formula of *Rhizobium* sp. The second factor was watering frequency, i.e.: (1) once-in-2-day, (2) once-in-4-day, and (3) once-in-6-day period.

### *Preparation of indigenous Rhizobium sp. inoculum*

The indigenous *Rhizobium* sp. isolates (B, E, and F isolates) were obtained from the collection in the Agrobiotechnology laboratory (Faculty of Agriculture, Universitas Muhammadiyah Yogyakarta, Indonesia). Each isolate was cultured in the Yeast Mannitol (YMC) broth and placed in a shaker for 48 hours. The isolates were characterized and counted for total microbial count using a total plate count (TPC) method on Yeast Mannitol Agar (YMA) media in 10-fold serial dilutions from $10^{-7}$ to $10^{-9}$ after 48 hours of incubation (Sutton, 2011). Then, 20% indigenous *Rhizobium* sp. inoculum was mixed with the carrier media from 90% Nano Peat + 5% Nano Biochar + 5% Nano Bone.

### *Preparation of mycorrhiza inoculum*

A mycorrhizal culture was produced using the trapping method in a pot culture on corn as a cultivated host for two months. Then, a period of stressing for a month was done to stimulate the formation of spores. The mycorrhizal replication results were assessed for colonization percentage using the Kormanik & McGraw method, with a threshold of above 80%. Additionally, the number of spores was determined using the approximation and wet filtration methodology, yielding a range of 50-60 spores per 100 grams of soil (Selvakumar *et al.*, 2016).

### *The planting of edamame soybeans*

To prepare the planting media, 10 kg of planting media was put in a polybag and added with 20 grams of mycorrhizal inoculum and a combination of 40 g manure and 1.5 g SP-36 fertilizer. The polybag was stored for one week. Then, 5 g of nano-formula indigenous *Rhizobium* sp. was mixed with 100 g of edamame soybeans seeds. Two seeds of treated edamame soybeans were put into each polybag. Watering was conducted per the treatment at intervals of 2, 4, and 6 days. Maintenance involves the regulation of pests, diseases, and weeds. The edamame soybeans were harvested between 63 and 68 days after planting.

### *Observed parameters*

The parameters that were observed in this study include the development of mycorrhizal fungi (vesicular-arbuscular mycorrhiza colonization and number of spores), root nodules (number of root nodules, nodule diameter, and nodule fresh weight), root attributes (root volume and root fresh weight), shoot development (shoot dry weight), and the yield of edamame soybeans (number of pods per plant, percentage of filled pods, fresh weight of soybeans per plant, and pod yield per unit area). A colony counter and light microscope were used to analyze bacterial viability. The culture media Yeast Mannitol Agar (YMA) + 1% congo red and mycorrhiza staining (KOH, HCl and acid fuchsin) was used to observe the mycorrhizal colonization.

### *Statistical analysis*

The presented study was analyzed using an ANOVA test with a 5% significance level. A post-hoc test with significantly different results was done using Duncan's Multiple Range Test to determine which treatments differed significantly. The plots were generated using R (4.1.3) in the R-Studio interface (version 2023.12.0) with the ggplot2 and cowplot packages.

## Results

### *The development of mycorrhizal colonization and spores with*

Result showed the relation between mycorrhizal colonization with *Rhizobium* sp. nano-formula after different watering frequency. In the treatment of mycorrhiza-indigenous *Rhizobium* sp. inoculum with six watering times a day, the highest colonization was obtained after five weeks (63.33 ± 7.26 %) (Figure1). However, samples treated with only mycorrhiza and watered once every six days showed no significant difference from those treated with the combination. The inoculum of mycorrhiza and indigenous *Rhizobium* sp. resulted in mycorrhizal colonization in a lower percentage, which might be related to mycorrhizal spores in the soil.

The number of produced nodes in different inoculum treatments and watering frequency can be seen in Figure 2. Although there was no interaction between watering frequency and type of inoculum, different inoculum types and watering frequencies showed significant differences. The most spores produced were seen in the treatment of mycorrhiza and indigenous *Rhizobium* sp. (46.89 ± 4.96 spores/100 g soil). The most frequent watering frequency displayed the highest produced spores with 41.89 ± 11.36 spores/100 g soil). Based on the response of edamame soybeans towards the number of spores and colonization percentage, a symbiotic relationship between edamame soybean and mycorrhizal can increase the spores and affect the plant's rhizosphere.

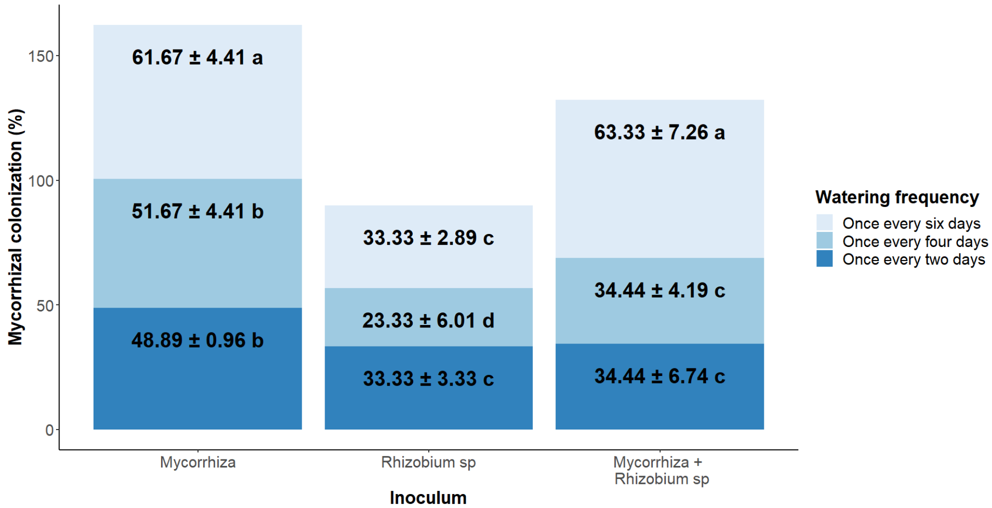


**Figure 1.** Mycorrhizal colonization on edamame soybeans with different inoculum and watering frequencies after five weeks (%): The same order and letter(s) indicated no significant difference between the mean values.

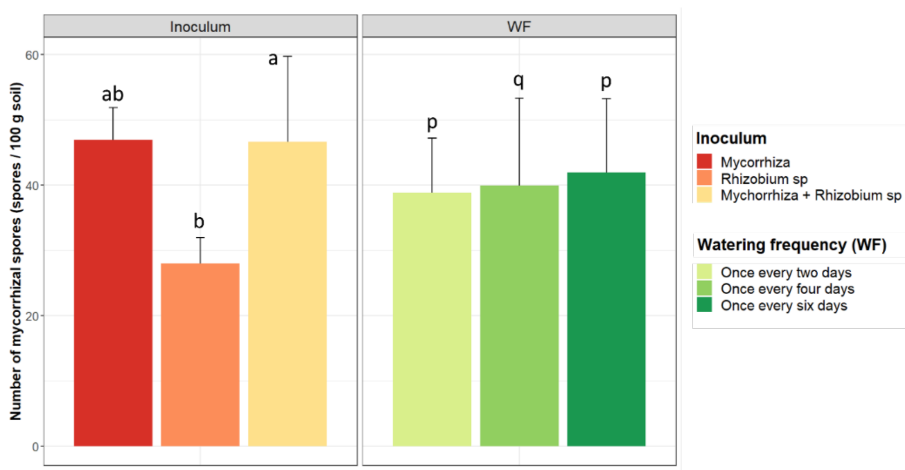


**Figure 2.** Number of mycorrhizal spores on edamame soybeans with different inoculum and watering frequencies after five weeks: The same order and letter(s) indicate no significant difference between the mean values.

***The effect of different inoculum and watering frequency on root nodules development of edamame soybeans***

The interaction between the type of inoculum and watering frequency on the number of nodules, nodule diameter, and fresh weight of the nodules is shown in Figure 3.

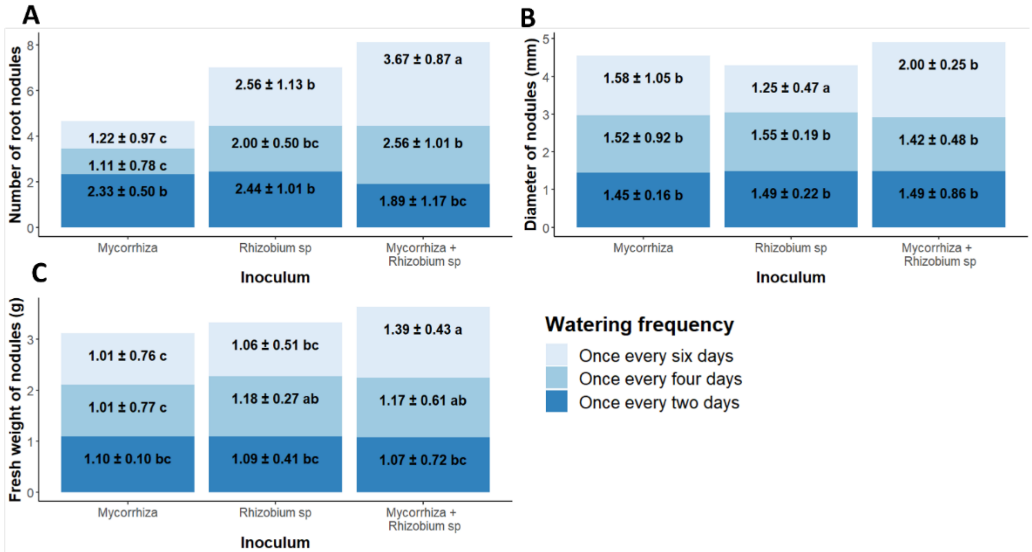


**Figure 3.** Number of root nodules(A), diameter of nodules in mm(B), fresh weight of nodules on edamame soybeans with different inoculum and watering frequencies after five weeks(C): The same order and letter(s) indicated no significant difference between the mean values.

Treatment of mycorrhiza and indigenous *Rhizobium* sp. with a watering frequency of once every six days produced the most nodules (3.67 ± 0.87 nodules) (Figure 3A) with the largest diameter of 2.00 ± 0.25 mm (Figure 3B) and highest fresh weight nodule of 1.39 ± 0.43 g (Figure 3C) as compared to single inoculum by mycorrhiza or indigenous *Rhizobium* sp. separately, even with less frequent watering. This result is supported by the significant colonization activity and spore production observed in enhanced nodulation stimulation of samples treated with mycorrhiza and indigenous *Rhizobium* sp. inoculum.

***Development of edamame soybean plants after different inoculum and watering frequency***

An interaction between the type of inoculum and watering frequency to the volume and fresh weight of roots in edamame plants were shown in Figure 4.

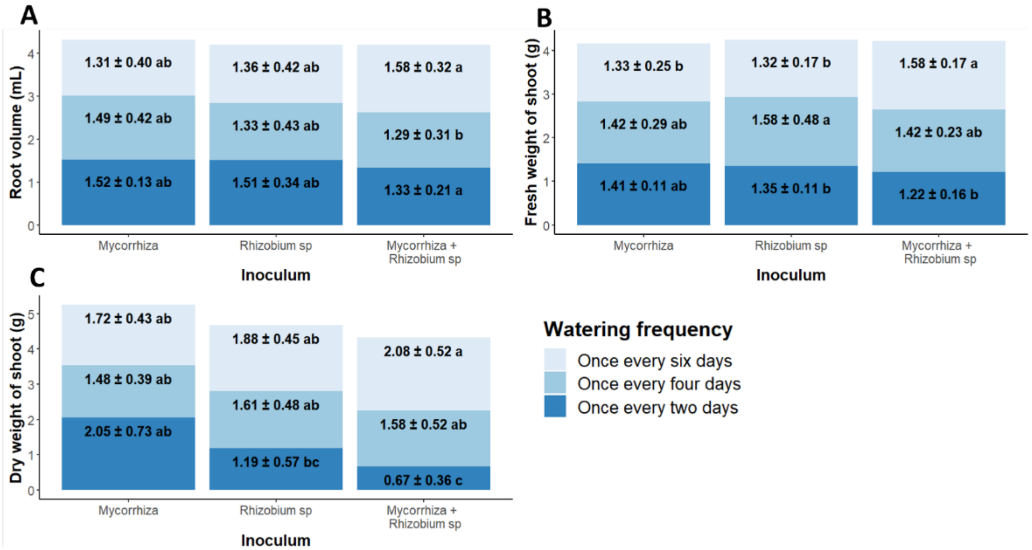


**Figure 4.** Volume of root in mL(A), fresh weight of shoot in gram, (B) dry weight of shoot in gram on edamame soybeans with different inoculum and watering frequencies after five weeks(C): The same order and letter(s) indicated no significant difference between the mean values.

When inoculum consisting of mycorrhiza and indigenous *Rhizobium* sp. with once per six days of watering frequency was applied, it resulted in the highest root volume of 1.58 ± 0.32 mL (Figure 4A) and heaviest fresh weight of 1.58 ± 0.17 g (Figure 4B). The combination inoculum leaded to more mycorrhiza, stimulating root branching and higher root volume. Additionally, the same treatment showed more root nodulation formation with a higher diameter, resulting in the nodules' heavier fresh weight. The inoculation with mycorrhiza and indigenous *Rhizobium* sp. Improved the root and weight of the roots.

***The effect of different inoculum and watering frequency on pod production of edamame soybeans***

The product of photosynthesis, or photosynthates, by soybean plants are collected in the pods containing macronutrients (carbohydrates, lipids, and proteins) and micronutrients (minerals and vitamins). However, this study found no interaction between the type of inoculum and watering frequency, as well as the different types of inoculums (Figure 5). On the contrary, various watering frequencies showed significantly different yield pods.

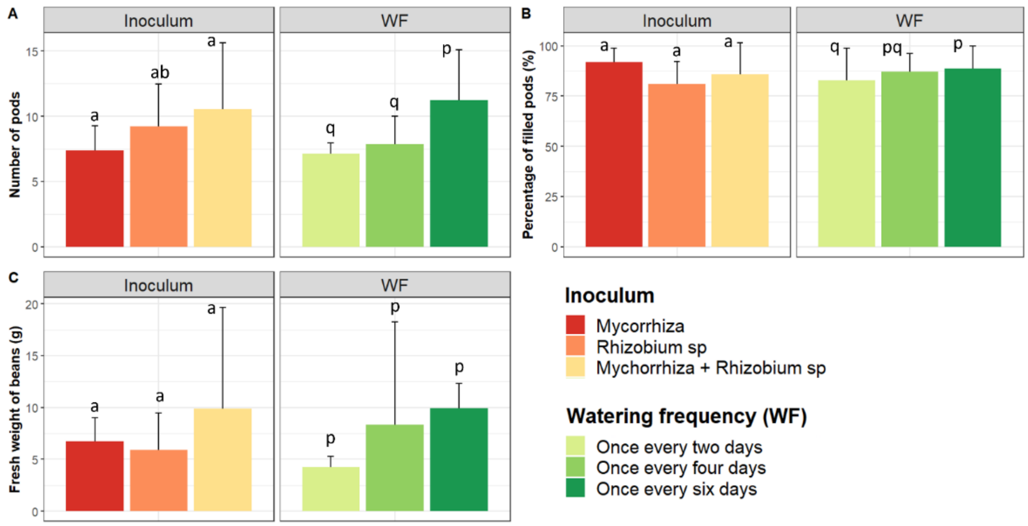


**Figure 5.** Number of pods per plant(A), filled pods percentage in %(B), fresh weight of beans per plant in gram on edamame soybeans with different inoculum and watering frequencies after five weeks(C): The same order and letter(s) indicated no significant difference between the mean values.

Watering once every six days produced more pods, with a fresh weight of beans of 11.26 ± 3.83 g per plant compared to other frequencies (Figure 5A). The activity of mycorrhiza and nano-formula indigenous *Rhizobium* sp. had shown significant differences towards the nodulation effectivity, improved rooting system, and growth on edamame soybeans, but no effect on the yields. This effect can be seen in the average of filled pods, which reached 92.00 ± 6.89 % (Figure 5B). Watering once every six days produced more pods, with a fresh bean weight of 9.91 ±2.41 g (Figure 5C) per plant compared to other frequencies.

However, the inoculum of mycorrhiza-nano-formula indigenous *Rhizobium* sp. tends to have a higher yield of soybeans (1.22 ± 0.26 tons/Ha) than other treatments (Figure 6). Watering frequency once per six days was significantly increased the produced pods, with a higher fresh weight of beans per plant, which is 1.21 ± 0.50 tons/Ha, compared to other frequencies.

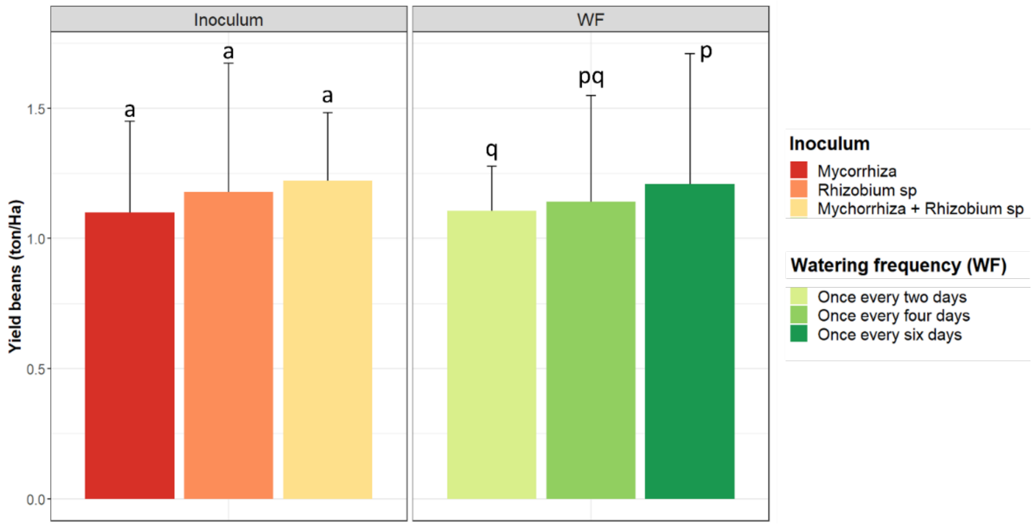


**Figure 6.** The yield of edamame soybeans with different inoculum and watering frequencies after five weeks: The same order and letter(s) indicated no significant difference between the mean values.

## Discussion

Vesicular-arbuscular mycorrhiza (VAM) is a symbiotic fungal that interacts with plant roots to increase nutrient absorption, improve plant durability from drought, and enhance water absorption capacity (Abdelmoneim *et al*., 2014). Mycorrhizal efficacy can be evaluated by the colonization percentage and number of spores on the root (Campbell *et al*., 1999). Inoculation of mycorrhiza colonized 30 – 54% of root systems in edamame soybeans.

In the treatment of edamame soybeans with the combination of mycorrhiza-indigenous *Rhizobium* sp. nano-inoculum and watering once every six days, the highest colonization was obtained after five weeks (63.33 ± 7.26 %). However, samples treated with only mycorrhiza and watered once every six days showed no significant difference from those treated with the combination.

The combination treatment of mycorrhizae and *Rhizobium* sp. nano-inoculant resulted in edamame soybeans with the highest mycorrhizal colonization and produced mycorrhizal spores after five weeks. Astuti *et al*. (2016) discussed that roots colonized with mycorrhiza will develop into internal hyphae, external hyphae, vesicles, and arbuscles on cortex tissues, thus indicating mycorrhizal colonization of root. The additional effect of less watering frequency was seen in the mycorrhizal colonization but not on the spore production, as no significant difference was seen between the edamame soybeans treated once every six days and those watered once every two days with the same mycorrhizae-*Rhizobium* sp. inoculum treatment.

The external hyphae can colonize roots and grow to produce mycorrhizal spores in soil (Campbell *et al*., 1999); thus, higher colonization resulted in more produced nodes. As mentioned by Abdelmoneim *et al.* (2014), the formation of spores by mycorrhizal is stimulated in drought-stress conditions. Based on the response of edamame soybeans towards the number of spores and colonization percentage, a symbiotic relationship between edamame soybean and mycorrhizal increased the spores and affected the plant's rhizosphere. Environmental factors affecting the spores' population included temperature, pH, soil moisture content, phosphorous, and nitrogen content (Astiko, 2021).

*Rhizobium* sp. is a symbiotic bacterium in Leguminosae roots with nitrogen-fixation ability; thus, it is heavily related to crop nitrogen availability. Various indicators, including the formation of nodules, the diameter of nodules, and the fresh weight of nodules determined the successful inoculation of *Rhizobium* sp. on soybean plants. These indicators imply that when nitrogen fixation becomes more active, it fulfils the plants' nitrogen requirements to a greater extent, leading to improve growth. Additionally, the increased chlorophyll activity and assimilated production enhanced plant development (Sari and Prayudyaningsih, 2015).

The application of *Rhizobium* sp. isolates significantly affected the diameter and root nodules (Sari and Prayudyaningsih, 2015). The research by Astuti *et al*. (2016) supported the findings in this study. The study discussed the inoculation of *Rhizobium* sp. and mycorrhiza on soybeans, which can increase nodule weight on sandy, stressed drought land. According to Diagne *et al*.(2020), mycorrhiza inoculation in Leguminosae with *Rhizobium* sp. can stimulate nodulation through secretion in drought-stress conditions.

The roots of Leguminosae inoculated with mycorrhiza and *Rhizobium* sp. will trigger tripartite interaction, which strongly affects root development. Therefore, the root's volume and fresh weight indicate the capacity to uptake nutrients and water from the soil, ensuring the plant's nutritional and hydration needs are fulfilled (Diagne *et al.*, 2020). The combination inoculum led to more mycorrhiza, stimulating root branching and higher root volume. Additionally, the same treatment showed more root nodulation formation with a higher diameter, resulting in the nodules' heavier fresh weight. The inoculation with mycorrhiza and indigenous *Rhizobium* sp. improved the root and weight of the roots.

According to Astuti *et al.* (2016), inoculation of *Rhizobium* sp. and mycorrhiza on soybeans can enhance root development in drought-stress environments. Mycorrhiza can interact with legumes to increase phosphate content and induce nitrogenase activity to increase branching development. Root development in drought stress may be due to the mechanism of the crop in managing root crown development. When the crop grows in a drought-stress environment, it will delay the growth of the shoot to increase the root growth rate.

The shoot's dry weight implies biomass's accumulated weight from photosynthesis results. Increased biomass production in plants ensures the smooth functioning of metabolic processes (Fahrudin, 2009). The inoculum of mycorrhiza and indigenous *Rhizobium* sp., with watering once every six days, resulted in the highest dry weight of the shoot, although no significant difference was seen compared to other different types of inoculums. This result aligned with the mycorrhizal colonization and nodulation of the combined inoculum that can improve root activity and led to enhance photosynthesis process and more biomass produced. On the other hand, drought-stress condition (watering frequency of once per six days) increased mycorrhiza, better absorption of nutrients and water intake and supporting photosynthesize.

Watering at a frequency of once every six days resulted in a higher yield of pods, with a greater fresh weight of beans per plant, compared to other watering frequencies. The mycorrhiza activity and the use of a nano-formula indigenous *Rhizobium* sp. showed notable variations in terms of their effectiveness in promoting nodulation, enhancing the root system, and facilitating the growth of edamame soybeans. However, these factors did not have any impact on the overall yield of the soybeans. This result is observable in the mean value of occupied pods, which had been attained. Watering at a frequency of once every six days resulted in more pods and a higher fresh weight of beans per plant than other watering frequencies. Nevertheless, introducing mycorrhiza-nano-formula indigenous *Rhizobium* sp. resulted in greater soybean production than alternative methods. Watering the plants every six days resulted in a considerable increase in the number of pods produced and a higher fresh weight of beans per plant compared to other watering frequencies.

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