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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

氏名：　Supriadi
Name

学位論文題目：　Study of biological nitrogen fixation in legume and non-legume plants
Title of Dissertation　（マメ科植物と非マメ科植物の生物的窒素固定の研究）

学位論文要約：
Dissertation Summary

Excess synthetic fertilizer usage caused emission since it produced from fossil fuel with high economic costs which pollute environment. Nitrogen fertilizer which is known as the biggest amount of synthetic fertilizer could be reduced with increase of biological nitrogen fixation. Understanding the mechanisms of biological nitrogen (N_2) fixation in legume and non-legume expects to decrease the synthetic nitrogen fertilizer consumption. The symbiotic relationship between rhizobia and legume contributed to the major source of biological nitrogen fixation (BNF). This symbiotic system plays a significant role in improving the fertility and productivity of low-N soils, and widely used for strategy of cropping system and plant rotation. Thus, the rhizobium-legume symbiosis has received most attention and has been studied extensively. On the other hand, the contribution of nitrogen fixation in non-legumes is limited. Different with that occurred in the root nodules of legumes, free living nitrogen-fixing bacteria (diazotrophs) and endophyte bacteria do not excrete mutualistic compounds to be directly used for host plant. Diazotrophic bacteria can enter through the sugarcane root by crack entry or by a Nod factor-independent process.

In this study, we used legume and non-legume plants to study the symbiotic nitrogen fixation. Firstly, we used *Lotus japonicus* as a model plant for genome studies in legumes. In the second study, we focused on BNF in non-legume plants which is related the potential of endophytic bacteria. In the third study, we focused on T3SS dependent nodulation on *Clitoria ternatea*.

Effect of ferritin in nitrogen fixation in *Lotus japonicus*

Legume nodule requires large amount of iron for complex of such as nitrogenase and leghemoglobin. In nodules, ferritin is accumulated at the initial stages of nodule development and increases at the nodule senescence stage. We investigated the effects of overexpressing and suppressing ferritin in *L. japonicus* nodule. Acetylene reduction activity (ARA) revealed nitrogen fixation was higher by the overexpression of ferritin under the higher iron concentrations, while ferritin-suppressed nodule by RNAi was higher nitrogen fixation under lower iron concentration. Our data suggest that suppressing ferritin in the nodules is effective under iron deficient conditions. Over accumulated ferritin in nodule is effective under the higher iron conditions. In conclusion, ferritin expression plays a role in nitrogen fixation activity in response to various iron concentrations. Ferritin is needed to help buffer the cell iron concentration for effective nitrogen fixation. At low iron concentrations, ferritin accumulation is not required by *L. japonicus* nodules.

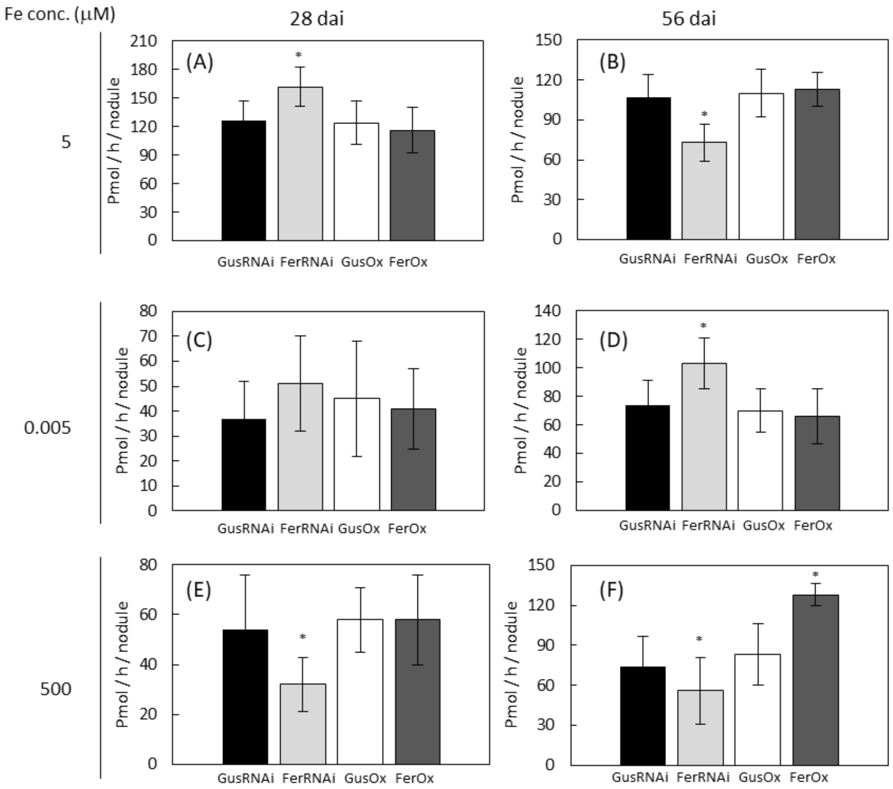

Figure 1 Acetylene reduction activity per nodule at 5 µM (A, B), 0.005 µM (C, D), and 500 µM (E, F) iron-citrate concentrations. The activities were measured at 28 and 56 days after inoculation (dai). Error bars indicate mean ± SD. Significant differences ($P < 0.05$) compared with the *Mesorhizobium loti*-inoculated nodules in GusRNAi and GusOx are indicated by asterisks.

Biological Nitrogen fixation in *Saccharum sinense*

BNF in sugarcane is widely studied, as it is environmentally and economically advantage. The aim of this study was to determine the bacterial diversity of the roots and soil using 16S rRNA gene sequencing. The sugarcane was sampled in two different fields: one field was where wheat was cultivated the previous year and the other field was where sugarcane has been grown continuously for 10 years in Kagawa Japan. Samples of sugeracane roots and soil were collected from three separate areas in each field. The sugarcane soil and roots from the field where wheat was cultivated last year were named soil-1 and root-1, respectively, while the soil and sugarcane roots from the field cultivated continuously with sugarcane for 10 years were named soil-10 and root-10, respectively. Bacterial community analysis suggested that the most abundant phyla in the roots and from the soil were *Proteobacteria*, *Verrucomicrobia*, *Acidobacteria*, and *Actinobacteria*, which represented more than 70% of the bacterial taxa in both the soil and roots. The microbial communities in soil-1 and soil-10 and in root-1 and root-10 were similar, indicating that the sugarcane continuous cropping did not change the core microbial community. Five classes were more dominant in the root bacterial populations than in the soil bacterial populations: *α-proteobacteria*, *β-proteobacteria*, *Cytophagia*, *Saprospirae*, and *Actinobacteria*. We speculated that these classes have a higher potential to be isolated as endophyte nitrogen-fixing bacteria. Moreover, *Sphingomonas*, *Asticcacaulis*, *Agrobacterium*, and *Rhizobium* species of the *α-Proteobacteria* that have been identified as important nitrogen-fixing bacteria also found in metagenomes data. Notably, not only *α-Proteobacteria* but also *β-Proteobacteria* increased as root bacteria. *β-Proteobacteria* were dominant in the roots at 26%, an increase of approximately 2–3 times higher compared with the 10% in the soil. The *β-Proteobacteria* identified in the sugarcane fields in Kagawa such as *Achromobacter*, *Burkholderia*, *Ralstonia*, and *Janthinobacterium* may be endogenous nitrogen-fixing bacteria. The sequencing data showed that not only *Rhizobium* sp. or *Bradyrhizobium* sp. but also *Agrobacterium* sp. contain *nifH* genes. The phylogenetic tree of 16SrRNA revealed that the isolated bacteria belong to *Agrobacterium* sp., *Bradyrhizobium* sp., and *Rhizobium* sp.

In conclusion, we identified the soil bacteria and endophyte bacteria in the roots of field-grown sugarcane in Kagawa. This report is the first on *S. sinense*, which is used to produce wasanbon. Diversity of bacteria in Kagawa's sugarcane was different from that in Brazilian and other Japanese sugarcane, *S. officinarum*, which might produce unique flavor for wasanbon sugar. Many *Rhizobium* sp. and *Agrobacterium* sp. with *nifH* are potential as nitrogen fixing bacteria.

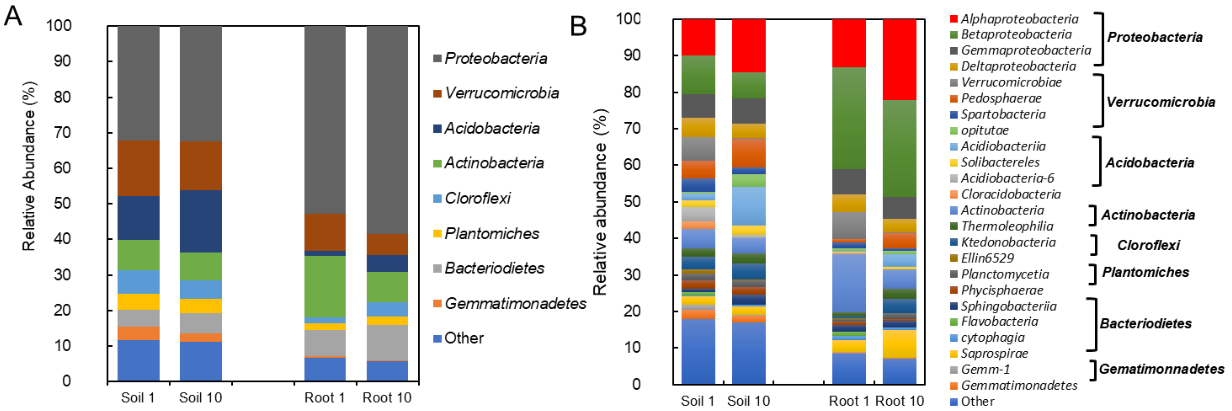

Figure 2 The relative abundance (%) for (A) phylum and (B) class of bacterial community in soil and root of sugarcane. The taxa represented accounted for less than 1% abundance in at least one sample. ‘Other’ is taxa with a maximum abundance of less than 1% in each sample.

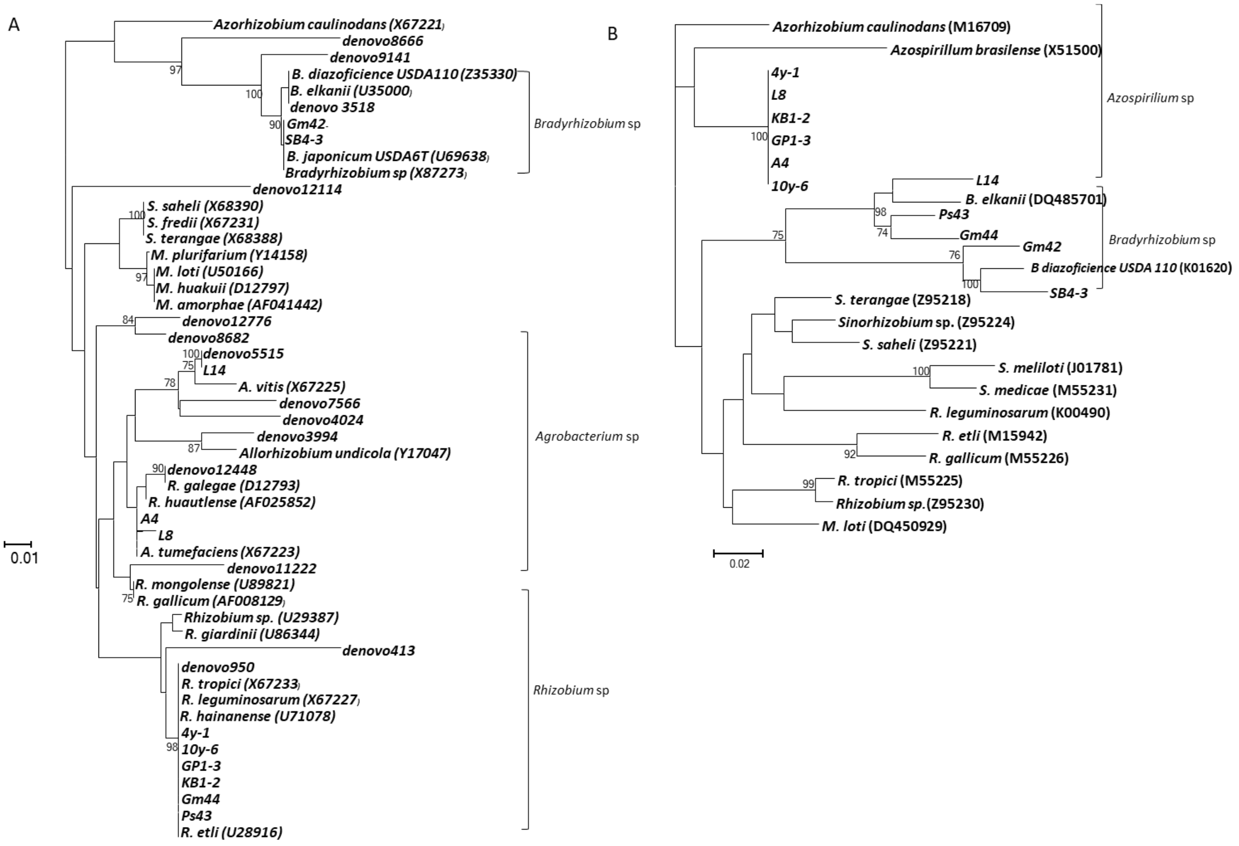

Figure 3 Phylogenetic (neighbor-joining) tree of Isolated bacteria from sugarcane based on 16SrRNA (A) and *nifH* (B) sequences. *A.*, *Agrobacterium*; *B.*, *Bradyrhizobium*; *M.*, *Mesorhizobium*; *R.*, *Rhizobium*; S., *Sinorhizobium*. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates). Evolutionary analyses were conducted in MEGA7.

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Symbiotic Nitrogen fixation in *Clitoria ternatea*

C. ternatea, also known as butterfly pea or blue pea, is a perennial plant belonged the family of *Fabaceae*. Some potential application of usage from *C. ternatea* are natural food colorants and antioxidants and always used as traditional medicine such as fever, inflammation, pain, and diabetes. In this study we aimed to understands the effect of type three secretion system (T3SS) of isolated bacteria. *C. ternatea* produced nodules with infection of *Bradyrhizobium elkanii* USDA 61, however did not with T3SS mutants. In this study we have used *Bradyrhizobium* strains which have been isolated from *C. ternatea* nodules grown in Thailand. Identification of the strains based on phylogenetic analysis of 16S rRNA and ITS showed that *B. elkanii* were major bacteria for plant growth and higher nitrogen-fixing activity in *C. ternatea*. We also produced T3SS mutants. As a result, *C. ternatea* produced nodules after infection with T3SS mutant, however produced Fix$^-$ nodules (lower nitrogen fixation). These data suggest the isolated bacteria have positive effectors for the nitrogen fixation in *C. ternatea* nodule.