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# *Association Of Nitrogen-Fixing Bacteria In Rice*

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## ABSTRAK

Penelitian ini bertujuan pertama: untuk memahami penanda GFP (protein fluoresen hijau) memberikan identitas bakteri penambat nitrogen yang jelas sebagai endofit ketika diaplikasikan pada jaringan tanaman. Penelitian ini bertujuan kedua: untuk memahami bakteri penambat nitrogen membuat kolonisasi khusus sebagai endofit menggunakan metode Seed dressing dan metode Dipping. Hasil penelitian berupa marka GFP yang menunjukkan kemampuannya untuk memberikan identitas yang jelas sebagai bakteri endofit ketika diaplikasikan dalam komuni mikroba alami. Selain itu, GFP juga stabil dalam aplikasinya pada jaringan tanaman padi dan mampu memberikan ekspresi yang cukup jelas. Bakteri Azospirillum lipoferum-1224, Azospirillum lipoferum-1247, Azorhizophilus paspali-2283, Azotobacter chroococum-2286 mampu mengkolonisasi jaringan tanaman padi baik menggunakan metode inokulasi Dipping atau Seed dressing.

## *ABSTRACT*

*The research was aimed first: to understandability of GFP marker (green fluorescent protein) gave identities nitrogen-fixing bacteria that clear as endophytic when was the application to plant tissue. The research was aimed second: to understandability of nitrogen-fixing bacteria make special colonization as endophytic use Seed dressing method and Dipping method. The results of the research are GFP markers that show its ability to provide a clear identity as an endophytic bacterium when applied in natural microbial communion. Besides that, GFP is also stable in the applications on rice plant tissue and is able to provide a fairly clear expression. The bacteria Azospirillum lipoferum-1224, Azospirillum lipoferum-1247, Azorhizophilus paspali-2283, Azotobacter chroococum-2286 were able to colonize the rice plant tissue using either the inoculation method of Dipping or Seed dressing.*

## INTRODUCTION

The special association system between plants with bacteria is very important for the significance of nitrogen contributions and consistently such as Acetobacter and Frankia which are specifically associated with sugar cane as endophytic bacteria (Kennedy et, al.,1997). Inoculation of bacteria in the plant to be successful following expectations, there are several factors that need

considering, among others; the first nitrogen-fixing activity by endophytic bacteria is determined by compatible associations to between bacteria and plants, so it was recommended by Watanabe (1986) for manipulation of bacteria and plant associations. To find out the special association between bacteria and plants a marker is needed in bacteria such as GFP (green fluorescent protein), in this study using GFP. second, bacteria is affected by competition between target bacteria and other bacteria or fungi to get nutrients (Kamuru, et, al., 1998). Third, bacteria are influenced by genetic factors both bacteria and plants (Rosenblueth and Romero, 2006). And the factor is further influenced by the environment (Lagreid, et, al., 1999). So as to anticipate the second and third problems that are suitable methods for bacterial inoculation. Thies, et, al., (1991) also to recommend the effectiveness of nitrogen-fixing bacteria inoculation in plants, an inoculation model is needed. in this study, two models of bacterial inoculation were carried out namely Seed dressing inoculation method (seed) and Dipping method (seedling).

## MATERIAL AND METHODS

To find out special associations between bacteria and plants are needed as a marker for bacteria such as GFP. The GFP transformation stages for bacteria will be explained as follows:

**Nitrogen-Fixing Bacteria**

In order to implement the transformation of genes, nitrogen-fixing bacteria (Azospirillum lipoferum-1224, Azospirillum lipoferum-1247, Azorhizophilus paspali-2283, Azotobacter chroococum-2286) ware tested for antibiotic resistance of kanamycin and ampicillin (50 and 100 µg/ml). Whereas the bacteria carrying the GFP gene (E. Coli 17.1) is resistant to the antibiotic kanamycin and ampicillin but not resistant to rifampicin. The next step is to test the resistance of nitrogen-fixing bacteria to rifampicin by growing bacteria in solid media LB that is containing rifampicin antibiotics 70 µg/ml. The growing colonies were then regenerated in solid LB medium containing rifampicin 50 µg/ml.

**The Bacteria Nitrogen-fixing Association Test in Rice**

The bacteria nitrogen-fixing association test on rice was held with two methods; the first inoculation of bacteria with seed dressing method, the second with the dipping method.

**Seed Dressing Inoculation Method**

Before the bacterial inoculation method using seed dressing was done, it first grew nitrogen-fixing bacteria that containing GFP marker ((Azospirillum lipoferum-1224, Azospirillum lipoferum-1247, Azorhizophilus paspali-2283, Azotobacter chroococum-2286), each bacterium stored in glycerol 12% - 15% was taken as much as 100 µl then put into an Erlenmeyer flask 250 ml which already contained 50 ml liquid LB medium containing 50 µg/mg kanamycin antibiotic, and shake at 6000 rpm for 48 hours. Then partially taken 1000 µl with micro pipet 200-1000 µl for counting bacteria by TPC (Total Plate Count) method, the next step centrifugation at 6000 rpm for 10 minutes, then the supernatant is removed, the pellet is taken and added 5 ml phosphate buffer then centrifuged again at 6000 rpm for 10 minutes, the supernatant was discarded while the pellet was taken and 5 ml phosphate buffer was added and then it was vortex until homogeneous.

The method of bacterial inoculation by means of seed dressing was carried out inoculating bacteria in rice seeds of approximately 2,9 x109 CFU/ml. Each homogenous bacteria was mixed with 10 gr of seed rice and soaked for 24 hours with 200 ml of sterile water in an Erlenmeyer flask, then the rice seeds were sown in a tray filled with soil. To test the endophytic properties of four nitrogen-fixing bacteria, bacterial re-isolation from two parts of seedling was carried out, including roots, stems, and leaf. Re-isolation is carried out on seedling aged 15 and 21 days after the nursery. The first step in doing re-isolation is to take the seed sample then clean it from the dirt that is attached using flowing tap water, the cut it with a sterile knife into two parts (root, stem + leaf), the results of cut are soaked with 10% sodium hypochlorite as much as 150 ml for 20 minutes, after washing with sterile water for five times. Each part was added with 1000 µl sterile water and then crushed sterile mortal, from the results of scouring taken 1000 µl with a micropipette and inserted into a test tube that had previously been prepared, the tube contained 9 ml sterile water, then diluted from 10-1 to 10-4.

The results of dilution, each taken as much as 100 µl using micro pipet to be grown by pour plate and spread plate method on solid LB media containing kanamycin 50 µg/ml, and bacteria incubated for 48 hours at room temperature. Observations of green fluorescent bacteria colonies were carried out under UV illuminators, if not present, the bacteria were considered not endophytic in rice plants, and if present,

these bacteria were considered endophytic in rice plants and then counted the number of colonies using the TPC method.

**Dipping Inoculation Method**

The method of bacterial inoculation using the dipping, the method was done inoculating bacteria by dipping the roots of rice seedling aged 21 days after the nursery approximately 23,5 X $10^9$ CFU/ml. Each of the four nitrogen-fixing bacteria (Azospirillum lipoferum 1224, Azospirillum lipoferum 1247, Azorhizophilus paspali 2283, Azotobacter chroococum 2286) which had been cultured fixed mixed with 200 ml sterile water in the Erlenmeyer flask. 21-old-day rice seedlings are removed from the nursery and the roots are cleaned of dirt and the next step is dipped in sterile water containing a mixture nitrogen-fixing bacteria that has been prepared beforehand, and silenced for 30 minutes.

Rice seeds that have been dipped in bacteria, then transplanted in a pot containing 5 kg of soil, and silenced the seed to grow for 7 days, then re-isolated.

Seed samples are taken and cleaned from the sticky dirt using flowing tap water, then cut with a sterile knife into three parts (roots, stems, and leaves), the cuttings are soaked with sodium hypochlorite 20% as much as 150 ml for 20 minutes, after washing by using sterile water for 5 times. Each of the three parts ware then crushed with mortar and pestle sterile and added 1000 µl of water sterile, the results of scouring ware taken 1000 µl and put into a test the tube that had previously been prepared to contain 9 ml of sterile water, then diluted from $10^{-1}$ to $10^{-4}$

The results of $10^{-2}$ dilution to $10^{-4}$ each were taken as much as 100 µl with a micro pipet to be grown on solid LB media containing Kanamisin 50 µg/ml, and bacteria incubated for 48 hours at room temperature. Observation on green fluorescent bacterial colonies ware carried out under UV illuminator, if not present, the bacteria ware considered not endophytic in rice plants, if present, these bacteria considered endophytic in rice plants and then counted the bacterial colony by the TPC method.

**The Observation**

1. Reisolation of four nitrogen-fixing bacteria using GFP (green fluorescent protein) markers in the dipping method.
2. Reisolation of four nitrogen-fixing bacteria using GFP (green fluorescent protein) markers in the seed dressing method.

## RESULTS AND DISCUSSION

**The Nitrogen-fixing Bacteria With GFP Marker**

The marker is needed to trace back the nitrogen-fixing bacteria to be studied, which is inoculated on rice plants. GFP marker is a molecular tagging that is currently widely used to observe gene expression and protein localization. The use of GFP as a marker does not require much preparation. GFP as a marker has the following advantages; its stability in relation to the shape of the structure, suitable for a species-independent prokaryote, or Eukaryota applications, does not interfere with analysis without the need for substrates or energy, while in vivo monitoring maintains the integrity of cell life. (Tsien. 1998).

In this study, the GFP marker was used to mark four bacterial inoculums that ware inoculated into rice tissue so that it would be easier to trace and bacteria could be re-isolated from the tissue without having to be mixed with indigenous bacteria which had naturally been in the rice tissue. Marking isolates with the GFP gene is done by transforming the genes contained in Escherichia coli PFAJ 1819 on the target bacteria chromosome so that recombinant bacteria that are able to express the GFP gene are produced. The success of the GFP gene transformation against a target, bacteria is characterized by green fluorescent emitted by bacteria when seen under UV light. The results of tracing bacteria using the GFP marker are shown in the figure below:

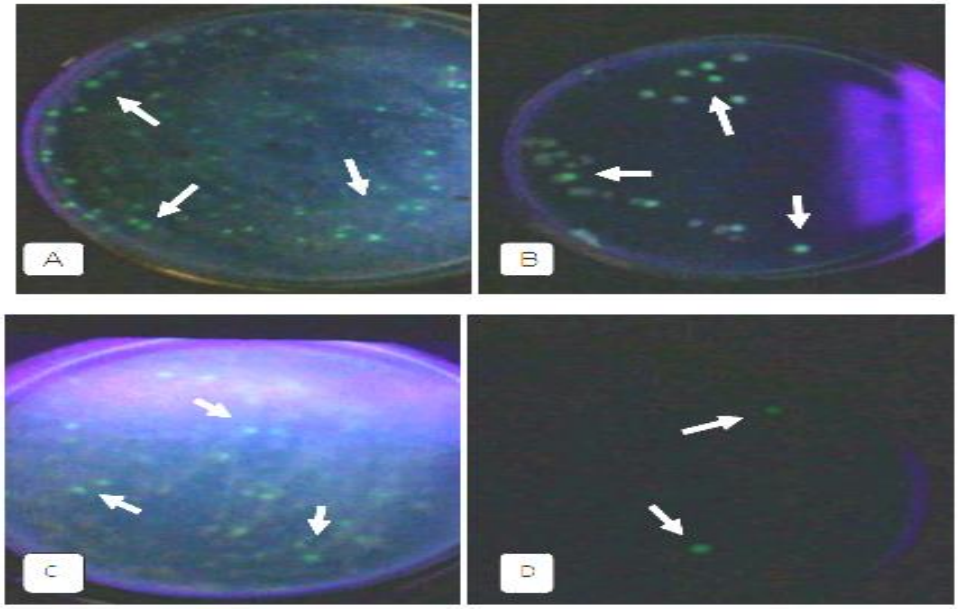


Figure 1. Nitrogen-fixing Bacteria with GFP markers, A : Azotobacteri chrococum-2286, B : Azorhizophilus paspali-2283, C : Azospirillum lipoferum-1224, D : Azospirillum lipoferum-1247

The results of the GFP marker on nitrogen-fixing bacteria that were tried in this study showed that GFP gene insertion occurred in the bacterial chromosome. GFP markers show its ability to provide a clear identity as an endophytic bacterium when applied in natural microbial communion. Besides that, GFP is also stable in the applications on rice plant tissue and can provide a fairly clear expression to see the picture above.

**The Results of Dipping Method**

Bacterial inoculation using the dipping method is done by dipping the roots of rice in bacteria. Bacterial inoculation using the dipping method from the experimental results showed four bacteria Azospirillum lipoferum - 1224, Azospirillum lipoferum - 1247, Azorhizophilus paspali - 2283, and Azotobacter chrocoocum – 2286 able to associate specifically, as endophytes within the rice, see figure 1.

Inoculation bacteria of a strain of Azospirillum lipoferum – 1224 can infect from roots, stems, and leaves. Bacteria that have been attached and colonized on the root surface can enter into the rice plant tissue through a gap formed in the lateral root crack and enter the epidermal tissue at the root tip and move through the xylem (Dobereiner et, al., 1995 in Dong et, al., 1997). The re-isolation of strain Azospirillum lipoferum-1224, 7 days after planting showed that the most colonies of bacteria were found in plant stem tissue, this result was also proven by Mano and Morisaki (2008) that endophytic azospirillum bacteria and Herbaspirillum bacteria were found in the root and stem tissue, most of these bacteria are found in the stem tissue of rice plants. The movement of bacteria from roots to stems and leaves are caused more by the environment in plant tissues, such as high osmotic pressure, changes in plant biomass and ecological systems between bacteria and plants.

The result of reisolation using the dipping method on bacteria strain of Azospirillum lipoferum – 1247 showed that bacteria colonization of plants was almost the same as other bacterial strains starting from roots, stems and leaves, but the strain of bacteria Azospirillum lipoferum – 1247 not found in the stem able to survive and does not macth the environmental conditions in the stem tissue. Colonization between Azospirillum lipoferum – 1247 bacteria in rice plants was very low compared to the other three bacterial strains. This showed that there was a discrepancy between bacterial strains and varietal rice plants, although the Azospirillum lipoferum – 1247 bacteria had a special relationship with rice plants as endophytes, but these bacteria were inside rice plant tissue cannot survive and the possibility of contributing nitrogen to the host plant will be small.

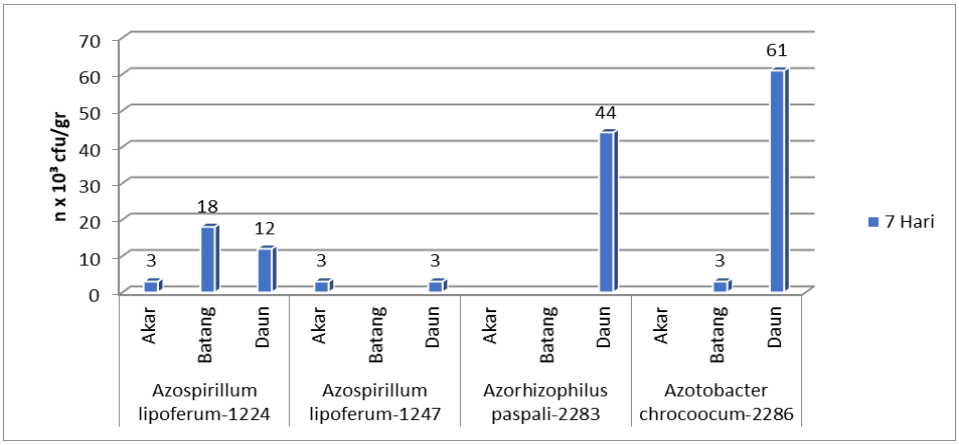


Figure 2. The results of reisolation of bacterial populations 7 days after planting

The results of reisolation bacteria of a strain Azorhizophilus paspali-2283 on seedlings showed that leaves tissue was a suitable place for strain of Azorhizophilus paspali-2283 bacteria in seedlings aged 7 days after planting. Although not found in the roots tissue and stems of bacterial colonies, it is suspected that bacteria also colonized starting from the root and then moving to the stem and then experiencing significant growth in leaves tissue.

The results of reisolation bacteria of strain Azotobacter chrococum – 2286 showed that conditions suitable for the growth of Azotobacter chrococum – 2286 bacteria were leaves and stem tissue at the age of seedlings 7 days after planting, colonization of Azotobacter chrococum – 2286 in plant seeds suspected to be with other bacteria through stem roots and then on the leaves.

The ability of Azotobacter chrococum – 2286 bacteria to multiple colonies in plant tissue are largely determined by bacterial strains with plant varieties and also environment changes in the plant tissues, this was also revealed by Dong at. al., (2003) in his research on the ability of the bacterium strain Klebsiella pneumoniae Kp342 to colonize several types of plants showed that the colonization process of a type of bacteria against plants is not a random process but an active process controlled by differences in the genetic material of bacteria at the strain level.

**The Results of Seed Dressing Method**

Bacterial inoculation using the seed dressing method was done soaking rice seeds with bacteria, bacterial inoculation using seed dressing method from the experimental results showed four bacteria *Azospirillum lipoferum-1224*, *Azospirillum lipoferum-1247*, *Azorhizophilus paspali-2283*, and *Azotobacter-2286* able to associate specifically as endophytic with rice plants. Reisolation of bacteria was carried out at seedling age 15 and 21 days after nursery. See figure 3.

The results of reisolation 15 days after the nursery generally showed a suitable place for all tested bacterial strains in the root tissues. The results of this reisolation were assumed that all strains of bacteria using the initial penetration seed dressing method were carried out on the rice seed embryo and then grew on the root tissue. According to Syamsudi (1997) bacteria will infect seeds that have a water content of more than 20% while the fungus will infect seeds that have a water content of 14%, with enough water content the seeds can provide many nutrients such as carbohydrates, a nitrogen compound, minerals and compounds addition to bacterial growth.

The bacterial population in rice plant tissue using seed dressing method was more than using the dipping method because bacteria inoculated using seed dressing were earlier adapted to plant tissue (initial penetration of rice seed embryos and later on root tissue) compared to dipping method (initial penetration at the root).

Bacterial strains of all tested were able to compete with bacteria and natural fungus that exist in plant tissue, although the response of each bacterium was different, an experiment from four bacteria tested by strains of *Azospirillum lipoferum* – 1247 which were later than other bacteria, the fastest response was by a bacterial strain of *Azotobacter chrococum* – 2286.

Reisolation of *Azospirillum lipoferum – 1224* strains at the age of 15 days of the seedling is suitble for the growth of these bacteria. At seed age 21 days after nursery strain *Azospirillum lipoferum*-1224 is still present in the roots even though the bacterial population is small compared to the age of the seedling 15 days after the nursery. Bacterial population 21 days after seedling on the stem begins to grow but in leaf tissue has not been found. The movement of bacteria from root tissue to stem and leaf is more

due to environmental conditions in plant tissue such as osmotic pressure, and the availability of nutrients in the tissue.

*Azospirillum lipoferum-1247* bacteria are based on reisolation in 15 days after the nursery is found in the root tissue. In the stem tissue and leaf tissue not found, then only reisolated 21 days after the nursery was found in the leaf tissue, but not found in the root tissue and stem tissue.

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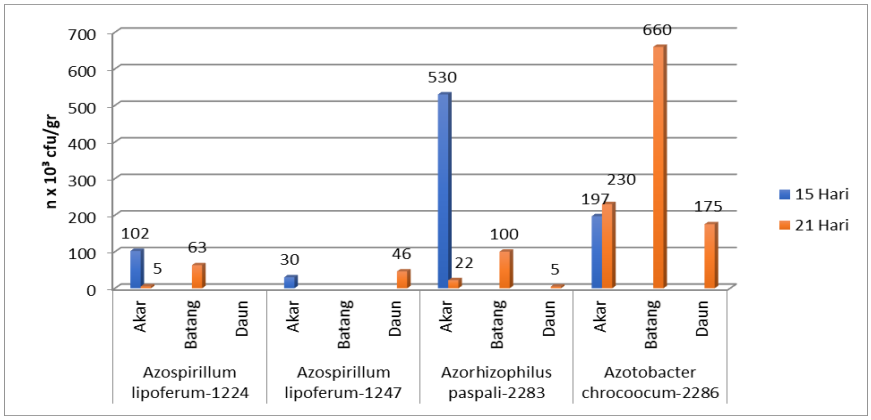


Figure 3. The results of reisolation of bacterial populations 15 and 21 days after planting

Reisolations of *Azorhizophilus paspali-2283* bacterial strain 15 days after nursery showed that the population in the root tissue was very high (530x103) but not found in the stem tissue and leaf tissue, then reisolated at 21 days after nursery, *Azorhizophilus paspali-2283* bacteria found in the root, stem and leaf tissue, from the three parts of the plant tissue the most population of these bacteria were found in the tissue leaf of plants.

Observation of the strain of *Azotobacter chrococum-2286* in reisolation 15 days after the nursery was only found in the roots, then reisolation 21 days after seedling of root tissue, stems, and leafs filled with a bacterial population of *Azotobacter chrococum-2286* strain but most of the bacterial population was found in the tissues stem, this shows that the stem tissue is very suitable for the growth of a bacterial strain of *Azotobacter chrococum-2286*. The colonization shown by the *Azotobacter chrococum-2286* strain using the seed dressing method reached a maximum value 105 cfu/gr as revealed by Dong et. al., (2003), even though there is also the colonization of bacteria in plant tissues reaching 107 CFU/gr bur rarely found. Gyaneswar el, al., (2001) in Widayati (2005) said the number of endophytic bacteria usually only ranged 104 CFU/gr to 105 CFU/gr. Whereas the pathogenic bacteria can reach 1010 CFU/gr.

Generally, the application of inoculation with two methods of dipping and seed dressing is able to provide special association conditions as endophytic for rice from four bacteria tested. The bacterial population, in general, using the seed dressing method is better than the dipping method, this fact is evidenced in the experiment of the seed dressing method the population in the plant tissue reaches 105 CFU/gr, while the dipping method is only 103 CFU/gr. Bacteria using the seed dressing method are thought to carry out initial penetration of the rice seed embryo and then develop on the roots when the roots begin to grow. The condition of wet seeds makes it easier to provide nutrients for bacterial farming, according to Syamsudi (1997) bacteria will infect seeds that have a water content of more than 20% with enough water content that the seeds can provide many nutrients such as carbohydrates, nitrogen compounds, minerals and additional compound for bacterial growth. The inoculation of bacteria strain of *Azospirillum lipoferum-1247* with all method tested showed that they were unable to adapt to the environment and competition between indigenous bacteria and fungi in the plant tissues, as reported by Kamuru, at. al., (1998) that bacterial penetration in plant tissue is influenced by competition between target bacteria and other bacteria of fungi to obtain nutrition. Rosenblueth and Romero (2006) bacteria are influenced by genetic factors both bacteria and plants. Bacteria are influenced by the environment both from inside and outside the plant tissue (Lagreid, et, al., 1999).

## CONCLUSION

The results of the research are GFP markers that show its ability to provide a clear identity as an endophytic bacterium when applied in natural microbial communion. Besides that, GFP is also stable in the applications on rice plant tissue and is able to provide a fairly clear expression. The bacteria Azospirillum lipoferum-1224, Azospirillum lipoferum-1247, Azorhizophilus paspali-2283, Azotobacter chroococum-2286 were able to colonize the rice plant tissue using either the inoculation method of Dipping or Seed dressing.

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