Characterization of *Rhizobium* Indigenous Isolates and Their Compatibility with Edamame Soybean

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**Abstract.** Three isolates were isolated from edamame soybean nodules and characterized as indigenous *Rhizobium* sp. compatible with Edamame soybean, showing the potential to be developed as an inoculum for biological fertilizers that can increase the efficiency of nitrogen fertilizers. All isolates grew on YMA + Congo Red medium, with colony characteristics of a circular shape, pink color, 1-3 mm in diameter with convex elevation, and slightly translucent (Opaque). The results of microscopic characterization showed that the bacilli-shaped cells were gram-negative. The growth curve showed that all isolates could grow optimally after 48 hours of culture. Physiologically, the three isolates were aerobic and catalase-producing bacteria. The re-inoculation of these Rhizobium isolates on edamame seeds proved its compatibility indicated by effective nodule formation up to 48.03 - 66.23%.

1. **Introduction**

Edamame soybeans (*Glycine max* L. Merr.), consumed in the form of snacks and processed products, contain a high nutritional value (11.9% protein), calcium, vitamins A, B1, C, and iron [1]. Edamame has the potential to be developed due to shorter anthesis period (±26 days), shorter harvesting age (65-68 days after planting), the heavier weight of 100 seeds (± 28 g), and higher production (3.5 tons/ha) compared to local varieties (1.7–3.2 tons/ha). Meanwhile, the Japanese export demand is 150,000-160,000 tons/year [2-3], and Indonesia can only fulfill 13.58% of it [4].

The main problem in Edamame soybean cultivation is the use of high doses of synthetic fertilizers (3-4 times the recommended dose), compared to local soybeans. The fertilizers used in Edamame soybean cultivation are 150 kg/ha urea, 150 kg/ha SP-36, and 100 kg/ha KCl [5]. This application can damage agricultural land and disturb the balance of nutrients in the soil, as well as endanger health [6]. Accordingly, we need a breakthrough in technological innovation using biological fertilizers of *Rhizobium* sp. that can bind Nitrogen as much as 50–75% of the N required by soybean [7].

*Rhizobium* sp. is rhizosphere bacteria that forms a symbiotic relationship with Leguminosae stimulating higher productivity, especially legumes. In the root nodule network, *Rhizobium* sp. fix nitrogen and convert it into ammonium and protein, which are then utilized by plants. The efficacy of legume-Rhizobium symbiosis is influenced by environmental factors such as pH, soil deterioration, stress conditions and extreme temperatures [8]. Interaction of *Rhizobium* sp. must compete with other strains in the rhizosphere to get a place to form nodules [9]. The success of the *Rhizobium* sp. to form nodules is very dependent on the number of indigenous *Rhizobium* sp. in the soil [10]. Previous studies revealed that several commercial *Rhizobium* sp. failed to form nodule in edamame soybean. This failure
was assumed to be associated with the origin of edamame as [11-13], the success of \textit{Rhizobium} sp. symbiosis with plants is strongly influenced by the compatibility between strains of \textit{Rhizobium} sp. and soybean varieties as well as soil environmental factors. The results of research by Astuti et al. (year of publish) had found the indigenous \textit{Rhizobium} sp. isolates (B, E, and F) showing symbiotic association with Edamame soybean cultivated in District of Tamantirto.

The potential of these indigenous isolates should be further characterized to identify the characteristics of each isolate as well as its compatibility level with edamame soybean. Therefore, this study was aimed to characterize the indigenous \textit{Rhizobium} sp. isolates and evaluate its nodule-forming capacity in Edamame soybean plants. This information would ascertain the potential of these indigenous isolates as source of biological fertilizer, thereby reducing the use of synthetic nitrogen fertilizers in Edamame cultivation.

2. Material and Methods
The isolates of indigenous \textit{Rhizobium} sp. used in this study were collected from previous study, namely B isolate, E isolate and F isolate [11]. Growth curve was performed in-vitro on Yeast Extract Mannitol Agar (YEMA) media. About 100 ml culture of the indigenous \textit{Rhizobium} sp. isolates was grown on YEMA media for seven days at room temperature. Growth was observed using Total Plate Count (TPC) method by measuring the cell density in dilution level of $10^7$, $10^8$ and $10^9$ with three replications in each dilution level.

Morphological characterization was performed through Gram staining, macroscopic and microscopic observations. Meanwhile, the physiology of each isolates was characterized based on its respiration mechanism and catalytic activity [14]. Each indigenous Rhizobium isolate was reproduced then inoculated on Edamame soybean seeds. The inoculated seeds were subsequently planted into ....... (please provide the soil mixture used in this study). The nodulation was observed weekly, including the nodule weight, diameter and effectiveness. Data of periodic observation was analyzed using standard error chart. Nodulation effectivity was analyzed using the F test. Significance among isolates was further tested using DMRT with a p<0.05.

3. Results and Discussion

3.1. The isolation of the indigenous \textit{Rhizobium} sp.
The isolates of \textit{Rhizobium} sp. were obtained by separating from the root nodules and the surrounding environment using a surface plating method on YEMA + Congo Red selective media and growing them as pure cultures using the streak plating method [13]. Six isolates were obtained. Screening, re-plating and characterization were performed, resulting in three isolates, namely B, E, and F (Figure 1).

![Figure 1. Indigenous Rhizobium sp. isolates (B, E, and F)](image)

3.2. The growth curve of the indigenous \textit{Rhizobium} sp. isolated form Edamame
The growth of isolate F increased rapidly after being inoculated and reached a maximum after day 1 ($4.43 \times 10^8$ CFU / ml) (Figure 2), then decreased until day 5. Meanwhile, the growth of isolates B and E had the same pattern, which was slower and reaching a maximum after incubation on day 2 ($7.1 \times 10^8$ CFU / ml). Considering the pattern resulted from growth curve, these three indigenous isolates of
Rhizobium sp. were categorized as fast-growing bacteria. The growth curve of bacteria consists of several following phases, started with adaptation phase, exponential phase, stationary phase until the death phase [15-16] mentioned two growth types of Rhizobium sp., namely fast-growing (3-5 days) and slow-growing (6-8 days).

![Figure 2](image)

**Figure 2.** Comparison of growth curve among three isolates of Edamame indigenous Rhizobium sp.

3.3. **Morphological and physiological characteristics of Edamame indigenous Rhizobium isolates**

**Table 1.** Comparison of macroscopic, microscopic and physiological characteristics among the indigenous Rhizobium sp. isolates

| Isolate | Morphology | Physiology |
|---------|------------|------------|
|         | Macroscopic | Microscopic |                        |
|         | Colony shape | Diameter | Elevation | Inner structure | Cell shape | Gram | Aerobic ability | Catalase |
| Isolat B | Circular | 1 mm | Convex | Opaque | Bacillus | Negative | Aerobic | Positive |
| Isolat E | Circular | 2 mm | Convex | Granular | Bacillus | Negative | Aerobic | Positive |
| Isolat F | Circular | 2 mm | Umbonate | Filamentous | Bacillus | Negative | Aerobic | Positive |

As presented in Table 1, the three indigenous isolates shared several similarities, such as its colony and cell shapes, Gram classification, respiration mechanism and catalytic activity. However, regarding its colony size, inner structure and elevation, those isolates showed distinguishable features (Table 1). These results were in line with [17] cribing that Edamame-isolated Rhizobium spp. had clear white and pink colony on YEMA + Congo Red selection medium. Other study highlighted that Rhizobium showed circular, transparent white or milky white-colored colony with either convex or umbonate surface [18]. Similarly, the elevation of this Gram-negative yet rod-shaped bacteria was commonly flat to convex and conical where its inner structure was either opaque yet translucent [19-20]. Diameter of Rhizobium sp. was ranging from 0.5 to 3.0 µm and this bacteria did not form any spore [19].

3.4. **Compatibility of the indigenous Rhizobium sp. isolates with Edamame soybean seeds**

Regarding the nodulation efficacy, there was no significant difference among the indigenous isolates of Rhizobium sp. in all parameters observed (Table 2). Based on the percentage of effective nodules, those three isolates showed compatibility with Edamame soybean where F isolate exhibited the highest
compatibility up to 66.23% (Table 2). However, considering the nodules weight, association of isolate E and Edamame resulted in the heaviest weight (17.7 gram) (Table 2).

Table 2. Comparison on compatibility between the indigenous *Rhizobium* sp. isolates with Edamame soybean.

| Isolates | Number of nodules | Nodule effectiveness (%) | Nodules weight (gram) |
|----------|-------------------|--------------------------|-----------------------|
| Isolate B | 5.40 ± 0.5 a      | 48.03 ± 1.9 a            | 12.93 ± 3.1 a         |
| Isolate E | 7.26 ± 0.4 a      | 55.05 ± 2.5 a            | 17.70 ± 1.4 a         |
| Isolate F | 8.19 ± 0.3 a      | 66.23 ± 2.3 a            | 13.33 ± 2.3 a         |

Values followed by the same letters in the same column are not significantly different according to DMRT with a p<0.05.

The formation of nodules on the Edamame soybean roots as a result of the re-inoculation of isolates B, E, and F prove that there is compatibility between these isolates and Edamame soybeans, supported by the activity of isolates B, E and F indicated by the effectiveness of nodules of 48.03 - 6.23% and weight of nodules 12.93-17.7 gram. Effective nodules is marked by reddish color due to the formation of Fe-rich leghemoglobin that regulates the oxygen content, thus promoting the nitrogen fixation activity. The amount of leghemoglobin in the root nodule correlated directly towards the amount of nitrogen fixed [21].

According to this study, all of these indigenous *Rhizobium* sp. isolates were potential be developed as biological fertilizers to reduce the dose of synthetic nitrogen fertilizers used in Edamame cultivation as well as increase its productivity. It was in line with [6] reporting that the application of 50% biological fertilizer successfully increased the Edamame productivity.

4. Conclusion
The colony characteristics of the indigenous *Rhizobium* sp. isolates (B, E, and F) from Tamantirto circular, pink in color, 1-3 mm in diameter with convex elevation, and slightly translucent (Opaque). The results of microscopic characterization showed that the cells were rod-shaped (bacillus) and gram-negative. The growth of isolates B, E, and F is fast growing. Meanwhile, the physiological observation showed that the three isolates were aerobic and catalase-positive. The results of the compatibility test with re-inoculation on Edamame seeds proved that the isolates were able to fix nitrogen with the formation of effective nodules of 48.03 - 6.23%.

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