Study of the presence of Rhizobium bacteria which were inserted into the cell tissue of soybean seed using vacuum technology

S Lekatompessy1, E Murniati2, T Kartika2, H I Sukimn1
1 Research Center for Biotechnology LIPI1
2 Bogor Agriculture University, Indonesia2

Email: sylviahrl@yahoo.com

Abstract. Rhizobium bacteria was known well as the bacteria which has potential on fixing nitrogen from the air and its widely used as biofertilizer. So far, the inoculants normally consist of Rhizobium bacteria which was packed in carrier material such as peat soil and applied to crop seed by mixing the seed with Rhizobium inoculant. This technique Rhizobium inoculants was not efficient anymore since much of bacteria cell was falling down, it did not completely sticked on the surface of seed. So this condition, resulted of un-effective function of the bacteria on running the nitrogen fixing process. To solve the problem, effort has been made to insert the bacteria inside the seed tissue using simple technology. This technology was developed for the target of symbiosis living between the bacteria and plants through optimal nitrogen fixing process. The purpose of this study is to confirm the presence of Rhizobium bacteria. That has been inserted into the soy bean seed using simple vacuum technology. The present of Rhizobium bacteria inside the seed tissue was observed from the preserved slices of seed tissue under amicroscope. The preserved slices of soybean seed tissue shown that the Rhizobium bacteria cell were able to reach the inside of seed tissue and fulfill the air space of palisade tissue.

1. Introduction

Glycine max (L.) Merrill or soybeans is the essential food source that has a protein content of ± 39%. Soybeans has an economic value in human life [6]. Soybeans is used for various purposes, especially for food and industrial raw materials [3]; [9]. Soybean has been consumed as the second staple food after rice since decade. People consume soybean mainly in the form of tempeh, traditional fermented food which made from soybean. Beside of that soybean was needed as raw material for making soy sauce, soybean milk, tofu, tauco and other soybean based snack. This condition made the requirement of soybean become very high. The national production of soybean in 2011 reached ± 870,000 tons of dry seeds per year, while the domestic demands of soybean about ± 2 million tons per year. The lack of national soybean production is caused by higher production cost, lower the productivity and lower selling price of soybeans which caused farmers disinterest in planting soybeans. Limited agricultural land also one of the constrain that the farmer did not very interesting on growing soybean. The figure in 2012 shows that the national production of soybean even worst than in 2012. The total production is only 783.158 tons per year. This production was resulted from 570.495 ha of agriculture land area. To overcome the national demands of soybeans, the government has to import soybean from other countries. Nowadays the government of Indonesia has to import the soybean from other countries such as Australia, United States, and neighbor countries in Asia up to 2 million tons annually.
One of the effort to improve the national production of soybean is extensification of soybean growing area. In 2013 the target of extensification area is 976.000 hectare for targeting production from 870.000 ton to 1.444.364 tons dried seed per year. This effort will be done by growing soybean in swamp peat soil area, dried land, and Perhutani area in total of 220.000 hectare beside the agricultural land which normally used to grow paddy rice.

In regard to grow soybean, the requirement of chemical fertilizer will also increase. As the program of green agriculture, it is recommended to use biofertilizer on growing crops including soybean. In this case it is well known that Rhizobium, nitrogen fixation bacteria, could be used as biofertilizer. So far the bacteria applied as inoculants to the seed of soybean. The application was done by mixing the seed with inoculants and hopefully the bacteria will covered the surface of seed and when the seed germinate the bacteria will start to infect the root of soybean. Normally the bacteria cell of Rhizobium was packed using carrier material. The carrier material is peat soil, the method of application sometimes was not efficient anymore because much of the bacteria did not stick longer on the surface of seed. The requirement of bacteria cell population is not enough to do the infection of root. As a result of that root nodules may fail to develop because of law population of Rhizobium cell which are available and stick on the surface of seed. This condition also provoke by the present of indigenous Rhizobium bacteria which might be much stronger than introduced Rhizobium bacteria. It is difficult to displace or replace them with the introduced strains. So large population of organism, around the developing seedling is important for rapid and effective nodulation [2]. In regard to the nutrient needed, a study of symbiosis living between bacteria and plants explained that attracted bacteria cell to the root plant depend on the type of root exudates produced by root of host plants. Beside of that nutrient is key constituent for nodule initiation, nodule development and nodule function. Most of the nutrient needed in nodulation process are Cobalt, Boron, Ferro, Molybdenum, Ca, and Cu. Those nutrient will be function on nodules formation.

LIPI has developed the technology which Rhizobium sp. bacteria cell could be inserted into soybean seeds. This technology actually aimed to save the cell bacteria from the influence of extreme condition in the soil when the seed planted in field. Soybean seed which was inserted by Rhizobium bacteria cell finally named as soybean plus LIPI. The plus soybean is the seed containing potential microbes in conducting to nitrogen fixation process. The soybean plus can be applied directly to the field by the farmer without any other treatment and did not require chemical fertilizers optimally. Soybean plus could reduced the requirement of chemical fertilizer up to 50%. So that the soil pollution could be reduce slowly. Sukiman [10] reported that the production of soybean plus in number of soybean growing area in Java could be twice than normal production. This result promoted the important of Rhizobium as biofertilizer substitute. It has reported also that the use of potential soil bacteria indirectly will support the availability of healthy food as it prepare through the green farming system. So, the establishment of optimizing the methodology of gaining the potential microbes on supporting the growth of crop is very crucial to be done. One of the innovation method is inserting the bacteria inside the seed tissue.

The presence of bacteria cell was study further and preserved slices seed tissue were prepared to confirm that innovation technology could be used for biofertilizer application. This innovation technology provides the efficient technique of application and guarantee the quality of product to be effective on supporting the growth of plants.

2. Material and Methods
2.1 Method of inserting the bacteria cell into the seed tissue.

The method of inserting the bacteria into the seed tissue was done as describe by Sukiman 2008. The inserting of bacteria was conducted using the engineered vacuum machine which developed by RC of Biotechnology LIPI. An amount of soybean seeds was mixed with suspension of Rhizobium sp. in population of about $10^9$ cells/ml. Seeds inserted into the vacuum device with a certain pressure. Further vacuum device suddenly opened so as to provide pressure to push the bacteria Rhizobium sp. Seed goes into the seed tissue (Figure 1). Expected from this method, the bacteria cell will be goes
forward to fill the air space area inside the seed tissue. The cell bacteria will penetrate the skin layer of seed with the help of air pressure in the vacuum device [8].

2.2. Preserved seed slices preparation

Preserved seed slide was prepared according to the method describes by Rijadi, S.J. 2008. At first soybean seed which was inserted by the bacteria and control seed was soaked inside the 70% alcohol and then connected with vacuum pump to removed the air inside the seed. The soaked seed was then incubate for overnight. After that the seed was treated by dehydration technique using range of alcohol concentration starting from 70%, 80% and finally 90% for 3 hours respectively. Following that, the seed then put into alcohol-xylene solution with different ratio for 3 hour respectively. Finally the seed was put into paraffin solution step by step starting from xylene paraffin solution with ratio 3:1, 1:1, and 1:3 and finally pure xylene. Seed then was put on block with holder.

Slicing the preserved seed was done using microtome on 10 micron (Figure 2). Slicing paraffin ribbon was the put into object glass which has applied with haup adhesive on the surface of hot plate at 40°C. The paraffin ribbon was incubated until enough dried for 3 days. Observation of seed slide preparation was done after the staining step. Staining step was done using safranin and fast green. Microscope observation was done to understand the position of bacteria cell inside the seed tissue.

Figure 1. The Rhizobium bacteria insertion into the seed tissue through the vacuum technology

Figure 2. Seed slide using paraffin method

2.3. Soybean seed storage methodology

An amount of soybean seed was treated by inserting Rhizobium bacteria cell into the seed tissue. The seeds were separated among these treatment with the control seed sand labeled. Seeds then packed into the plastic subsequently sealed and stored in the cans. Effective plastic packaging was aimed to retard water content during storage. Seeds stored on the room temperature range 24°C-31°C. This conditions applied actually to adjust with method of seed treatment normally done by the farmer since the farmers did not have sophisticated facilities and non-treated seed (KO) were stored and regularly during range period of storage 0 – 4 moths, seeds were took off and preserved seed slice was prepare as describes by [7] method.
3. Result and Discussion
The use of nitrogen-fixing bacteria inoculant Rhizobium sp. for growing for seed storage. Treated seed (K+) soybean has been known since decade. Rhizobium bacteria recognized as beneficial bacteria since it can run the nitrogen fixing process and supplying sufficient nitrogen for optimal plant performance[4]. In addition to that the use of potential microbes on supporting the growth of plants could be minimizing pollution from excessive nitrogen application. Pollution because of chemical fertilizer gave an effect of poison for human being life.

The promotion of using potential microbes as biofertilizer substitue is become important. It is well known that the green technology were the major force in the bringing the increasing of crop yields. One option of this technology is to involved mainly the use of biofertilizer especially nitrogen (N). Range of N$_2$ Fixing organisms which could contribute nitrogen to agriculture are well identified. Heterotrop organism (e.g. Rhizobium, Azotobacter, Azospirillum and autotrop (e.g Rhodospirillum, Anabaena, Cyanobacteria) microorganism have been surely confirmed can fix nitrogen from the air [5].

Symbiotic N$_2$ fixing is dependent on establishment of a successful association between the host species and N$_2$ fixing organism. The N$_2$-fixing organism must be present and able to develop symbiosis living with the host species. Inoculation usually involves addition for the organisms either directly to soil or coated onto seed, however sometimes it was not very effective caused of many technical aspect concerned. Farmer education level become one of the serious constrain on successfully the application of inoculant. To solve those constrains, LIPI developed the technology namely soybean plus technology.

Soybean plus seeds are seeds that has potential microbes which able to conduct the process of biological nitrogen fixing Soybean plus seeds can be directly applied to the field by farmers and does not require chemical fertilizers optimally because nitrogen required by the plants is already available from the result of fixing nitrogen from the air.

![Figure 3](image)

**Figure 3.** Soybean seed slices stained using fast green staining
A: Bacterial Rhizobium inserted soybean seed slices tissue (K+)
B: Control soybean seed slices tissue (KO)

Confirmation of vacuum technology is subjected into seed with insertion of Rhizobium sp. (K+) and without insertion (KO). The result of observation under the microscope shown that the bacteria could goes through the inside of seed tissue that is the palisade tissue. Figure 3 indicated the horizontal seed slices preparation stained with the fast green dye. It could be seen that at treatment K+, the bacteria cells was found placed the air space of seed tissue that is at palisade tissue. Observation under the microscope show that the bacteria cell looks in irregular shape. This condition happened since the vacuum treatment which was exposed to the bacteria cell might gave an shock so then the position of cell become irregular. Treatment KO explained that air space of seed tissue did not filled out by the bacteria cell, since before vacuum treatment there is no bacteria added to the seed. Similarly, figure 4 shown the result of horizontal slice seed preparation using red safranin dye. Staining with red safranin dye provided clear figure compare to the fast green dye. The red safranin provide the color of
bacteria similar to Gram stain, the cell even clearly visible located in palisade tissue. It is proved that the bacteria cell can be inserted to soybean seed through the vacuum technology.

The existence of the bacteria inside the seed tissue was evaluated in regard to the length of storage. Soybean seed normally is viable only up to 3 months storage, however after that; the viability of seed will be decrease. The optimal percentage of germination recommended for planting usually is above 90%. Below 90%, the success of germination could not be much guarantee. Meanwhile since the stock of soybean seed sometimes is limited so the evaluation of the seed survival and viability during the period of storage is very important. Study the viability of soybean seed which has inserted by the bacteria was conducted in regard to the viability of *Rhizobium* cell when it sowing in the field.

The experiment result showed that the bacteria which has inserted into the soybean seed and storage for a certain period of time still viable and the characterization of the bacteria itself was not changes. Figure 3 shows the presence of bacterial cell (*Rhizobium* sp.) inside the seed tissue and indicates that fast green staining does not provide clearly visible figure to distinguished the present of bacteria cell compared to control seed.

In contrast, Figure 4 shown the soybean seed slice which is staining with safranin. It is clearly seen that the bacteria cell are present inside the seed tissue and coloring as Gram stain. Red Safranin dye is more suitable to use for detail observation on the presence of bacteria. The bacteria are visible even in irregular form. This result proved that the vacuum technology could be work effectively to insert *Rhizobium* sp. into the soybean seeds. Slices of preserved soybean seeds indicate that the bacteria *Rhizobium* sp. could get through into the palisade tissue.

![Figure 4. Soybean seed slices using safranin staining](image)

A: Bacterial *Rhizobium* inserted soybean seed slices tissue (K+)
B: Control soybean seed slices tissue (KO)
Figure 5. The bacteria remained viable inside the seed tissue during 0 – 4 months storage period. Note: (1) surface of the seed coat ; (2) pa: palisade tissue ; hy: hourglass; co: cotyledons ; pr: parenchym

Figure 5 shows a mounted seed slice preparations of soybean inserted seed which have stores on the period of 0 to 4 months. Preserved slices of soybean seeds were compared with seeds slice of reference [1]. The experimental results clearly shown that the bacterial cell is located in the palisade tissue. The effect of storage did not influence the viability of bacterial inside the seed. The viability of bacteria remained stable and could actively infect the root plant even after 4 months storage. The figure of seed slices during period of storage indicate the same condition of bacteria cell, stable and the population of bacterial cell are steady. This result promote that the inserted bacteria through the vacuum technology could be state as one of the short cut technology on microorganism application and cover the problem of reducing number of cell during application. The slices intact seeds were seen starting from the seed surface, palisade tissue, tissue “hour glass layer” and cotyledons similar as shown in Figure slice of reference (f).

Proving the existence of bacteria in the seed tissue, Figure 6 shows the pattern of bacteria which are not regular in shape instead of rod-shaped form. The irregular form pattern was present because when the bacterial cell was applied by the vacuum device, it will resulted randomized irregular form. The seed slices which consist irregular form of bacterial cell was shown in comparison with the microscopic observation of fresh cell.

Figure 6. Microscopic analysis indicated that the cell shape of *Rhizobium* bacteriain the seeds same as in pure culture
The cell population of *Rhizobium* sp. inside the seed is in the range of $6.6 \times 10^5$ cells/ml. Pressure is used to enter the bacteria do not harm the skin of soybean seed. Insertion of bacteria to seed plus provide a significant influence on the amount of cell populations *Rhizobium* sp. compared to control seeds, since the seeds controls did not find any bacterial cell *Rhizobium* sp. Cell populations of *Rhizobium* sp. which are inserted into the soybean seeds during seed storage ranging from 0-4 months was shown in Figure 6. The result indicates that the population of *Rhizobium* sp. cell during the period of seed storage ranging from 0-4 months can be maintained in the range of $6.6 \times 10^5$ cells/ml. Those results of population of *Rhizobium* sp. cell supported the observations that have been made in Figure 4 and Figure 5.

In this case it is confirmed that bacteria cell could be applied by inserted the cell into the seed tissue and during the storage period the number of cell could be maintain stable at the requirement of number of cell needed for infection the root plant.

### 4. Conclusion

This research study proved that the vacuum technology could be used for inserting the bacteria cell into the seed tissue. The seed which consist of bacterial cell could store until 4 months without losing their viability and more over the bacteria which was inserted into the tissue of seed remained stable and viable until the time they start to infect the root plant. The population of bacterial cell could be maintain viable at $6.6 \times 10^5$ cell per ml and it was inserted inside the seed. The amount of cell of bacteria inside the seed, it was same the amount of cell of bacteria which it was freshly inserted. Histological study of preserved of seed slice indicated that for staining red safranin dye provide detail figure of seed slices which support to identified the location of bacterial cell. Bacterial cell actually located at palisade tissue of seed. Innovation technology of inserted the bacterial cell into the seed could be promote the effective, efficient way of microbes inoculation and this technology could be socialize and recommended to be use further by the soybean farmers.

### 5. Acknowledgement

The authors would like say thanks to Harmastini Sukiman, M.Agr and all member of Plant Symbiotic Microbe.

### 6. References

[1] Agarwal VK and James BS 1997 *Principles of Seed Pathology* Second edition Lewis Publishers New York
[2] Bacon PE 1995 Nitrogen Fertilization in the Environment. Publishers Marcel Dekker Inc. New York
[3] Damardjati DS, Marwoto DKS, Swastika D M, Arsyad Y and Hilman 2005 Prospek dan Arah Pengembangan Agribisnis Kedelai Badan Penelitian dan Pengembangan Pertanian Departemen Pertanian Jakarta
[4] Dudeja SS, Singh NP, Sharma P, Gupta SC, Chandra R, Dhar B, Bansal RK, Brahmaprakash GP, Potdukhe SR, Gundappagol RC, Gaikawad BG and Nagaraj KS 2011 Springer
[5] Ledgard S F and Peoples M B 1988 Measurement of nitrogen in the field. In: Advances in nitrogen cycling in agricultural ecosystems Wallingford UK CAB International
[6] Prentis S 1990 In Biotechnology: A New Industrial Revolution George Brazziler Inc. New York
[7] Rijadi S J 2008 Pelatihan Singkat Kerja Histologi, Sitologi dan Perkecambahan Polen Pusat Penelitian Bioteknologi LIPI Cibinong
[8] Subagio M 1999 Petunjuk Pemakaian Alat Pembuat Bibit Unggul Kedelai (Mixer Vacuum Device) Pusat Penelitian dan Pengembangan Kalibrasi Instrumentasi dan Metrologi LIPI Serpong
[9] Sudaryanto T D K S and Swastika 2007 Ekonomi kedelai di Indonesia Di dalam Kedelai: Teknik Produksi dan Pengembangan Pertanian Bogor
[10] Sukiman H I 2008 Kedelai Plus Pusat Penelitian Bioteknologi LIPI Konferensi Press