Colonization and Performance of Diazotroph Endophytic Bacteria on Palm Oil (Elaeis guineensis Jacq L.) Leaves

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Abstract. Excessive and continuous use of inorganic fertilizer can lead to negative impact on the environment qualities, therefore microbial biochemical approaches which are more friendly need to be considered. The absorption of nitrogen elements has been widely known through the roots and then transported to the leaves before chlorophyll performing the works on the photosynthesis process. However, the discovery of many diazotroph endophytic bacteria in the leaves suggests that nitrogen is not only supplied through the soil but it can be fixed by the diazotroph endophytic bacteria through the leaves. This study aims to evaluate the colonization and performance of the diazotroph endophytic bacteria on palm oil leaves. A total of 42 isolates of endophytic bacteria isolated by Mardiah (2014) were selected based on their ability to fix nitrogen qualitatively on nitrogen free medium and quantitatively based on Nessler reagent. One of the selected isolates with the highest nitrogen fixation capability is KSD2. The sequencing results showed that KSD2 isolate is closely related to Bacillus cereus. Colonization and performance evaluation of Bacillus cereus on palm oil seedlings were performed at 0, 2, and 4 weeks after inoculation. Treatments included Co (control/untreated), Ta (inoculation via soil), and Da (inoculation via leaves) with 7 replications. Data were evaluated using SPSS program with Univariate Analysis of Variance (significance level >95%). The colonization of Bacillus cereus was evaluated with PCR method approach using a specific primer (BCFomp1/ BCRomp1). PCR results showed that Bacillus cereus successfully colonized palm oil leaves as evidenced by the target sequences of 575 bp long.

1. Introduction
The nitrogen element is absorbed by the plant in the form of nitrate compounds (NO₃⁻) and ammonium (NH₄⁺). Biologically, this element is obtained by certain microorganisms known as nitrogen-fixing bacteria or better known as diazotroph bacteria. It has been reported that nitrogen fixation by diazotroph microorganisms is a major source of nitrogen addition in forest ecosystems [1]. Diazotroph bacteria have been widely isolated from rhizosphere and rhizoplane of non-leguminous crops [2]. However, the holding efficiency of N₂ is lower when compared with the diazotroph endophytic bacteria. This is because the availability of photosynthates for rhizosphere and rhizoplane bacteria are limited [3].

Based on its potential, this study is more focused on diazotroph endophytic bacteria. Diazotroph endophytic bacteria lives on plant tissues and have the ability to fix nitrogen. The advantages of diazotroph endophytic bacteria is the plants directly provide photosynthates as nutrients for bacterial
growth, and also provide an environment with low oxygen levels, thus spurring the expression of the nitrogenase enzyme responsible for nitrogen fixation. In addition, endophytic bacteria also do not have to compete with other soil microbes to obtain root exudates for their survival [3-4]. The advantages possessed by the diazotroph endophytic bacteria make the N potential donated by the bacteria is larger than the diazotroph nonendophytic bacteria. Based on research in sugar cane, diazotroph endophytic bacteria were able to increase root dry weight and plant height compared to rhizosphere bacteria [5]. In addition, the potential of N contributed by diazotroph endophytic bacteria is greater than diazotroph non-endophytic bacteria, due to their presence in the intercellular tissues of plants that are not easily lost, while the free N nutrients in nature are highly labile, rain-washed and eroded, and easily evaporate into the air [5].

Leaves are the plant organ known to be colonized by diazotroph endophytic bacteria. During this time, the absorption of nitrogen elements has been widely described through the roots and will furtherly be transported to the leaves as chlorophyll forming that works on the photosynthesis process. The discovery of many diazotroph endophytic bacteria in the leaves suggest that nitrogen is not only supplied through the soil but can be fixed by the diazotroph endophytic bacteria through the leaves. Further, the nitrogen pathway to the leaves can be shortened and more efficient so it is useful for faster photosynthesis. Inoculation of the diazotroph endophytic bacteria in leaves is expected to increase the N element as chlorophyll forming. In the end, the photosynthesis process will immediately increase the plant growth. Therefore, the colonization of the diazotroph endophytic bacteria in leaves needs to be further evaluated.

Successful inoculation of diazotroph endophytic bacteria in leaves is identified by its colonization in plant tissues. The detection, visualization, and quantification of inoculated bacteria on the surface and into plant tissues during the plant growth cycle are supportive in the direct study of in vivo bacterial activity. In addition, the ability to compete with native bacteria can also be revealed. Species and cultivars of host plants, endophytic species and strains, inoculum concentrations, age and growth conditions of host plants are known as limiting factors of endophytic introduction [6-8].

So far, the endophytic bacteria capability resulting from the palm oil network in fixing nitrogen and its role in palm oil nurseries is less understood. Therefore, the ability of colonization and subsequent fixation of nitrogen from endophytic bacteria inoculated on leaves of palm seedlings should be extracted to minimize use of chemical fertilizers. In this study, inoculation of leaves and soil to introduce diazotroph endophytic bacteria into palm oil seedlings was performed. The colonization was detected using PCR. Increased bacterial colonization and the capacity of certain bacteria to modulate plant metabolism are key issues for further study, as this may provide insight into the intercropping-endophytic relationship of plants.

2. Materials and Methods
2.1. Qualitative Selection of Nitrogen Fixation Capabilities with NfB (Nitrogen free Bromtimol) Medium

Forty two endophytic bacteria isolates used for this research isolated from PT Astra Agro Lestari plantation (PT Agro Menara Rahmat, Central Kalimantan). This isolation has been done by Mardiah [9]. Isolation of endophytic bacteria from palm oil is done by taking healthy tissues in the roots, stems, leaves, and fruits aseptically. Each tissues were surface sterilized with 70% ethanol and 2% sodium hypochloride, then planted in Nutrient Agar (NA) medium aseptically. After 48 hours of incubation, bacterial isolates that grew on the media purified and the pure culture stored in a reaction tube containing NA media as stock culture [9]. Qualitative Selection of Nitrogen Fixation Capabilities with NfB started by culturing the isolates on 10 ml of NB medium for 24 h at room temperature. A total of 1 ml bacterial cultures were introduced into Mannitol NfB semi solid medium and incubated for 24 hours. The positive bacteria binding to nitrogen was observed from blue to yellow change of colour in the media and also the formation of the pellicle on the surface of the media [10].
2.2. Quantitative Selection of Nitrogen Fixation Capabilities with Nessler reagent
Bacteria that have been grown on NB media for 24 hours were centrifuged for 10 minutes, 4000 rpm. A total of 1 ml of culture supernatant was diluted with 20 µl of seignette salt and 20 µl of nessler reagent before incubation for 5 minutes. The mixture of a solution exhibiting a yellow color indicates the presence of NH$_4^+$ is formed. The formed NH$_4^+$ was calculated by spectrophotometer at a wavelength of 425 nm [11-12]. The supernatant absorbance values obtained were then converted to ppm by the formula (supernatant absorbance = 1.7594 * ppm NH$_4$Cl + 0.0175) with reference to standardized NH$_4^+$ curves.

2.3. In Planta Test with Nessler Reagent
A total of 1 ml bacterial culture was inoculated on the leaf that has been first injured with a sterile needle. After one week, the part of the leaf that has been inoculated by the bacteria was cut off then surface sterilized and crushed. The scours were centrifuged for 10 minutes at a speed of 4000 rpm. A total of 1 ml centrifugation supernatant was diluted 10x and tested its ammoniac uptake by adding 20 µl of seignette salt and 20 µl of Nessler reagent. Next is calculated the absorbance at λ 425 nm [11-12]. The supernatant absorbance value obtained was then converted to ppm by the formula (supernatant absorbance = 1.7594*ppm NH$_4$Cl + 0.0175) with reference to NH$_4^+$ standard curve.

2.4. Identification of Morphological Character of Colonies and Microscopic Observations
The morphology of the colony was observed visually in petri dishes against the shape, color, edges, surface, and colony growth patterns. Microscopic observations of bacterial cells were carried out for cell shape and Gram reaction. The technique of gram staining was done in the following protocols: the first stage created a smear by dripping aquades as much as 3 drops then putting as much as one bacterial colony, leveling it, and passing it on fire quickly to dry. The isolate smears were dabbed with the basic dye of crystal violet and left for 1 minute, then washed with water flowing carefully. The swab is then streaked with Lugol or Gram Iodine and left for 1 minute. The excess reagents were removed and the smear soaked in 96% alcohol for 30 seconds. The swabs were washed with water flowing carefully. The smear was coloured with safranin for 1 minute, washed again with water flowing carefully and then dried. The smear was observed with a microscope.

2.5. Molecular Identification of 16S rRNA
The amplification of 16S rRNA gene was carried out using PCR colony method. The pure bacterial isolates were grown on solid NA medium and extracted using ose into sterile deionized water and homogenized and then boiled for template in the PCR process. Five µL of bacterial suspension was used as template and mixed with 2.5 µL primer 785F 16S rRNA (5' AGAGTTGTATCCTGGCTCAG- 3'), 2.5 µL reverse primer 907R 16S rRNA (5' GGTGACCTTGTACGACTTTAGG- 3'), and 15 µL sterile deion, and 25 µL PCR mix in PCR tube. The PCR tube containing 50 µL of mixed material was then homogenized using a vortex prior to PCR. The amplification process was carried out at an initial denaturation temperature of 95°C for 3 minutes with 1 cycle, 95°C denaturation for 30 seconds, annealing 60°C for 30 seconds, and extension 72°C for 30 seconds with 30 cycles, end extension 72°C for 7 minutes with 1 cycle.

2.6. Sequencing
Samples are sequenced using Macrogen services in Korea. Sequencing results were chromatogram data (file with extension ".ab1"). The sequencing data was further analyzed by the BLAST (Basic Local Alignment Search Tool) software from the NCBI website (www.ncbi.nlm.nih.gov/BLAST) and BioEdit.

2.7. Phylogenetic Tree Analysis
The result of sequencing was edited using Bioedit program. Reference species were searched using BLASTn nucleotide search (BLASTn) of NCBI. The sequence of isolates was aligned with some
reference species using the CLUSTAL_W program version 2.0.11. Sequence alignment was re-edited to remove unreadable gaps or nucleotides during sequencing. The phylogenetic tree was constructed based on distance analysis using neighbour-joining method. The phylogenetic tree was constructed using the MEGA 6 program. The trust values of each established branch were determined using bootstrap analysis based on re-sampling of 1,000 times.

2.8. Preparation of Liquid Inoculant
The pure culture of the diazotroph endophytic bacteria on the agar was tilted and rejuvenated on the NA medium. A total of 1 colony was grown into 50 ml of NB under agitation for 24 hours. One ml of the liquid colonies was taken and was poured into 100 ml of NB medium. This culture was grown in reciprocal shaking incubators at room temperature until it reaches the maximum growth rate, for incubation of the used velocity culture of 150 rpm.

2.9. Inoculation of Diazotroph Endophytic Bacteria In Palm Oil
The used Saplings Seeds were 6 months year old of unfertilized palm oil seeds. Palm oil seedlings were first selected based on the parameters of relatively uniform with an average height of 20-22 cm and has 5-6 leaf blade. The planting medium was 5 kg of sterilized soil. The inoculation was done by injuring the leaves and by spraying the liquid inoculant on the wounded leaf. Plants inoculated with \(10^{11}\) bacterial cell crops. To remove excess nutrients, the cells were washed twice with sterile water followed by centrifugation of 8000 g for 20 minutes prior to inoculation. The control plants were uniquely inoculated with 5 ml of sterile water. Sampling was carried out on week 0, 2\textsuperscript{nd}, and 4\textsuperscript{th} after inoculation of one plant per replication.

2.10. DNA extraction
The DNA was extracted from 100 mg of leaves using the Dneasy Plant Mini Kit (QIAGEN). The amount of DNA was photometrically calculated at 260 nm wave lengths. The quality of the extracted DNA in photometric checks with A260 / A280 calculation ratio should be above 1.9 and A320 close to 0.

2.11. Evaluation of Colonization by PCR method
Primers used in this PCR targeted gene fragments of \(\text{motB}\) on \(\text{Bacillus cereus}\), \(\text{BCFomp1}\) forward primers (5'-ATCGGCTCGTGGATGACGA-3'), and reverse \(\text{BCRomp1}\) primer (5'-CTGCATATCCCTACCGCAGCTA-3'). The length of the PCR product was 575 bp. The amplification process was performed at 94°C initial denaturation temperature for 3 minutes with 1 cycle, denaturation 94°C for 30 seconds, annealing 54.5 °C for 1 minute, and extension 72°C for 1 minute with 30 cycles, end extension 72°C for 7 minutes with 1 cycle [25].

2.12. Measurement of Chlorophyll Content
A total of 0.1 gram of leaves were cut into small pieces followed by an addition of 10 ml of 80% acetone, crushed for 5 minutes until smooth for extraction. The extraction results were filtered and 80% acetone were added to the volume limit of 10 ml. Calculation of chlorophyll content was done at \(\lambda\) 645 nm and \(\lambda\) 663 nm, with the following calculation:

\[
\text{Total chlorophyll (mg / L)} = 20.2 \ D645 + 0.02 \ D663 \\
\text{Chlorophyll a} = 12.7 \ D663 - 2.69 \ D645 \\
\text{Chlorophyll b} = 22.9 \ D645 - 4.68 \ D663
\]

2.13. Statistical Analysis
Statistical analysis to determine the absorption of ammonium and total chlorophyll was performed by univariate analysis (ANOVA) in randomized block design (RAK) at 95% confidence level. If there is any real difference, Post Hoc Test DMRT (Duncan's Multiple Range Test) at 95% confidence level is
performed. The statistical test was carried out using SPSS statistical (Statistical Product and Servie Solution) program for Window release 16.

3. Results and Discussion

3.1. Qualitative Test Result of Diazotroph Endophytic Bacteria Isolate

Forty-two Mardiah’s endophytic bacterial isolates (2014) were selected based on their qualitative nitrogen fixing ability by growing it on a semi-solid Nitrogen Bromtimole (NFB) medium (0.5% agar content). In a qualitative test, of the 42 endophytic bacteria positively capable to fix nitrogen, twenty three isolates or 54.8% showed the change of color medium from blue to yellow (Figure 1). The formation of pellicle as well as discoloration of the medium from a bluish green color to yellow was growth characteristics and nitrogenase activity of non-symbiotic nitrogen-blocking (N) bacteria [13]. Bacteria capable of living on nitrogen-free media will secrete organic acids causing the discoloration of the media [9]. In addition, the semi-solid media allow non-symbiotic nitrogen-fixe r bacteria to thrive at low partial of O2 pressures that favorable this bacterium at the time of nitrogen fixation [14].

Figure 1. The result of NFB medium test by diazotroph endophytic bacteria, a) the medium becomes yellow due to nitrogen fixation activity, b) the medium unchanged

3.2. Quantitative Test Result of Diazotroph Endophytic Bacteria Isolate

In this research, the result of linear regression for NH4+ standard curve was 0.989. From the standard curve, the value of ammonia concentration in each sample can be calculated. The magnitude of the nitrogen fixation rate in the sample within 24 hours based on the calculation obtained the following results (Table 1). Based on these data, it is known that the highest level of nitrogen fixation was measured in KSD2 isolate. This is also in accordance with the results of qualitative tests showing that KSD2 isolates have the ability to fix nitrogen.

In this test, there were several isolates capable to fixing nitrogen well on NfB medium but showed low result on test with Nessler reagent or vice versa. It is assumed that the C source used in these two tests is different. In NfB medium used C source of mannitol while in Nessler test used C source of glucose. Both qualitative and quantitative results showed that KSD2 isolate has the highest N fixation level compared to other isolates. This is presumably because the isolates of KSD2 are able to use carbon sources of mannitol and glucose well for optimization of nitrogenase activity. The substrate type affects the maximum activity of nitrogenase enzyme from Nostoc linckia bacteria [15].
Table 1. Nitrogen fixation level of pure culture endophytic bacteria isolated from palm oil

| Colony Code | Average of Ammoniac Uptake (ppm) | Colony Code | Average of Ammoniac Uptake (ppm) |
|-------------|----------------------------------|-------------|----------------------------------|
| KSAK1       | 49.7420*                         | KST11       | 33.5768                           |
| KSAK2       | 20.5807                          | KST12       | 49.0378*                         |
| KSAK3       | 33.4168                          | KST13       | 25.5832                           |
| KSAK4       | 14.8347                          | KST14       | 24.1018                           |
| KSAK5       | 39.4378*                         | KST15       | 23.3976                           |
| KSAK6       | 34.9853                          | KST17       | 45.8688*                         |
| KSAK7       | 37.9942                          | KST18       | 39.7297                           |
| KSAK8       | 23.8777                          | KSBga       | 36.2337                           |
| KSAK9       | 36.0416                          | KSBgB       | 43.9481*                         |
| KSAK10      | 50.3502*                         | KSB1        | 13.4744                           |
| KSAK11      | 16.0032                          | KSB4        | 24.0058                           |
| KST1        | 35.4974                          | KSB14       | 32.4885                           |
| KST2        | 23.8137                          | KSD2        | 55.8560*                         |
| KST3        | 45.4526*                         | KSD3        | 38.4104                           |
| KST4        | 39.4027                          | KSD6        | 0.9264                            |
| KST5        | 18.8201                          | KSBK1       | 39.3707                           |
| KST6        | 23.7497                          | KSBK3       | 32.3604                           |
| KST7        | 40.1389*                         | KSBK4       | 18.3200                           |
| KST8        | 29.5115                          | KSA1        | 21.4449                           |
| KST9        | 33.8219                          | KSA3        | 39.9469                           |
| KST10       | 28.9673                          | BKA17       | 33.1287                           |

*Note: the above value is ppm of ammoniac uptake compared with negative control (n = 3)
(*) Isolates with ammoniac uptake more than 40 ppm

3.3. *In Planta* Diazotroph Endophytic Bacteria Test Result

The results of *In Planta* test on 42 isolates of endophytic bacteria in N fixation showed that KSD2 isolate bacteria had the highest nitrogen fixing ability compared to other endophytic bacteria isolates of 46.65 ppm (Table 2). This result is likewise consistent with qualitative and quantitative results of pure culture which also shows that KSD2 isolate has the highest nitrogen fixation potential. Based on the results of nitrogen fixation test qualitatively, quantitatively, and *In Planta*, the KSD2 isolates were further selected as inoculants to be tested for their ability in fixing nitrogen on palm oil leaf saplings.

There are endophytic bacteria in rice and corn plants that are capable for N₂ fixation in the air. A total of 142 isolates of endophytic bacteria were isolated consisting of 95 isolates of endophytic bacteria from rice and 47 isolates from maize from West Java, East Java, and South Kalimantan [16]. A total of 20 isolates of endophytic bacteria were isolated from palm root. Three of these bacteria have the ability to fix nitrogen namely NFB 2, NFB 3 and NFB 4 with ARA 2.10, 2.78, and 3.13 ppm/hour activities respectively. The isolates are also known to increase plant height, number of leaves, and number of root of palm oil seedling [17]. The combination of diazotroph endophytic bacteria isolate inoculation with no nitrogen fertilization gave the best vegetative growth yields for palm oil seedlings, i.e., the diameter of congestion, seedling height, and dry weight of palm oil seedlings [18].

Table 2. Levels of Nitrogen Fixation of Endophytic Bacteria in Palm Oil Leaf

| Colony Code | Average of Ammoniac Uptake (ppm) | Colony Code | Average of Ammoniac Uptake (ppm) |
|-------------|----------------------------------|-------------|----------------------------------|
| KSAK1       | 41.5890 de                      | KST11       | 40.7599 ed                      |
| KSAK2       | 41.4033 de                      | KST12       | 46.6626 de                      |
| KSAK3       | 46.5122 de                      | KST13       | 39.4251 a                       |
| KSAK4       | 44.0218 de                      | KST14       | 45.8688 de                      |
The above value is ppm of ammoniac uptake compared with negative control (n = 2). The numbers followed by the same letter show no significant difference based on the Duncan test at a 95% confidence interval.

3.4. Morphological Identification of Diazotroph Endophytic Bacteria Isolate

Morphological identification was done by visualizing colony character and cell shape observation using light microscope with 10 x 20 magnifications, after gram staining. There is also a KOH test for confirmation. The results showed that KSD2 colony visualized morphology of white milk, irregular colonies, edges of filamentous colony, smooth surface, with a colony diameter of about 2.5 mm. Gram staining results showed that the isolates were a type of gram (+) bacteria, rod-shaped, and not slimy when tested for KOH (Figure 2).

3.5. Molecular Identification and Sequencing of 16S gene rRNA

The pairwise alignment and multiple sequences were performed on the KSD2 sequence and some of the subject sequences obtained from BLASTn using the Mega 6 program with E. coli as an outgroup. The result of phylogenetic tree construction with neighbour-joining test (Figure 3) shows that KSD2 isolate belongs to Bacillus cereus group. So, it can be assumed that KSD2 isolate closely related with B. cereus species. B. cereus is a rod-shaped bacteria found in the ground. These bacteria can adapt to extreme environments and resistant to hot temperatures, cold temperatures, and radiation as they can form endospore [19]. These bacteria are both aerobic and anaerobic and can metabolize carbohydrates, proteins, peptides, and amino acids during the production of L-lactate, acetate, formate, succinate, ethanol, and carbondioxide [20]. This bacterium is also a nitrogen fixer on the soil. Nitrogenase enzyme activity was detected in B. megaterium, B. cereus, B. pumilus, B. circulans, B. licheniformis, B. subtilis, B. brevis, and B. firmus [21]. Biological nitrogen activity was detected in B. mariflavi and Paenibacillus massiliensis while also identifying nifH gene fragments in B. megaterium and B. cereus [22]. Some strains of B. subtilis and B. cereus have been isolated and selected from China based on their ability to stimulate plant growth and disease control caused by fungi affecting wheat roots [23]. The widely use of B. cereus A47 strain in the country is able to stimulate wheat production about 11%. Some species of Bacillus are also able to invade tissue in various plant species where these bacteria have an important role in plant protection and growth spurts. This ability belongs to a species commonly recognized as a living organism free from soil, including B. cereus [24].

3.6. Colonization of Endophytic Bacteria in Leaves of Palm Oil

The association ability of KSD2 isolate with palm seedlings is characterized by the presence of this isolate within the plant tissue. The PCR results (Figure 4) showed that the inoculation through the leaf...
at 4th week after inoculation (Lane 2) contained a band at 575 bp corresponding to the reference (Lane 9). The reference is a gDNA isolated from a pure culture of KSD2 isolate.

![Figure 2](image.png)

**Figure 2.** Morphology of KSD2 isolate (A) Colony form of KSD2 isolate, (B) Gram Staining Cell Form

The higher number of sequences that can be amplified by the primary electrophoration results will show increasingly thick band. The band thickness of gDNA on lane 2 indicated that there was much colonization of *Bacillus cereus* in the 4th week after inoculation through the leaf. This is because the PCR uses a specific BCFompF1 and BCFompR1 primer that amplifies the *motB* gene encoding the outer membrane protein in *B. cereus* [25] while also showing the same position with gDNA’s pure isolate of KSD2 on lane 9. Meanwhile, both inoculation of leaves, soil, and control (Lane 5, Lane 6, and Lane 7) did not show any bands on the 2th week after treatment. This is due to the gDNA that is not well insulated. Manzano et.al (2003) used specific primers in the PCR process to detect *B. cereus* in coffee concentrate samples. The obtained data indicated the specificity and primary sensitivity that can be used to check for the presence of *B. cereus* in different food products, and to avoid the need for labor and time consumption [26]. PCR method was similar to the one used to detect the presence of *B. cereus* in raw milk samples [27]. First, endophytic bacteria usually penetrate through secondary roots...
by secreting cellulase or pectinase enzymes [28], or upper parts of plants such as stems, flowers, sprouts radicals, stomata or cotyledon and leaves torns [29].

**Figure 4.** Results of PCR of palm leaves gDNA to gDNA of KSD2 isolate using *Bacillus cereus* specific primer

The bacteria colonize at the point where it enters or spreads throughout the plant living in cells, intercellular space or in vascular systems [30]. Compliance of existing compounds on palm leaf sapling is believed to be one of the keys to successful entry of isolate KSD2 into the palm oil network. KSD2 isolates were isolated from palm leaf tissue so that the compounds present in the palm oil leaves were in accordance with their nutritional sources.

3.7. Leaf Nitrogen Fixation Ability

The univariate of analysis variance showed that inoculation of isolate KSD2 (*B. cereus*) on palm oil seedlings was significant (p > 95%) at 4th week after inoculation compared to control and inoculation via soil (Figure 5). The results of Hino and Wilson showed that bacteria from the *Bacillus* genus were able to fix nitrogen. Bacillus has a nitrate reductase enzyme; this enzyme acts as an electron acceptor [31]. Treatments at 0 and 2nd week were not significant either in control, inoculation via soil, or inoculation via leaves. The insignificant inactivation of *B. cereus* in leaves at 2nd weeks was suspected because bacteria were still in the process of adaptation to the environment and still in the process of penetration into plant cells or in other words, the bacteria had not entered as endophytes. This condition causes the working of nitrogenase enzyme in *B. cereus* to be ineffective because of exposure to free oxygen. The presence of contacts between endophytic bacteria isolates and free oxygen enables the destruction of nitrogenase-enzyme complexes that will inhibit the nitrogen-blocking process by endophytic bacteria [32]. The nitrogenase enzyme will become inactive and experience an irreversible reaction when in contact with free oxygen, because free oxygen will react with the metal group in the enzyme nitrogenase [33].
Figure 5. Nitrogen fixation In Planta

The treatment of *B. cereus* inoculation via soil at week 0 (before inoculation), 2\textsuperscript{nd} week, and 4\textsuperscript{th} week was not significantly suspected due to competition with other soil microbes. In addition, the presence of certain organic acids in the soil, either from the metabolite results of other microorganisms in the soil and from the root palm exudate itself, is also suspected to inhibit the work of nitrogenase enzymes that exist in *B. cereus* if absorbed into the cell. The presence of glutamine, glutamic acid, asparagine, alanine, and tironin can decrease the activity of nitrogenase 50-98\% [34]. Temperature and soil moisture greatly affect the ability of bacteria in mineralizing organic N to ammonia. The maximum mineralization process of organic N by bacteria occurs at a temperature of 30-35°C and its activity is not maximal if dry soil conditions and oxygen quantities are very limited [35].

3.8. Chlorophyll Content

The result of univariate of analysis variance showed that inoculation of KSD2 isolate (*B. cereus*) both via soil and through leaves on palm oil seedlings at weeks 0, 2, and 4 was not significant compared to control (p <95\%) (Figure 6). This is assumed because the key of chlorophyll formation is not only determined by nitrogen but also by other elements, whereas in this research there is no fertilization at all so it is assumed that other chlorophyll-forming elements are not fulfilled. Chlorophyll is commonly synthesized in leaves to capture different amounts of sunlight in each species depending on environmental and genetic factors. Factors affecting chlorophyll synthesis include: light, sugar or carbohydrates, water, temperature, genetic factors and elements of nitrogen, magnesium, iron, manganese, Cu, Zn, sulfur, and oxygen.

Based on Susilowati (2003) study, JCBd 2.1 and JLkCN 2.3 isolates significantly influence plant growth height compared to control. However, the inoculation treatment did not differ significantly in stem diameter, leaf number, wet canopy weight, crown dry weight, root dry weight, chlorophyll content, and N content [15]. In soybeans, nitrogen fixation is highly dependent on the number of photosynthesis products and the maximum activity of nitrogenase which is often associated with high photosynthetic activity. Previous reports have shown that the large amount of chlorophyll is one indicator of photosynthesis. Chlorophyll is the key biochemical component responsible for photosynthesis. Chlorophyll biosynthesis is performed by certain genes within the chromosome. These genes play a role in coding enzymes involved in the biosynthetic pathway tetrapirrol (porpirin nucleus) as the structural center of chlorophyll [36-37]. The main factor of chlorophyll forming is nitrogen (N).

4. Conclusions

Endophytic bacterial isolates with the best nitrogen fixation ability in palm oil leaf were *B. cereus* of 46,6562 ppm. PCR results showed that *B. cereus* application on palm oil leaves was able to colonize by visualizing 575 bp DNA bands on PCR results. *B. cereus* application in leaves can increase 39.03\% of leaf ammonia levels at 4\textsuperscript{th} week after inoculation.
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