An evaluation of phosphorus uptake in *Sesbania cannabina* when ferric phosphate is applied in the presence of phosphate-solubilizing rhizobia

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**ABSTRACT**

Deficient available phosphorus (P) in soils can majorly limit crop production. Furthermore, nodule formation in legumes is inhibited in P-deficient soil. Phosphate-solubilizing rhizobia are supposed to improve the plant P uptake even in insoluble P accumulated soil. We investigated the utilization of insoluble ferric phosphate, which is generally less available for agricultural crops, by *Sesbania cannabina* inoculated with phosphate-solubilizing rhizobia. Our evaluation of the P-solubilizing capacity showed that P was mineralized from ferric phosphate in inoculated rhizobia. In the soluble P treatment, nodule dry weight was significantly correlated with shoot dry weight and P content. Nodule dry weight and nitrogenase activity in *S. cannabina* supplied with ferric phosphate were significantly higher than those in the soluble P treatment. But, these increases were not necessarily effective on P status in the plants supplied with ferric phosphate.

**Key words:** Green manure, Nodule, Phosphorus mineralization, Rhizobia.

**INTRODUCTION**

Modern agriculture is dependent on regular inputs of phosphate fertilizer as well as other chemical fertilizers. Most phosphate fertilizers have been manufactured by treating rock phosphate, a non-renewable resource, (estimated to last approximately 30-300 years; Reijnders, 2014; Mew, 2016). Phosphorus (P) is an essential element classified as a macronutrient because of the relatively large amounts of P required by plants. Although agricultural soil has large P reserves, much of the P exists in forms unavailable for plant uptake. Namely, most P in soil is present as insoluble inorganic P or organic P, both of which are released into the soil solution very slowly. In acidic soil, ferric phosphate (FePO₄) and aluminium phosphate (AlPO₄) are the main product. P deficiency can severely limit plant growth and productivity. In the case of legume plants, more P is generally required for legume-rhizobia symbiosis. Robson *et al.* (1981) reported that P deficiencies result in deleterious effects on legume nodulation, development and function.

Several bacterial and fungal species act as phosphate-solubilizing microorganisms and their potential to mobilize soil P has been well studied (Chuang *et al.*, 2007; Hariprasad and Niranjana, 2009; Bahadur and Tiwari, 2014). Some rhizobia can solubilize organic and inorganic phosphates in soil (Alikhani *et al.*, 2006). Rhizobia with phosphate-solubilizing ability would provide nutritional benefits to the plant by mobilizing P and fixing N₂ (Peix *et al.*, 2001). While the symbiosis with compatible P-solubilizing rhizobia is expected to increase the P uptake of host plants, it is unknown how these rhizobia would affect host plant growth.

*Sesbania* species, which are introduced into temperate regions as green manure, have been found to exhibit a strong P acquisition ability: for example, *S. rostrata* is known to exude organic acids such as citrate, malate, succinate, and fumarate from its roots (Aono *et al.*, 2001). These organic acids are the major factors responsible for P solubilization from insoluble inorganic phosphates. The *Rhizobium* sp. associated with *S. cannabina* also showed an ability to solubilize tricalcium phosphate (Daimon *et al.*, 2006). These studies show the beneficial effect that the *Rhizobium* sp. associated with *S. cannabina* has on nodule formation and P uptake in the host plant under low P availability conditions.

Our study had two objectives: 1) to investigate P mineralization from insoluble ferric phosphate by the *Rhizobium* sp. strain U-9709-SC associated with *S. cannabina* and 2) to investigate the effects of P sources including ferric phosphate on the growth and P uptake of *S. cannabina*. Our aim was to determine the correlation between nodules/plant growth and P uptake and to determine the P mineralization from the above-mentioned P sources by the *Rhizobium* sp. associated with *S. cannabina*. 

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MATERIALS AND METHODS

Experiment 1: The evaluation of the capacity of phosphate solubilization from insoluble inorganic phosphate (FePO₄) by Rhizobium sp. U-9709-SC: The phosphate-solubilizing ability of Rhizobium sp. strain U-9709-SC associated with S. cannabina (Daimon et al., 2006) was tested in flasks containing 20 mL of YM liquid medium supplemented with 100 mg P L⁻¹ of ferric phosphate (FePO₄; Kanto chemical CO., INC) as insoluble phosphate (Plessner et al., 2007). No P was added to the control treatment (0-P).

The pH was adjusted in each treatment to 6.8 (0-P) and 5.8 (Fe-P), respectively. Two treatments were established: inoculated with 20 μL of the inoculant (10⁶ cells mL⁻¹) of Rhizobium sp. strain U-9709-SC associated with S. cannabina or uninoculated in each media. Treatments were established with five replicates per treatment. The flasks were incubated at 26°C on a rotary shaker (120 rpm) for 5 days. The supernatant was separated from the bacterial cells and insoluble phosphates by centrifugation (10000 rpm, 10 min). The final pH of the supernatant was measured with a pH meter (Horiba, B-212) and the liberated P was estimated with the molybdenum blue method.

Experiment 2: Comparison of nodulation and P uptake of S. cannabina under different P sources: Plants were grown in a pot filled with 470 g of soil mixture [Akadam soil (reddish soil; Heiwa, Japan): fertilized granulated soil (0.4 g N kg⁻¹, 1.0 g P₂O₅ kg⁻¹, and 0.6 g K₂O kg⁻¹; Kureha, Japan) = 20:1(v/v)]. The soil properties were as follows: pH (H₂O) 5.9, EC 0.015 mSm⁻¹, organic matter 17.2 mgg⁻¹, total P 1.42 gkg⁻¹, available P content (Bay2) 7.4 mgkg⁻¹, phosphate absorption coefficient 2256mg100g⁻¹, total nitrogen 1.37 gkg⁻¹, ammonium N 35.0 mgkg⁻¹, and nitrate N 31.0 mgkg⁻¹. Basal nutrients (171 mg NH₄NO₃, 446 mg K₂SO₄, 370 mg MgSO₄·7H₂O, and 440 mg CaCl₂·2H₂O) were added to each pot. Three treatments were established with five replications: 1) Soluble P treatment, NaH₂PO₄ (Wako pure chemical industries, LTD) as a soluble inorganic source of P (Cui et al., 2017). 2) Insoluble P treatment, ferric phosphate (FePO₄) as an insoluble inorganic source of P, and 3) Control, No P was added to the soil. P treated treatment was added each P source in a solid form at a rate of 100 mg P kg⁻¹.

Three S. cannabina seeds were sown into each pot. After emergence, the seedlings were thinned to one plant per pot. Two milliliters of liquid Rhizobium sp. strain U-9709-SC (10⁶ cells mL⁻¹) inoculant was added to each pot once a week for three weeks after sowing. Plants were grown in a greenhouse without temperature control. Plants were harvested 42 days after sowing and the dry weight (DW) of shoot, root, and nodule was measured, additionally, we measured the nitrogenase activity (Daimon et al., 1999). For evaluation of N and P status, chlorophyll content of leaves (Arnon, 1949) and P content by the vanado molybdate colorimetric method were investigated, respectively.

Statistical analysis: Statistical analysis was conducted using IBM SPSS Statistics software, Version 19. A Student’s t test was used to determine the statistical difference in P concentration between treatments in Experiment 1. The data were analyzed using one-way analysis of variance where F ratios were significant at P<0.05 in Experiment 2. Data from Experiment 2 were analyzed using Tukey’s test when P values were considered significant. Pearson’s correlation coefficient analysis and simple regression were used to assess the relationships between growth, P uptake parameters (DW and P content of shoots and roots), and nodule DW. A Student’s t test was also used to compare the treatments in Experiment 2: the correlation coefficients of the regression lines were compared between shoot DW and nodule DW.

RESULTS AND DISCUSSION

The evaluation of the capacity of phosphate solubilization from insoluble inorganic phosphate (FePO₄) by Rhizobium sp. U-9709-SC: The medium pH decreased with Rhizobium sp. strain U-9709-SC inoculation in both treatments. The pH values of uninoculated and inoculated samples after 5 days were 6.8 and 6.1 in the control treatment (initial pH = 6.8), and 5.1 and 4.9 in the FePO₄ treatment (initial pH = 5.8). The P-solubilizing and mineralizing ability of Rhizobium sp. strain U-9709-SC is shown in Fig. 1. Rhizobium sp. strain U-9709-SC cultured in the control treatment showed negative values because P was consumed from the basal media. On the other hand, Rhizobium sp. strain U-9709-SC was found to have the ability to mineralize P from FePO₄. The solubilized P from FePO₄ was approximately 3.96 μg P mL⁻¹ (7.86 μg (inoculated) – 3.90 μg (uninoculated) in the culture solution).

Several studies have shown that rhizobia can mobilize P from insoluble inorganic and organic phosphates (Alikhani et al. 2006; Daimon et al. 2006; Sridi and Mallaiah 2009). Furthermore, Rhizobium sp. strain U-9709-SC could solubilize P from FePO₄ in the present experiment. Stumm and Morgan (1995) reported that below approximately pH 5.0, the solubility of Fe (III) phosphate minerals in solution increases as the pH decreases. In our experiment, the pH value decreased from 5.8 (initial pH) to 5.1 and 4.9 in uninoculated and inoculated treatments, respectively. Therefore, Fe bounded Fe was somewhat mineralized during the incubation. However, an approximately two-fold increase in P concentration was observed in solutions inoculated with Rhizobium sp. strain U-9709-SC. Siderophores, which are known to be iron-chelating agents, are secreted by some microorganisms (Plessner et al. 1993). Therefore, P mineralization in the FePO₄ treatment by Rhizobium sp. strain U-9709-SC might be due to the production of siderophores.
The asterisk (*) indicates the statistical significance at $P<0.05$.

**Comparison of nodulation and P uptake of S. cannabina under different P sources:** In Experiment 2, the soluble P ($\text{NaH}_2\text{PO}_4$) supplement clearly increased shoot DW: there was a two-fold increase compared to that in the Insoluble P treatment (Table 1). Root DW was also significantly increased ($P<0.05$) in plants supplied with the soluble P. A significant difference was not observed in the root-shoot ratio between Soluble P and Insoluble P treatments, although both treatments had significantly lower ratios than P free treatment. In contrast, the nodule DW was highest in plants supplied with ferric phosphate ($\text{FePO}_4$), resulting in a greater than three-fold increase in nodule DW compared to that in Soluble P treatment. Insoluble P treatment also showed highest value in the nitrogenase activity. P contents of shoots and roots were significantly higher in plants supplied with the soluble P ($\text{NaH}_2\text{PO}_4$). But, the P content of nodules was significantly higher (approximately three-fold) in Insoluble P treatment than in the Soluble P treatment. The chlorophyll content in Insoluble P treatment was significantly lower than the other treatments. These results indicate that $\text{FePO}_4$ application showed an inhibitory effect on the shoot growth, but not on nodule formation and development. Kleinert et al. (2014) suggested that P partitioning within the rhizobia-symbiotic plant tissue was not evenly distributed in P-limited plants as compared to control plants supplied with adequate P. The P concentration was not notably higher in the nodules than in any other tissues of P-stressed plants with concentrations up to three-fold higher in some plants (Sa and Israel, 1991; Kousa et al., 2005; Schulze et al., 2006). Sulaiman and Tran (2015) suggested that nodules represent a preferentially strong sink for P incorporation among various plant tissues when P is limited. Our results were consistent with those of previous studies.

Positive correlations were found between nodule DW and shoot DW in Soluble P and Insoluble P treatment ($\text{NaH}_2\text{PO}_4$: $r = 0.85$, $P = 0.016$, $\text{FePO}_4$: $r = 0.87$, $P = 0.011$) (Table 2). The regression equations were as follows for Soluble P treatment and Insoluble P treatment, respectively: $y = 30.98x + 2.58$ ($P<0.05$) and $y = 10.43x + 0.63$ ($P<0.05$) where shoot DW is the outcome variable ($y$) and nodule DW is the predictor variable ($x$). The regression coefficient of Soluble P treatment was significantly greater than that of Insoluble P treatment ($t = 2.26$, $P<0.05$). The $\text{NaH}_2\text{PO}_4$ application also showed a significant positive correlation between nodule DW and shoot P content; $\text{FePO}_4$ application did not show a significant correlation.

Greater biomass allocation to below-ground plant parts in soil with low P availability could result in a greater number of nodules and development. But, these increases do not necessarily lead to increase in shoot P content. Photosynthetic allocation to below-ground parts and Fe supply may explain the increase and development of nodules in Insoluble P treatment. Fe is necessary for nitrogen fixation because it is an essential component of key compounds (leghemoglobin and ferredoxin) required for the fixation process. O’Hara et al. (1988) reported that peanut plant root nodule masses significantly increased in size after application of iron. It is suggested that there are two main pathways of P flow inside the symbiotic tissues: one is the direct pathway, where phosphate is absorbed on the surface of the nodule,

**Table 1:** Effect of different P treatments on dry weight, root-shoot ratio, P content, chlorophyll content and nitrogenase activity of S. cannabina.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dry weight (g)</th>
<th>Root-shoot ratio (g/plant$^4$)</th>
<th>P content (mg)</th>
<th>Chlorophyll content (mg/g FW) ($\mu$mol plant$^4$ hr$^{-1}$)</th>
<th>Nitrogenase activity (umol plant$^4$ hr$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shoot (g)</td>
<td>Root (g)</td>
<td>Nodule (g)</td>
<td>Shoot (mg)</td>
<td>Root (mg)</td>
</tr>
<tr>
<td>P free</td>
<td>1.0±0.1 b</td>
<td>0.5±0.1 b</td>
<td>0.02±0.01 b</td>
<td>0.52±0.04 a</td>
<td>0.7±0.1 c</td>
</tr>
<tr>
<td>Soluble P</td>
<td>3.4±0.3 a</td>
<td>1.3±0.2 a</td>
<td>0.03±0.01 b</td>
<td>0.39±0.02 b</td>
<td>3.7±0.3 a</td>
</tr>
<tr>
<td>Insoluble P</td>
<td>1.6±0.1 b</td>
<td>0.6±0.0 b</td>
<td>0.09±0.01 a</td>
<td>0.40±0.02 b</td>
<td>1.6±0.2 b</td>
</tr>
</tbody>
</table>

Soluble P and insoluble P was supplied $\text{NaH}_2\text{PO}_4$ and $\text{FePO}_4$ as a P source, respectively. Different letters for each variable indicate significant differences (Tukey $P<0.05$) between treatments.
and the other is an indirect pathway through the roots via the vascular network (Al-Niemi et al., 1998; Qin et al., 2012). An efficient Fe transport in the nodules would allow the plant to increase nodule biomass. The assimilation and subsequent partitioning of carbon between shoots and belowground tissues are affected by soil P availability. Generally, the shoot to root ratio is decreased under P-deficient conditions. In our experiment, the plants in P free treatment had a significantly lower shoot to root ratio. However, there was no significant difference in shoot to root ratios between Soluble P (NaH$_4$PO$_4$) treatment and Insoluble P (FePO$_4$) treatment. Therefore, considerable carbon from aboveground was allocated to nodules, leading to higher nodule development, which resulted in a significantly lower regression coefficient of shoot DW in Insoluble P treatment than that in Soluble P treatment.

### REFERENCES


### CONCLUSION

Shoot growth and P uptake in *S. cannabina* are still inhibited following ferric phosphate application even though associated rhizobia have P-solubilization ability. While, the number of nodules and nodule development was stimulated by ferric phosphate application. Qin et al. (2012) suggested that a higher P concentration in the nodules allows a plant to adapt to a P deficiency in the soil. Because we investigated relatively early growth response of *S. cannabina*, our future research efforts will examine how the accumulated P in nodules is used at a later growth stage.

### ACKNOWLEDGEMENT

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