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Test of the resistance of rhizobium bacteria to salinity for the development of food legume plants in coastal areas

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Abstract. Soil salinity is one of serious environmental problems. Rhizobium is a mutualistic bacterium capable of symbiosis with legumes. The aim of the study was to test the resistance of rhizobium bacteria isolated from root nodules of food legumes against saline media. Root nodules were collected from coastal areas of the district of Tegal, Pemalang, Semarang, Pati, and Rembang in Central Java. Rhizobium bacteria were isolated from the acquired root nodules, and then their identity was confirmed by biochemical tests. Rhizobium bacteria resistance to salinity was determined by growing isolated colonies on a medium with different salt content. Results showed that food crop legumes found from coastal area were soybeans (*Glycine max* L. Merr), peanuts (*Vigna sinensis* L.), green beans (*V. radiata* L.), and long beans (*V. unguiculata* L.). There were 6 types of Rhizobium bacterial isolated from the following food crop legumes: 1) soybean isolated from Pati, 2) soybean isolated from Rembang, 3) peanut isolated from Pemalang, 4) green bean isolated from Pemalang, 5) long bean isolated from Semarang, and 6) longbean isolated from Tegal. The salinity resistance was in the following order: rhizobium bacterial isolated from green beans, soybeans, peanuts, and long beans, respectively.

1. Introduction

Increasing soil salinity, soils high in soluble salts, and sodicity soils high in exchangeable sodium, are serious land degradation issues worldwide, and are predicted to increase in the future. 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. Salt-affected soil, saline and sodic soil generally occur in arid and semiarid climate where rainfall is insufficient to leach soluble salts from soil profile [1]. The saline soil may also occur in dry areas where soil drainage is impaired, evaporation increases, and rainfall is low. Salinity problems also occur in irrigated lands, particularly when irrigation water is of marginal quality [2]. 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.

The salinity stress is one of the most influential factors in limiting agricultural production [3]. The term saline is used in conjunction with a soil having a conductivity of more than 4 mmhos/cm at 25°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 referred as 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 [4]. The response of plants to the salinity or electrical conductivity (EC) levels expressed in mmhos/cm at 25°C is different. Tree legumes *Sesbania grandiflora* and *Leucaena leucocephala* exhibited higher salinity resistance than the cover crops legumes *Calopogonium mucunoides* and *Centrosema pubescens* [5]. Plant growing on saline soil is inhibited due to sodium ion poisoning (Na). The results of Sopandie [6] 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 [6]. Fuskhah [7] 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. Salinity may act as a water stress, which affects the photosynthetic rate, or may affect nodule metabolism directly [8].

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 [9] 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. Saline soils are generally deficient in nutrients, microbial activates and population is low [10]. 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. Isolation, and identification of rhizobium bacteria from food legumes

Isolation and identification of rhizobium bacteria was aimed to obtain rhizobium bacteria isolate from food legumes. The isolation and identification used root nodules of food legumes from north central coast area of Central Java namely Tegal, Pemalang, Semarang, Pati, Rembang, 95% ethanol, 0.1% sublimate, aquadest, yeast mannitol agar medium (YMA), congo red (CR) bromine thymol blue (BTB), NaCl, and tools for picking up root nodules. The root aggregates were collected, then sterilized, isolated, and identified according to Vincent [11].

2.1.1. Collection of root nodules. The root nodules were collected by separating the root nodule, by cutting 0.5 cm root on each side of the root nodules.

2.1.2. Sterilization of root nodule. Root nodules was immersed in a 95% ethanol solution for 5-10 seconds, then sterilant was immersed in a 0.1% sublimate solution for 3-5 minutes, then washed five times with sterile water.

2.1.3. Isolation of rhizobium bacteria. The sterile root of the 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°C) [11].

2.1.4. 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.

2.2. Selection and resistance test of rhizobium bacteria isolated from food legume against salinity. These activities aimed to obtain rhizobium bacterial isolates from food plants that are resistant to high levels of salinity, and find out how much the level of resistance of the rhizobium bacteria isolates to the level of media salinity.

2.2.1. Test the resistance of rhizobium bacteria to salinity. Basal medium, NaCl was sterilized at 121° C for 15 minutes. Addition of low concentrations of NaCl, then gradually increase the NaCl level gradually. As a control, basal medium was not added with NaCl. The prepared medium was then inoculated with Rhizobium bacterial isolates and incubated for 5 days at room temperature (28° C). After incubation, the number of Rhizobium bacteria colonies that were still able to grow were calculated.

2.2.2. Increased resistance of rhizobium bacteria to salinity. Colonies that were still able to grow were grown on a medium which gradually increases its NaCl concentration. And so on until Rhizobium isolates were found that were truly resistant to high salinity. Criteria for resistance to salinity were the number of colonies that grow. The parameters observed were the number of Rhizobium colonies.

3. Results and discussions

3.1. Isolation and identification of rhizobium bacteria from food legumes

Results showed that food crop legumes that can be found from coastal area of Central Java were soybeans (*Glycine max* L. Merr), peanuts (*Vigna sinensis* L.), green beans (*V. radiate* L.), and long beans (*V. unguiculata* L.). Effective root nodules, which were fresh, non-wrinkled and non-dried were taken for further sterilization and isolation. The laboratory analysis revealed 6 types of Rhizobium bacterial isolates from the following food crop legumes: 1) soybean isolated from Pati, 2) soybean isolated from Rembang, 3) peanut isolated from Pemalang, 4) green bean isolated from Pemalang, 5) Long bean isolated from Semarang, 6) long bean isolated from Tegal. The biochemical and Gram test of Rhizobium bacteria is presented in **Table 1**.

Table 1. The biochemical and Gram test of rhizobium bacteria

Isolates	Gram	YMA + CR	YMA + BTB
Long bean isolated from Tegal	negative	Pink	Yellow
Peanut isolated from Pemalang	negative	Pink	Yellow
Green bean isolated from Pemalang	negative	Pink	Yellow
Long bean isolated from Semarang	negative	Pink	Yellow
Soybean isolated from Pati	negative	Pink	Blue
Soybean isolated from Rembang	negative	Pink	Blue

Six purified isolates showed the same colony characteristic after 3-5 days incubation. The colonies milky white, translucent, glistening, and circular in shape, shiny, raised and 2-4 mm in diameter in YMA [12, 13]. The colonies were Gram-negative. All of the isolates obtained grew well on YMA + CR medium, white colony color and medium color remained pink. The colonies grown on the medium of YMA + BTB grows well, the medium color of green turn bluish after rhizobium grows for soybean isolated from Pati and soybean isolated from Rembang. Another 4 types of rhizobium bacterial colonies grown on the medium of YMA + BTB grows well, the medium color of green turns yellowish

after rhizobium grows. This indicates that rhizobium bacterial isolates that isolated from soybean from Pati and Rembang were included slow growing bacteria, but the other were included fast growing bacteria and acid producers [14]. Microbes have no real anatomical features, so bacterial identification is based on morphology, culture properties and biochemical properties. Morphology of microorganism based on shape and size is not sufficient to identify. Other features such as staining properties, colony growth pattern, growth reactions to carbohydrates, and the use of amino acids are helpful in the identification of microbe [15].

3.2. Selection and resistance test of rhizobium bacteria isolated from food legume against salinity

Rhizobium isolates tested for their salinity resistance were rhizobium bacterial isolates from the following food crop legumes: 1) soybean isolated from Pati, 2) soybean isolated from Rembang, 3) peanut isolated from Pemalang, 4) green bean isolated from Pemalang, 5) Long bean isolated from Semarang, 6) long bean isolated from Tegal. The number of rhizobium bacterial colonies (log CFU x 10⁶) each isolate at various levels of salinity media as in **Table 2**.

Table 2. Number of colonies (log CFU x 10⁶) of rhizobium isolates in agar media containing NaCl

Isolates	Level of Salinity (ppm NaCl)				
	0	2500	500	7500	10000
Long bean isolated from Tegal	0.34	0.31	-	-	-
Peanut isolated from Pemalang	1.20	2.06	2.53	0.81	0.90
Green bean isolated from Pemalang	2.30	2.30	2.30	2.00	3.87
Long bean isolated from Semarang	3.61	-	-	-	-
Soybean isolated from Pati	3.38	-	-	-	-
Soybean isolated from Rembang	2.62	2.48	2.48	2.00	2.85

Table 2 shows that the tested rhizobium isolates showed resistance at various levels of salinity. Rhizobium bacteria isolated from Pemalang green bean root nodules showed their resistance to very high levels of salinity which is still able to grow on media with a concentration of 10000 ppm NaCl or equivalent to electrical conductivity (EC) 17.5 mmhos/cm [16]. The level of salinity is divided into 5 categories based on the value of DHL (electrical conductivity) or EC in mmhos / cm which are very low (0-2), low (2-4), medium (4 - 8), high (8-16), and very high (> 16) [17]. Rhizobium bacterial isolates isolated from Rembang soybean root nodules and rhizobium bacterial isolates isolated from Pemalang peanut root nodules also showed very high salinity resistance of 10000 ppm NaCl compared to other rhizobium isolates tested. When viewed from the number of colonies that are still able to grow in media with high salinity levels, the results showed that the order of resistance to salinity began with the most resistant rhizobium isolates isolated from green bean nodules, followed by soybeans, peanuts and long beans.

Rhizobium bacteria that are grown at low salinity levels, then grown again at a level of salinity that is gradually increased will also show their resistance at a high level of salinity to a certain extent. The resistance of Rhizobium bacteria obtained at high salinity levels is presumably also caused by the origin of isolates taken from the coastal area. Gilmour (1990) in Atlas and Bartha [18] states that there are microorganisms that are tolerant of high salt levels called halotolerant and there are microorganisms that require high salt concentrations called halophilic. Rhizobium obtained may include halotolerant that are still able to live at high salt concentrations. In addition to influencing osmotic pressure, high salt concentrations tend to change the nature of proteins by disrupting protein structures that are important for enzymatic activity. The mechanism of resistance of halotolerant microorganisms is that these microorganisms tend to excrete sodium ion concentrations that are relatively toxic and high from the inside of the cell to achieve osmotic balance [18].

4. Conclusion

Food crop legumes that can be found from coastal area of Central Java were soybeans (*Glycine max* L. Merr), peanuts (*Vigna sinensis* L.), green beans (*V. radiate* L.), and long beans (*V. unguiculata* L.). The laboratory analysis revealed 6 types of rhizobium bacterial isolates from the following food crop legumes: 1) soybean isolated from Pati, 2) soybean isolated from Rembang, 3) peanut isolated from Pemalang, 4) green bean isolated from Pemalang, 5) Long bean isolated from Semarang, 6) long bean isolated from Tegal. The most resistant sequence of resistance to salinity was rhizobium bacterial isolates from green beans, soybeans, peanuts, and long beans respectively.

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