Effect of dose and time of saline-tolerant \textit{Rhizobium} \textit{sp.} inoculation to productivity of black soybean (\textit{Glycine soja} (L) merrit) on saline media

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Abstract. Black soybeans are a food plant commodity that is used as raw material for making soy sauce because it can give a natural black color. This study aims to examine the effect of various doses and times of inoculation of saline-tolerant \textit{Rhizobium} \textit{sp.} inoculum against the production of black soybean (\textit{Glycine soja} (L) Merrit) on saline media. The research was conducted on 25 September 2019 - 20 February 2020 at the Green House and the Laboratory of Ecology and Plant Production, Faculty of Animal and Agriculture, Diponegoro University, Semarang, Central Java. The study used a completely randomized design with a 4x3 factorial pattern with 3 replications. The first factor is the inoculum dose, namely 0, 5, 10, and 15 mg / plant. The second factor is the time of inoculation, that are at planting, 7 and 14 Days After Sowing (DAS). The results showed that the inoculum dose of 15 mg / plant gave the highest yield on the variables of plant height, number of leaves, weight of seeds and weight of 100 seeds. Inoculum application at planting gave the best results on the variable number of leaves and seed weight. The interaction between dose and time of administration of \textit{Rhizobium} \textit{sp.} effect on pod and seeds weight.

1. Introduction

Black soybean is one of the food commodities in Indonesia which is used as raw material for making soy sauce. The use of black soybeans can give soy sauce a natural black color. The use of yellow soybeans is an option for soy sauce producers because of the low production of black soybeans. Black soybeans production in Indonesia in 2015 only reached 15.68 quintal / ha. DETAM 1 variety of black soybean is a superior variety with a large seed size of 14.84 grams / 100 seeds and a potential yield of 2.51 tons / ha.

Utilization of marginal land such as saline soil can be carried out as an effort to expand the cultivation area of black soybean to increase black soybean productivity. Salinity can cause stress in plants so that physiology, production and quality of crop
yields can be disturbed. Appropriate use of saline-tolerant *Rhizobium* bacteria inoculums as biological fertilizers can improve soil quality and crop yields. Saline land is one of the suboptimal land types that have not been widely used in plant cultivation because of its toxic effects on plants. Saline soil is a soil with a high salt and low nutrient. Saline land area in Indonesia reaches 440,300 ha and can continue to grow due to rising sea levels. High salinity in the soil can cause low osmotic pressure in plants, low content of N and K nutrients in the soil, high soil pH, and high Na + content. Efforts to manage saline soil can be done through the provision of biological agents, namely *Rhizobium* bacteria and manure. Giving *Rhizobium* bacteria inoculum and organic matter can increase the available N nutrient for plants, reduce pH, increase nodulation and soybean yield in saline land. One of the efforts to make plants grow in saline land is by inoculating saline-tolerant *Rhizobium sp.* bacteria as a biological fertilizer. The presence of *Rhizobium sp.* Bacteria in soybean plants it can cause the formation of nodules that are able to fix free nitrogen in the air so that it is available to plants. The availability of N for plants is expected to increase black soybean crop production. This study aims to examine the effect of the appropriate dose treatment and timing of saline-tolerant *Rhizobium* bacteria inoculum to increase the production and quality of black soybean on saline media.

2. Materials and Methods
The research was conducted in the Greenhouse and the Laboratory of Ecology and Plant Production, Faculty of Animal Husbandry and Agriculture, Diponegoro University. The materials needed are black soybean seed DETAM-1 variety, saline soil with an Electrical Conductivity (EC) value of 5.18 dS/m with a pH of 8.5, YEMA + CR (Yeast Extract Mannitol Agar + Congo Red) medium, YEM medium, distilled water, 70% and 96% alcohol, cow manure, urea, SP36, and KCl, saline-tolerant *Rhizobium* bacteria inoculum. The tools used are shovels, plastic bags, markers and label paper, digital scales, ruler, camera micropipettes and microtips, spectrophotometer, erlenmeyer, petri dishes, test tube, mortar, autoclave, oven and laminair air flow. The experimental design of this research was a Completely Randomized Design (CRD) factorial with two factors. The first factor is the inoculum dose, namely K0 = Control, K1 = 5 mg/plant, K2 = 10 mg/plant, K3 = 15 mg/plant and the second factor is the time of the inoculation which consists of P1 = 0 DAS, P2 = 7 DAS P3 = 14 DAS. This research conducted in three replications so that there are 36 units.

3. Result and Discussion
*Rhizobium*-legume symbiosis sensitive with osmotic stress that caused by high salinity. This is in accordance with the research by Boulbaba et al. [3] which states nodul initiation which occurred *Rhizobium*-legume symbiosis high sensitivity with osmotic stress, and high salinity can decrease the growth of plant. The results of the analysis of variance showed that the treatment of giving various inoculum doses of *Rhizobium sp.* saline resistance has a significant effect, while the interaction between treatments of various inoculum doses of saline-tolerant *Rhizobium sp.* and the time of inoculation and the treatment time of inoculation of *Rhizobium sp.* both different saline resistance had no significant effect on the height parameters of black soybean.
Table 1. Height of Plant of Black Soybean (*Glycine soja* (L) Merrit) at 8 Weeks After Sowing (WAS) Dosage and Time of Inoculation.

| Rhizobium Inoculant Bacteria | Time of Inoculation | Mean     |
|------------------------------|---------------------|----------|
|                              | P1 (0 DAS)          | P2 (7 DAS) | P3 (14 DAS) |
| K0 (control)                 | 38,67               | 41,33     | 38,00       | 39,33<sup>b</sup> |
| K1 (5 mg/plant)              | 126,33              | 129,67    | 129,00      | 128,33<sup>+</sup> |
| K2 (10 mg/plant)             | 122,67              | 142,33    | 138,33      | 134,44<sup>+</sup> |
| K3 (15 mg/plant)             | 137,67              | 143,33    | 119,67      | 133,56<sup>+</sup> |
| Mean                         | 106,33              | 114,17    | 106,25      | (-)            |

Different superscript letters in the same row indicate significant differences based on DMRT (p <0.05)

Figure 1. Height of plant of black soybean at dose and time of inoculation.
Table 1 and Figure 1 shows that the plant height without inoculum (K0) treatment gave the lowest yield with an average of 39.33 cm and had a lower value than the height of the DETAM 1 black soybean plant cultivated in optimum conditions (58 cm). The planting media used was saline soil with a moderate category, that is 5.18 dS/m which could have a negative impact on plant growth and reduce plant yields. Saline soils with EC above 4 dS/m are categorized as high salinity [8]. Plant height that given the inoculum of saline-tolerant Rhizobium sp. is higher than the treatment without inoculum. Symbiosis between legume and saline-resistant Rhizobium shown positivity synergistic in the ability of legume to survive under salt-stress condition and be able to increase the growth of legume. The association of stress-tolerant legume and saline-resistant Rhizobia shown the positive result synergistic advantage in the ability legume to grow in under salt stress condition [2].

Based on Table 1 it is known that the treatment of various doses and times of inoculation of saline-resistant rhizobium inoculum was not significantly different to the height parameters of black soybean plants.

| Rhizobium Inoculant Bacteria | Time of Inoculation | Mean     |
|------------------------------|---------------------|----------|
|                              | P1 (0 DAS)          | P2 (7 DAS) | P3(14 DAS) |
| K0 (control)                 | 35,33               | 32,33     | 31,00      | 32,89\(^c\) |
| K1 (5 mg/plant)              | 57,67               | 54,00     | 59,67      | 57,11\(^b\) |
| K2 (10 mg/plant)             | 62,67               | 68,33     | 54,00      | 61,67\(^{ab}\) |
| K3 (15 mg/plant)             | 75,33               | 65,33     | 54,00      | 64,89\(^a\) |
| Mean                         | 57,75\(^{a}\)       | 55,00\(^{ab}\) | 49,67\(^b\) | (-) |

Different superscript letters in the same row and column indicate significant differences based on DMRT (p <0.05)

Based on Table 2 it was obtained that the inoculum dose treatment of Rhizobium sp. 15 mg saline resistance / plant (K3) gave significantly more leaf count compared to other dose treatments, namely 64.89 leaves, but not significantly different from the 10 mg / plant (K2) dose treatment, that is 61.67 leaves. Inoculation of Rhizobium sp bacteria with different concentrations, namely 0 g / kg, 5 g / kg, 10 g / kg and 15 g / kg of seeds in soybean plants affected the increase in plant height, leaf number and wet biomass of the plant [16]. The treatment of inoculum during planting had the highest number of leaves compared to other treatments, namely 57.75 leaves, but not significantly different from the treatment of Rhizobium sp. at 7 DAS with a mean of 55 leaves. Rhizobium bacteria inoculation is carried out by mixing it with soybean seeds before planting, so that when the seeds have germinated, there will be Rhizobium bacteria which will be in symbiosis with soybean plants [13].
Based on the results of analysis of variance, it shows that the treatment of various doses and times of inoculation of *Rhizobium* sp., both of them showed significant effect ($p < 0.05$) on the parameter of the number of leaves of black soybean at 8 WAS, but the interaction between the two treatments did not have a significant effect.

**Table 3.** The number of effective root nodules of black soybean (*Glycine Soja* (L) Merrit) at the dosage and time of inoculation.

| *Rhizobium* Inoculant Bacteria | Time of Inoculation | Mean   |
|-------------------------------|---------------------|--------|
|                               | P1 (0 DAS) | P2 (7 DAS) | P3 (14 DAS) |
| K0 (control)                  | 38.67       | 32.00      | 47.00       | 39.22<sup>b</sup> |
| K1 (5 mg/plant)               | 118.33      | 131.33     | 154.33      | 134.67<sup>a</sup> |
| K2 (10 mg/plant)              | 138.00      | 108.33     | 178.67      | 141.67<sup>a</sup> |
| K3 (15 mg/plant)              | 165.67      | 112.67     | 97.67       | 125.33<sup>a</sup> |
| Mean                          | 115.17      | 96.08      | 119.42      |                  |

Different superscript letters in the same column indicate significant differences based on DMRT ($p < 0.05$)

**Figure 2.** Number of pods of black soybean at dose and time of inoculation.

Table 3 shows that the number of root nodules was effective by inoculation of *Rhizobium* sp. saline resistance gave higher yields compared to the treatment without
inoculum (K0).\textit{Rhizobium sp} bacteria inoculation on the parameter of the number of nodules of black soybean plants gave a significant effect. Giving inoculums could increase the population of \textit{Rhizobium sp} bacteria which was characterized by colonization so that the root area of soybean plants was infected and formed root nodules [10]. The different times of inoculation and the interaction between the two treatments were not significantly different. The detrimental effects in plants and \textit{Rhizobium} nodulation are caused by salt concentration that accumulated in the tissues of plants. Salinity can be understood as abiotic stress that can decrease productivity of plants and affects the nodulation [6]. Based on the analysis of variance, it shows that the treatment of giving various inoculum doses of \textit{Rhizobium sp}. saline resistance showed a significant effect (p <0.05), while the different treatment times of inoculation and their interactions had no significant effect.

Table 4. Number of pods of black soybean (\textit{Glycine soja} (L) Merrit) at the dosage And time of inoculation

| \textit{Rhizobium} Inoculant Bacteria | Time of Inoculation | Mean  |
|-------------------------------------|---------------------|-------|
|                                     | P1 (0 DAS)          | P2 (7 DAS) | P3(14 DAS) |
| K0 (control)                        | 39,33               | 42,00    | 44,33      | 41,89\textsuperscript{b} |
| K1 (5 mg/plant)                     | 101,67              | 97,00    | 122,67     | 107,11\textsuperscript{a} |
| K2 (10 mg/plant)                    | 100,33              | 92,33    | 111,67     | 101,44\textsuperscript{a} |
| K3 (15 mg/plant)                    | 126,33              | 95,00    | 94,33      | 105,22\textsuperscript{a} |
| Mean                                | 91,92               | 81,58    | 93,25      |         |

Different superscript letters in the same column indicate significant differences based on DMRT (p <0.05)

Based on Table 4 and Figure 2 it is known that the number of pods of black soybean plants in the treatment of \textit{Rhizobium sp}. saline resistance gave higher yields than without inoculum (K0). This is due to the inoculum of saline-tolerant \textit{Rhizobium sp}. can help plant growth in saline soils, in addition, the presence of \textit{Rhizobium sp} bacteria can increase the number of root nodules which affect the number of pods of soybean plants. \textit{Rhizobium sp} bacteria inoculation in legume plants could increase the number of root nodules which affected the number of pods [11].

Based on the analysis of variance, it shows that the treatment of giving various inoculum doses of saline-tolerant \textit{Rhizobium sp}. showed a significant effect (p <0.05), but the different times of inoculation and the interaction between the two treatments had no significant effect.
Table 5. Weight of pods of black soybean (*Glycine soja* (L) Merrit) at dosage and time of inoculation.

| Inoculant Bacteria | Time of Inoculation | Mean |
|--------------------|---------------------|------|
|                    | P1 (0 DAS)          | P2 (7 DAS) | P3(14 DAS) |
| K0 (control)       | 9.30^c             | 10.87^c  | 11.00^c    | 10.39^c |
| K1 (5 mg/plant)    | 29.67^d            | 56.99^bc | 59.37^abc  | 48.68^b |
| K2 (10 mg/plant)   | 55.64^bc           | 53.34^bc | 71.09^a    | 60.02^a |
| K3 (15 mg/plant)   | 67.55^ab           | 47.07^c  | 56.59^bc   | 57.07^a |
| Mean               | 40.54^b            | 42.07^ab | 49.51^a    |      |

Different superscript letters on the interaction matrix, the same column and row shows significant differences based on DMRT (p <0.05)

Based on the results in Table 5 it is known that there is an interaction between the treatment of various doses and the time of inoculation on weight of pod variable. Treatment of inoculum dose 10 mg / plant saline resistance at 14 DAS (K2P3) significantly gave the highest yield, namely 71.09 grams but not significantly different from the treatment 15 mg / plant at planting and treatment dose 5 mg / plant at 14 DAS. The availability of nitrogen nutrients can increase the formation of amino acids and proteins in the seeds so that the pods can be filled completely. Symbiosis *Rhizobium*-legume can fix atmospheric nitrogen for increased vegetative growth and pod production [5]. The pods of soybean plants that have been formed then fill with N as the pods age. Additional *Rhizobium* bacteria that were inoculated on plants had to first adjust to the new habitat and each inoculant had a different adaptability. Different results because inoculants need time to adapt to new habitats, whereas each inoculant has different adaptability [9].

Table 6. Number of black soybean seeds (*Glycine soja* (L) Merrit) at the dose and time of inoculation.

| Inoculant Bacteria | Time of Inoculation | Mean |
|--------------------|---------------------|------|
|                    | P1 (0 DAS)          | P2 (7 DAS) | P3(14 DAS) |
| K0 (control)       | 81.67               | 79.67   | 91.33      | 84.22^b |
| K1 (5 mg/plant)    | 203.67              | 200.00  | 237.67     | 213.78^a |
| K2 (10 mg/plant)   | 221.33              | 188.33  | 229.33     | 213.00^a |
| K3 (15 mg/plant)   | 256.33              | 206.67  | 208.33     | 223.78^a |
| Mean               | 190.75              | 168.67  | 191.67     |      |

Different superscript letters in the same column indicate significant differences based on DMRT (p <0.05)
Based on the results in Table 6 it is known that the number of seeds in the treatment given the *Rhizobium* sp. saline resistance gave higher yields than without inoculum (K0). This is because inoculation of *Rhizobium* sp bacteria in legume plants gives a good effect from the symbiosis of mutualism and stimulating the growth of plant and root. *Rhizobium* sp biological fertilizer increase the bean yield in under optimal condition with increasing the efficiency fixation of nitrogen that led increase rooth and bean grain yield under optimal conditions [12].

Based on Table 6 it is known that the treatment of various inoculum doses of *Rhizobium* sp. had a significant effect on the parameter of the number of black soybean seeds (p <0.05), while the treatment time of inoculation and the interaction between the two treatments had no significant effect.

Table 7. Seed weight of black soybean (*Glycine soja* (L) Merrit) at the dose and time of inoculation

| Rhizobium Inoculant Bacteria | Time of Inoculation | Mean |
|------------------------------|--------------------|------|
|                              | P1 (0 DAS)         | P2 (7 DAS) | P3(14 DAS) |      |
| K0 (control)                 | 3.16<sup>f</sup>   | 3.58<sup>f</sup> | 5.30<sup>ef</sup> | 4.01<sup>d</sup> |
| K1 (5 mg/plant)              | 13.29<sup>cd</sup> | 13.37<sup>cd</sup> | 9.69<sup>de</sup> | 12.12<sup>c</sup> |
| K2 (10 mg/plant)             | 24.85<sup>ab</sup> | 17.63<sup>bc</sup> | 11.22<sup>de</sup> | 17.90<sup>b</sup> |
| K3 (15 mg/plant)             | 28.96<sup>a</sup>  | 24.31<sup>ab</sup> | 12.56<sup>cd</sup> | 21.94<sup>a</sup> |
| Mean                         | 17.57<sup>a</sup>  | 14.72<sup>a</sup> | 9.69<sup>b</sup>  | (-)  |

Different superscript letters on the interaction matrix, the same column and row show significant differences based on DMRT (p <0.05)
Based on the results in Table 7 it is known that the interaction between the inoculum dose treatment of *Rhizobium sp*. 15 mg / plant and time of inoculation during planting gave the best results on the weight parameter of black soybean seeds, it is 28.96 grams, but it was not significantly different from the 15 mg / plant inoculum treatment given at 7 DAS (K3P2). The time of inoculation with higher doses had a good impact on plant yields and the inoculation time at planting increased the effectiveness of *Rhizobium* bacteria in fixing nitrogen. The higher concentration effective *Rhizobium* inoculant that inoculate at planting time enhanced the growth of plant, grain and biomass yield in legume [14]. High concentration of *Rhizobium* inoculums had the potential to increase levels of N which could be absorbed by plants so that it had an effect on crop yield. Treatment of various inoculum doses was significantly different in the weight parameter of black soybean seeds. The yield of black soybean seed given the inoculum of *Rhizobium sp*. saline resistance is higher than the yield of black soybean seeds cultivated in optimum conditions without additional inoculums, it is 2.51 tonnes / ha or 15.06 g / plant. The inoculation of *Rhizobium sp* bacteria could increase seed yield [15]. Inoculation time at planting (P1) gave the best results on the weight parameter of black soybean seeds with an average of 17.56 grams but not significantly different from the treatment of inoculation time at 7 DAS. Young roots have nutrients that can meet the needs of bacteria and can help the reproduction of bacteria in the roots. The root hairs in young roots provide nutrients for bacterial growth [7].

**Table 8.** Weight of 100 seeds of black soybean (*Glycine soja* (L) Merrit) at the dose and time of inoculation

| *Rhizobium* Inoculant Bacteria | Time of Inoculation | Mean     |
|-------------------------------|---------------------|----------|
|                               | P1 (0 DAS)          | P2 (7 DAS) | P3 (14 DAS) |
| K0 (control)                  | 4.35                | 4.65      | 5.11        | 4.70c         |
| K1 (5 mg/plant)               | 12.95               | 18.84     | 15.07       | 15.62b        |
| K2 (10 mg/plant)              | 15.94               | 17.10     | 16.89       | 16.64ab       |
| K3 (15 mg/plant)              | 18.11               | 15.62     | 19.67       | 17.80a        |
| Mean                          | 12.84               | 14.05     | 14.19       |               |

Different superscript letters in the same column indicate significant differences based on DMRT (p < 0.05)

Based on the results in Table 8 it is known that the highest yield of the weight parameter of 100 seeds was found in the treatment of *Rhizobium sp*. 15 mg / plant (K3) saline resistance, that is 17.80 grams, which was not significantly different from the 10 mg / plant (K2), but significantly different from the 5 mg / plant (K1) and without inoculation of *Rhizobium sp*. (K0). The lowest yield on the weight of 100 seeds was found in the treatment without inoculum, that is 4.70 grams which has a lower value than the weight of 100 black soybean seeds DETAM 1 cultivated in optimum conditions (14.84 grams). Inoculation with saline-resistant *Rhizobium* can reduced the effect of salinity and increased the growth and seed yield. This is in accordance with Ahmad *et al*. [1] reasearch which stated the mungbean that inoculated with *Rhizobium* reduced the effect...
of salinity on physiological variable thus improving the photosynthetic rate which led increase growth and seed yield. Inoculate *Rhizobium* sp bacteria inoculums with high doses was able to increase plant growth and yield on saline soils. The high concentration of *Rhizobium* sp inoculums was able to provide nitrogen for plants and increase plant growth and yield on saline soils [16]. Based on Table 8 it is known that the different times of inoculation and the interaction of the two treatments showed no significant difference.

4. Conclusion

The conclusion that can be drawn is the inoculum of *Rhizobium* sp. saline resistance at a dose of 15 mg / plant given during sowing gives the highest yield of DETAM-1 black soybean in saline soils.

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