Isolation of Rhizobium Bacteria from Forage Legumes for the Development of Ruminant Feed

E. Fuskhah[1]*, E.D. Purbajanti[1], S. Anwar[1]

[1]Faculty of Animal and Agricultural Sciences, Diponegoro University, Semarang, Central Java, Indonesia.

Corresponding E-mail: eny_fuskhah@yahoo.com

Abstract

The aimed of the study was to explore the presence of Rhizobium bacteria along the northern coast of Central Java, to develop a saline-resistant legumes. Rhizobium bacteria is a mutualistic bacterium capable of symbiosis with legumes so that legumes crop yields increase. The research begins with sampling of soil and root nodule of forage legumes along the Northern Coast of Central Java including Tegal, Pekalongan, Semarang, Demak, Pati. Soil samples were analysed for salinity, Total Dissolved Solids, and pH. Rhizobium bacteria were isolated from the acquired root nodule, then identified by biochemical test to ensure that the isolates obtained were Rhizobium bacteria. The results showed that the five districts/municipal sites sampled by the soil have very low salinity to very high levels. The highest level of soil salinity was found in Demak (Sayung) which has an electrical conductivity value (EC) of 17.77 mmhos/cm. The EC values of legumes overgrown soils showed a low salinity level while bare soils have high salinity levels. Feed crops legumes that could be found in the northern coast of Central Java were *Centrosema pubescens, Calopogonium mucunoides, Leucaena leucocephala,* and *Sesbania grandiflora.* The study obtained 6 kinds of isolates of rhizobium bacteria isolated from forage legumes, included 1) *Centrosema pubescens isolated from* Pekalongan, 2) *Centrosema pubescens isolated from* Tegal, 3) *Calopogonium mucunoides isolated from* Pekalongan, 4) *Leucaenaleucocephala* isolated from Tegal, 5) *Leucaena leucocephala* isolated from Semarang, 6) *Sesbania grandiflora* isolated from Tegal.

1. Introduction

Indonesia is the largest archipelagic country in the world with a total of about 13,667 islands. Between one island and another island is separated by the sea. Such conditions resulted in the Indonesian state having thousands of hectares of saline coastal land. The saline soil may also occur in dry areas where soil drainage is impaired, evaporation increases, and rainfall is low. Saline land in Indonesia which is thousands of hectares is a very potential land if managed properly, so it can be useful optimally. One of the alternative uses is to grow feed crops. As is known the availability of feed crops is declining, along with reduced land for feed crops and seasonal constraints. Reduced land for feed crops, among others, due to displace by food crops and settlements. Attention to the marginal lands such as saline land is now getting bigger. On the other hand, the need for animal protein is increasing as the population increases. Ruminant livestock producing meat and milk is a potential animal source of animal protein. Feed plants are an important factor for the development of ruminant livestock, as most of the feed for these animals is forage. For that reason, it is necessary to use the saline field to grow the feed crops. Efforts to grow feed crops and raise ruminant livestock in coastal areas are also an alternative of business diversification to improve the living standards of fishermen around the coast who are generally low income.

The salinity stress is one of the most influential factors in limiting agricultural production [1]. The term saline is used in conjunction with a soil having a conductivity of more than 4 mmhos/cm at 25^{o}C, having the percentage of sodium exchangeable less than 15 and a pH less than 8.5. Water-soluble salts tend to accumulate in the top layer or ground surface. The process of soluble salt accumulation in the soil is called salinization. The accumulation of a number of salts such as chloride, sulphate, sodium chloride, calcium and magnesium salts are present in the saline soil. Sodium chloride (NaCl) is the dominant salt in saline soils in coastal areas [2]. The response of plants to the salinity or electrical conductivity (EC) levels expressed in mmhos/cm at 25^{o}C is different. Tree legumes *Sesbania grandiflora* and *Leucaena leucocephala* exhibited higher salinity resistance than the cover crops legumes *Calopogonium mucunoides* and *Centrosema pubescens* [3]. Plant growing on saline soil is inhibited due to sodium ion poisoning (Na). The results of Sopandie [4] showed that with increasing NaCl concentration (up to 250 mM) will increase Na content in canopy and roots of barley and peanut plants. In addition, nutrient uptake is inhibited in saline soil. The addition of 2.0 mM calcium (Ca) will lower the Na content in the canopy of the plant. Calcium in saline conditions has contributed to the ion translocation regulation, thus reducing the toxicity of Na + ions while increasing K + ion uptake. In other words, the selectivity of K + / Na + in the membrane is influenced by calcium [4]. Fuskhah [5] showed that high salinity decreased nitrogenase activity of *Caliandra callothyrsus*. Efforts to increase the productivity of saline can be achieved through the cultivation of salt tolerant plants and reclamation

Legumes have mutualistic symbiotic ability with Rhizobium sp. bacteria that grow in the root region. The existence of this bacteria causes the formation of root nodules that are able to fix the free nitrogen from the air so as to supply the plant needs for nitrogen. The symbiotic result is expected to increase the production of forage plants. Fuskhah [6] demonstrated that the use of Rhizobium inoculum from 20-60 g / kg of seeds combined with phosphorus fertilization can increase the production of the dry matter forage Centrosema pubescens Benth. The ability to fix nitrogen can reduce the cost of purchasing artificial N-fertilizers, so the application of Rhizobium inoculation in legumes becomes very important to spur nitrogen fixation. For application in saline soils it is necessary to look for efforts to obtain saline-resistant Rhizobium isolates that can be applied to legumes in saline coastal areas. Based on these descriptions, then attempts to obtain saline-resistant Rhizobium strains are important to improve association success with forage legumes in coastal areas.

2. Materials and Methods

2.1. Analysis of Land Salinity of North Coast of Central Java

The objective of this study was to obtain information about salinity level of land area of North Coast of Central Java. This study used soil from North Coast of Central Java, plastic bags, chemicals and tools needed for pH analysis, total dissolved solids (TDS) and electrical conductivity (EC). The selection of soil sampling sites is based on the classification of climate according to Oldeman. Of the 11 districts along the North Coast of Central Java, most have a C climate of 7 districts. Climate D as many as 3 districts, and climate E as much as 2 districts. There are 1 districts with part of climate D and some having climate E. Thus, soil samples from C climate region were 3 districts, 1 location having D climate, and 1 location having E climate. Parameter observed is the pH, TDS, and EC of each soil sample.

2.2. *Isolation, and Identification of Rhizobium Bacteria from Forage Legumes*

The materials used in this study were root nodules of forage legumes from North Central Coast area of Central Java, 95% ethanol, 0.1% sublimate, distilled water, yeast mannitol agar medium (YMA), congo red (CR) bromine thymol blue (BTB), NaCl, tools for picking up root nodules, aluminium foil, rubber bands, cotton, cover paper, plastic, test tube, tweezers, erlenmeyer, stirring rod, petri dish, ‘ose’, oven, autoclave.

The sample of root nodules was taken from the North Coast of Central Java. The root aggregates were collected, then sterilization, isolation, identification according to [7].

1. Collection of root nodules
   The root nodules were collected by separating the root nodule and cutting 0.5 cm root on each side of the root nodules.
2. Sterilization of root nodule
   Root nodules was immersed in a 95% ethanol solution for 5-10 seconds, then immersed in a 0.1% sublimate solution for 3-5 minutes, then washed five times with sterile water.
3. Isolation of Rhizobium bacteria
   The sterile root was crushed with tweezers, then the root nodules were grown on a plate medium YMA + CR, incubated for 3-5 days at room temperature (28^0C) [7].
4. Identification of Rhizobium bacteria
   The colonies that grew apart and identified as Rhizobium bacteria were subsequently grown on the identification medium, using the inclined medium YMA + CR, YMA + BTB. Next, the bacteria were stained with Gram dye. All the acquired Rhizobium bacteria are collected. Pure culture obtained can then be stored as a Rhizobium culture stock, and may be used for further research.

3. Results and Discussion

3.1. Analysis of land salinity of north coast region of central java

The samples soil were obtained from Tegal, Pekalongan, Semarang, Demak, Pati during the dry season (August-September 2017). Based on the results of the survey and laboratory analysis, the results obtained as listed in Table 1.

Table 1 shows that from the five districts/municipal locations sampled the soils have very low to very high salinity levels. The salinity level is divided into 5 categories based on EC values in mmhos/cm that are very low (0-2), low (2 - 4), medium (4 - 8), high (8 - 16), and very high (> 16). The salinity level of the land usually changes according to the season. In the dry season, the level of soil salinity usually increases as the amount of water derived from rain is not enough to neutralize the amount of water lost by evapotranspiration, so that when water is evaporated into the atmosphere it causes the soluble salts to accumulate to the soil surface [8]. Causes the soil to be saline are :
(1) the soil has a salt-containing material [9], (2) sea water intrusion, the accumulation of salt from the irrigation water used or the groundwater motion reclaimed from the seabed [8], (3) high evapotranspiration rate with low rainfall so that minerals are not completely washed [10].

The highest level of soil salinity was achieved by Demak (Sayung) which has an electrical conductivity value (EC) of 17.77 mmhos / cm. EC values of legumes overgrown soils show a low salinity level while bare soils have high salinity levels. This shows that legumes are not able to live on soils that have high salinity levels. Feed crops legumes that can be found in the northern coast of Central Java are *Centrosema pubescens, Calopogonium mucunoides, Leucaena leucocephala*, and *Sesbania grandiflora*

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Table 1. Soil pH, TDS, and EC Analysis Results of North Coast Region of Central Java

Java Location (Plants)	TDS (ppm)	EC (mmhos/cm)	pH
Tegal (*Centrosema*)	241	1.545	8.58
Tegal (*Leucaena*)	152	0.31	**6.93**
Tegal (*Sesbania*)	458	0.911	9.00
Pekalongan (*Calopogonium*)	372	0.729	6.46
Semarang (-)	7.83	3.15	6.08
Demak (-)	8840	17.77	7.39
Pati (-)	3.97	7.89	1950

Description: (-) no legume

3.2. Isolation and identification of Rhizobium bacteria

3.2.1. Collection of root nodules, based on the observation, it can be said that the nodule of the roots have a variety of shapes and sizes. Most root nodules in all species lie at the root of the root. In transverse slices, the root nodules appear pink. This shows that the root nodule is effective. In accordance with the opinion of [11], which stated that effective root nodules are pink, because they contain leghaemoglobin. A red pigment called leghaemoglobin is encountered in a root nodule between the bacterium and the membrane surrounding the membrane. The number of leghaemoglobin in the root nodule has a direct relationship with the fixed amount of nitrogen [12].

3.2.2. Isolation and identification of Rhizobium bacteria, effective root nodules, which are fresh, non-wrinkled and non-dried are taken for further sterilization and isolation. The results of laboratory analysis, obtained 6 kinds of isolates of rhizobium bacteria isolated from forage legumes were: 1) Centrosema pubescens isolated from Pekalongan, 2) Centrosema pubescens isolated from Tegal, 3) Calopogonium mucunoides isolated from Pekalongan, 4) Leucaena leucocephala isolated from Tegal, 5) Leucaena leucocephala isolated from Semarang, 6) Sesbania grandiflora isolated from Tegal. The growth of rhizobium bacteria as in Table 2.

Table 2. Growth of rhizobium bacteria

Various isolates	Density	YMA + CR	YMA + BTB
Centrosema isolated from Pekalongan	++	pink	yellow
Centrosema isolated from Tegal	+	pink	yellow
Calopogonium isolated fromPekalongan	++	pink	yellow
Leucaena isolated from Tegal	+++	pink	yellow
Leucaena isolated from Semarang	+	pink	yellow
Sesbania isolated from Tegal	++	pink	yellow

Description: + = grow solid, ++ = grows very solid. +++ = grow very dense

The obtained isolates were then tested biochemically. After incubation for 5 days, all of the isolates obtained grew well on YMA + CR medium, white colony color and medium color remained pink. The colony grown on the medium of YMA + BTB grows well, the medium color of green turns yellowish after Rhizobium grows. This indicates that all of the isolates obtained are included fast growing bacteria. Microbes have no real anatomical features, so bacterial identification is based on morphology, culture properties and biochemical properties. Morphology of microorganisms based on shape and size is not sufficient to identify. Other features such as staining properties, colony growth patterns, growth reactions to carbohydrates, and the use of amino acids are helpful in the identification of microbes [13].

4. Conclusion

The conclusions that can be drawn was that the five districts/municipal sites sampled by the soil have very low salinity to very high levels. The highest level of soil salinity is achieved by Demak (Sayung) which has an electrical conductivity value (EC) of 17,77 mmhos/cm. The EC values of legumes overgrown soils show a low salinity level while bare soils have high salinity levels. Feed crops legumes that can be found in the northern coast of Central Java were *Centrosema pubescens*, Calopogonium mucunoides, *Leucaena leucocephala*, and *Sesbania grandiflora*. The results of laboratory analysis, obtained 6 kinds of isolates of rhizobium bacteria isolated from forage legumes were: 1) *Centrosema pubescens* isolated from Pekalongan, 2) *Centrosema pubescens* isolated from Tegal, 3) *Calopogonium mucunoides* isolated from Pekalongan, 4) *Leucaena leucocephala* isolated from Tegal, 5) *Leucaena leucocephala* isolated from Semarang, 6) *Sesbania grandiflora* isolated from Tegal.

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