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CO-INOCULATION EFFECT OF *BACILLUS PUMILUS* TUAT1 AND *BRADYRHIZOBIUM JAPONICUM* USDA110 ON GROWTH PARAMETERS OF SOYBEAN*

Phoo Wint Yee Thaw[1], Shin OKAZAKI[2]

Abstract

Enhancement of legume nitrogen fixation by co-inoculation of plant-growth-promoting rhizobacteria (PGPR) with rhizobia is an alternative way to improve the availability of nitrogen in a sustainable agricultural system. This study was conducted to evaluate the co-inoculation effect of *Bacillus pumilus* TUAT1 and *Bradyrhizobium japonicum* USDA110 on soybean (Glycine max L.) cv. Enrei by using the plant box experiment and examined the growth parameters (plant weight, root weight, nodules number and nodule weight). The experiment was performed in the Plant Microbiology Laboratory, Department of International Environmental and Agricultural Science, Tokyo University of Agriculture and Technology, Japan in 2018. The growth parameters were measured after 15 days, and 30 days of inoculation period and the results indicated an increase number for all parameters in the dual inoculation than the single inoculation. The plants inoculated with *B. pumilus* TUAT1, *B. japonicum* USDA110 and co-inoculation (TUAT1 + USDA110) promoted the plant weight and root weight of soybean compared to un-inoculated plants in both inoculation periods. The nodule formation was not observed in the plants inoculated with *B. pumilus* TUAT1 alone and un-inoculated (control) at 15 days post inoculation, when co-inoculated and USDA110 inoculated plants provided the highest number of nodules and nodules weight after 30 days. The results proved the potential benefits of increasing nodulation and promoting plant growth through the synergetic effect of TUAT1 and USDA110.

Keywords: *Bacillus pumilus* TUAT1, *Bradyrhizobium japonicum* USDA110, co-inoculation, soybean

Introduction

Soybean (*Glycine max* L. Merr.) is an important legume plant for human consumption, which has high protein content and rich nitrogen because it can fix nitrogen in the atmosphere. Some legumes that form nodules, such as soybean, peanut, and common bean are important crop plants that are difficult to grow under stress (Barea *et al*., 2005; Esitken *et al*., 2006). Likewise, the growth of soybean also has many limitations, which cause various problems and yield losses. The direct or indirect use of beneficial microorganisms such as plant growth-promoting rhizobia (PGPR) can promote plant growth because they have multiple mechanisms and play a key role in modern agriculture in developing countries (Glick, 1995).

The formation of symbioses with a variety of nitrogen-fixing soil bacteria (called rhizobia) makes its own fertilizers in the legumes. In agricultural production, the symbiotic relationship between legumes and rhizobia has become an important sector. Rhizobia are very important for crop production due to biological nitrogen fixation, wherein atmospheric elemental N_2 is converted to ammonia (NH_3), provides the required nitrogen to legumes because 65% of the nitrogen applied in agriculture (Matiru and Dakora 2004; Franche *et al*., 2009). In addition, it is a simple low-cost method to maintain soil fertility and increase crop yield through legume–rhizobium symbiosis. Therefore, inoculation with rhizobium has become a popular agronomic preparation, which can provide sufficient nitrogen for legumes instead of using nitrogen fertilizer.

[1] Assistant Lecturer, Department of Botany, University of Yangon

[2] Professor, Graduate School of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Japan

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Bradyrhizobium japonicum is a gram-negative, rod-shaped bacterium that performs root nodulating and nitrogen-fixing in legume plants. In 2015, Hungria *et al*. reported that dual inoculation of nitrogen-fixing bacteria and plant growth-promoting bacteria (PGPB) is more popular than single inoculation because this combination increases soybean yield and improves agriculture development. The combination of *Bacillus* strains and *B. japonicum* provided the increased number of nodules, weight of nodule, shoot, and root, total nitrogen together with grain yield in soybean (Bai *et al*., 2003 and Elkoca *et al*., 2008). Therefore, the improvement of plant's performance in the agricultural system benefits crop growth when inoculated with PGPR and rhizobial strains.

Among PGPR strains, *Bacillus* species are gram-positive, endospore-forming bacteria, which competitively colonize the roots and supply crop productivity directly or indirectly. In this study, one PGPR strain, *B. pumilus* TUAT1 was selected, which was isolated from the field of Tokyo University of Agriculture and Technology (Fuchu, Japan) and it has the plant growth-promoting effect because it promoted the rice yield up to 20-30% (Khin Thuzar Win *et al*., 2018). The aim of the study was to evaluate the single-inoculation and co-inoculation effect of *B. pumilus* TUAT1 with *B. japonicum* USDA110 on some growth parameters of soybean.

Materials and Methods

(i) Bacterial strains and growth conditions

Bacillus pumilus TUAT1 strain was grown in Luria-Bertani (LB) medium (Green *et al*., 2012) at 28°C and *Bradyrhizobium japonicum* USDA110 (current name *B. diazoefficiens)* (Krause, 2002) strain was grown at 28°C in arabinose-gluconate (AG) medium (Sadowsky *et al*., 1987). Both strains were provided from the Plant Microbiology Laboratory, Tokyo University of Agriculture and Technology.

(ii) Inoculum preparation

B. pumilus TUAT1 strain was incubated in LB broth for 24 h and *B. japonicum* USDA110 strain was incubated in AG broth for 72 h at 28°C under shaker condition. Then, cell suspensions were prepared containing 1 x 10^7 CFU ml^{-1} for each isolate.

(iii) Experimental design and inoculation test for plant assay

The box experiment was conducted in the Plant Microbiology Laboratory, Department of International Environmental and Agricultural Science, Tokyo University of Agriculture and Technology, Japan. The soybean (*Glycine max* L.) cv. Enrei seeds were sterilized with 70% ethanol for 30 seconds (s) and then with 1% sodium hypochlorite for 30 s. Seeds were rinsed with sterilized distilled water for 10 times, placed the seeds on the plates containing the autoclaved tissue paper. Then, it covered with double layers aluminium foil and incubated at 25°C for 2 days under dark conditions. After 2 days of incubation, the germinated seedlings were transferred to plant box (CUL-JAR300; Iwaki, Tokyo, Japan) containing sterile vermiculite. Each plant box containing 1 seed of Enrei was inoculated with 1 ml/10^7 cells of bacterial strains and watering with B & D (Broughton and Dilworth, 1971) nitrogen-free plant nutrient solution. The plant boxes were cultivated in a plant growth cabinet (LPH-410SP; NK Systems Co. Ltd., Osaka, Japan) at 25 °C and 70% humidity under a 16/8h day/night cycle (Faruque *et al*., 2015). The four treatments were performed: 1. No inoculation (Control), 2. *B. pumilus* TUAT1 alone, 3. *B. japonicum* USDA110 alone, and 4. *B. pumilus* TUAT1 and *B. japonicum* USDA110 (co-inoculation) respectively with three replications per treatment. Plant weight, root weight, nodules number, and nodule weight were evaluated at 15 days and 30 days post-inoculation (dpi).

Results

Effect of single and co-inoculation on soybean growth

The evaluation of single inoculation and co-inoculation effect on soybean seedling growth was recorded at 15 days and 30 days after sowing. In 15 days, the plant weight and root fresh weight were not significantly different in all treatments, while it was moderately changed in these parameters at 30 days (Fig.1 and 2). Remarkably, no significant differences were observed in the plant weight of controls, TUAT1 and USDA110 inoculated plants, whereas it was slightly improved in co-inoculation treatment at 15 days (Fig.3A). Similarly, root fresh weight remained stable in all inoculated plants, while a small increase recorded in co-inoculated plants (Fig.4A). Interestingly, the co-inoculated and TUAT1 inoculated plants indicated the high branches of the leaf at 15 days post-inoculation (Fig.1).

In 30 days, the highest plant weight was obtained in the plants inoculated with USDA110 and co-inoculated plants with significantly different, whereas the same results were not recorded in control and TUAT1 inoculated plants (Fig.3B). The fresh weight increased in roots of co-inoculated plants as compared to other treatments, but no significant differences were observed between them (Fig.4B).

In nodulation performance, the number of nodules was variable based on each inoculates (Fig. 1 and 2). As expected, uninoculated plants did not show any nodules at both incubation period. In 15 days after sowing, no nodules were formed in TUAT1 treatment, but nodules appeared in the other two treatments (Fig. 5A and 6A). However, the number of nodules and nodule weight were significantly different in co-inoculation as compared with other treatments after 30 days post-inoculation (Fig. 5B and 6B). Small nodules were observed in the secondary root of TUAT1 while the big nodules were enhanced in the main root of USDA110 treatment. Both sizes of nodules were formed in co-inoculation treatment with the highest nodules number (Fig. 2).

Another interesting point was the color of leaves, which is one of the important parameters to determine the level of nitrogen fixation. The color of leaves was not shown significance in 15 days (Fig. 1), although it was changed into yellow color in control and TUAT1 treatments at 30 days post-inoculation. However, in the plants inoculated with USDA110 and co-inoculated ones, the color of the green leaves did not change. (Fig. 2). That means USDA110 affected the concentration of chlorophyll.

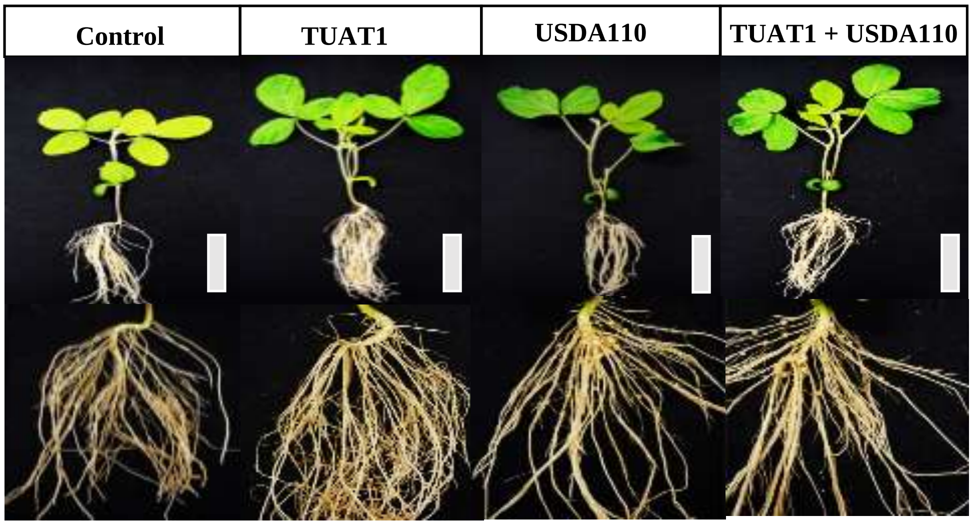

Figure 1 Comparison of plant growth and nodulation of *Glycine max* cv. Enrei plants, roots, and nodules inoculated with no-inoculation (control), *B. pumilus* TUAT1, *B. japonicum* USDA110, and *B. pumilus* TUAT1 and *B. japonicum* USDA110. Plants were photographed at 15 days post-inoculation (dpi).

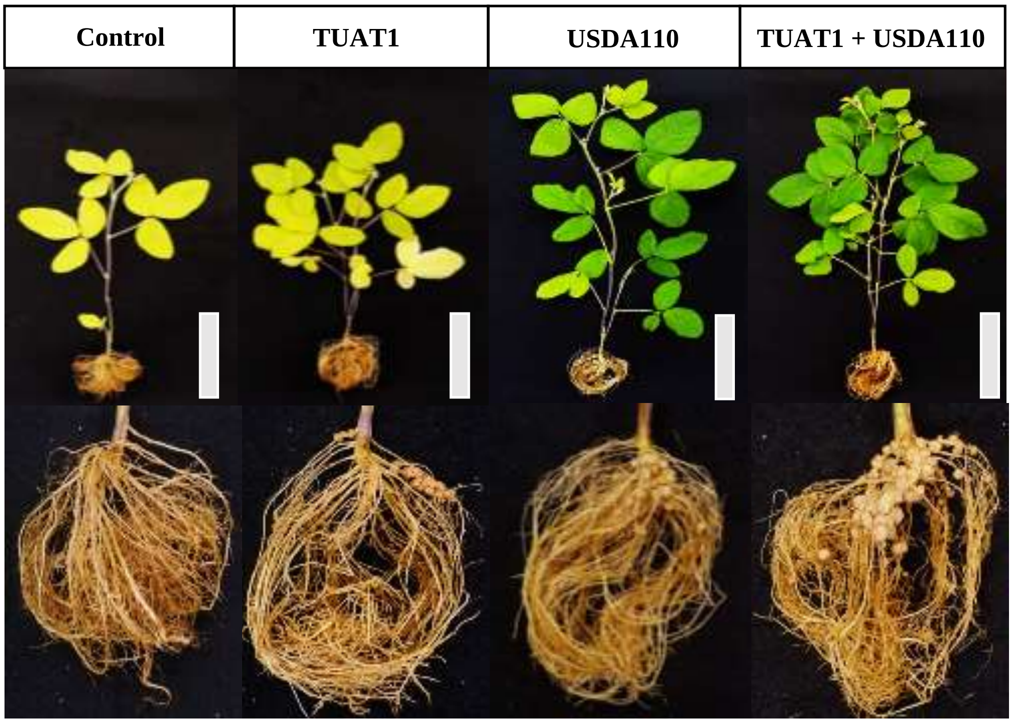

Figure 2 Comparison of plant growth and nodulation of *Glycine max* cv. Enrei plants, roots, and nodules inoculated with no-inoculation (control), *B. pumilus* TUAT1, *B. japonicum* USDA110, and *B. pumilus* TUAT1 and *B. japonicum* USDA110. Plants were photographed at 30 days post-inoculation (dpi).

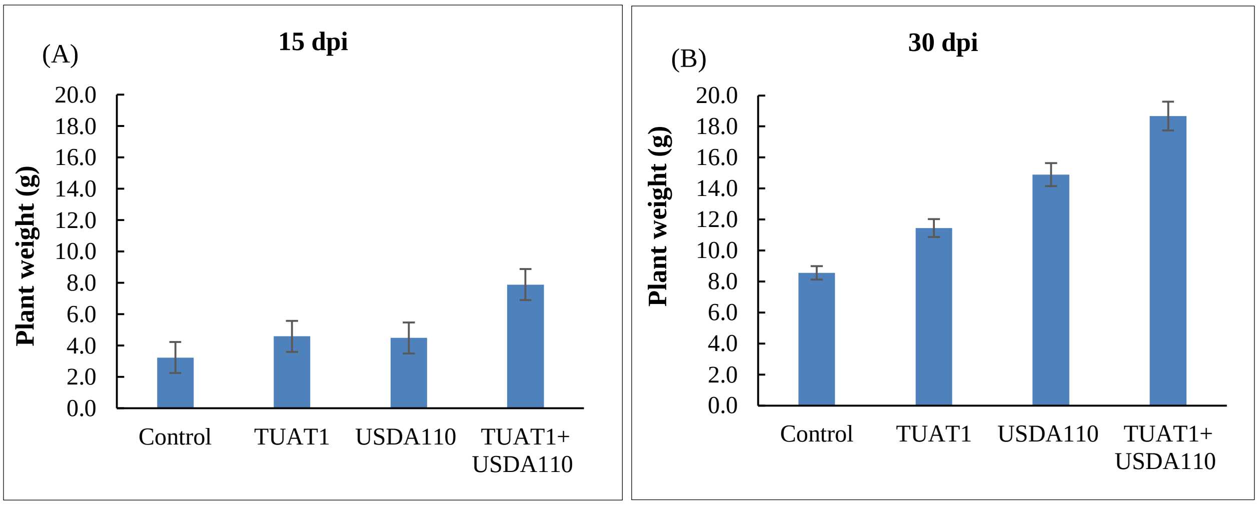

Figure 3 Fresh plant weight of soybean seedling at (A) 15 days and (B) 30 days after sowing. The histograms at each treatment are not significantly different at $P < 0.05$ (t-test). The bar on each histogram indicates standard deviation (SD).

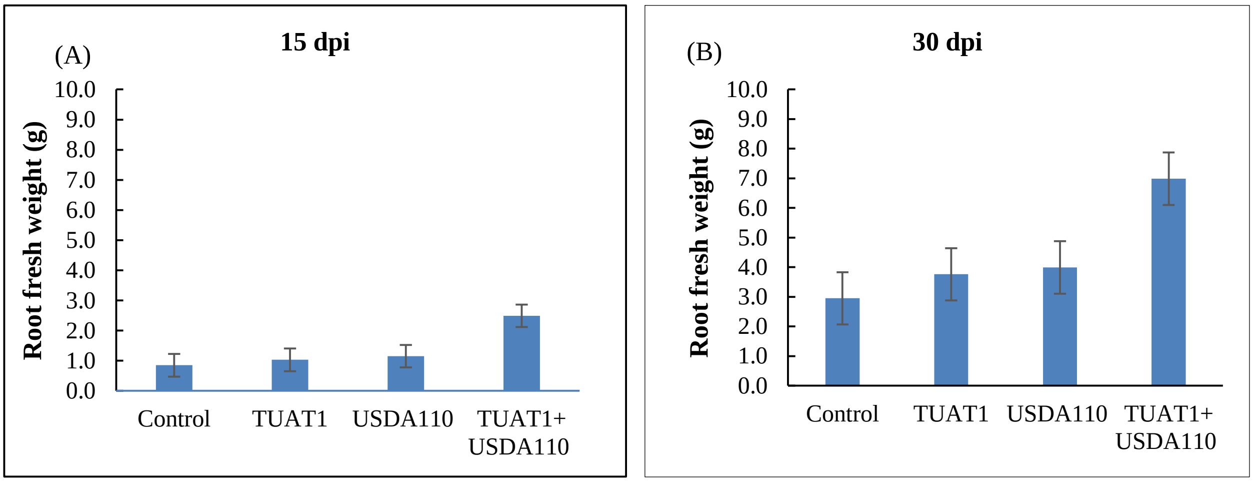

Figure 4 Root fresh weight of soybean seedling at (A) 15 days and (B) after sowing. The histograms at each treatment are not significantly different at $P < 0.05$ (t-test). The bar on each histogram indicates standard deviation (SD).

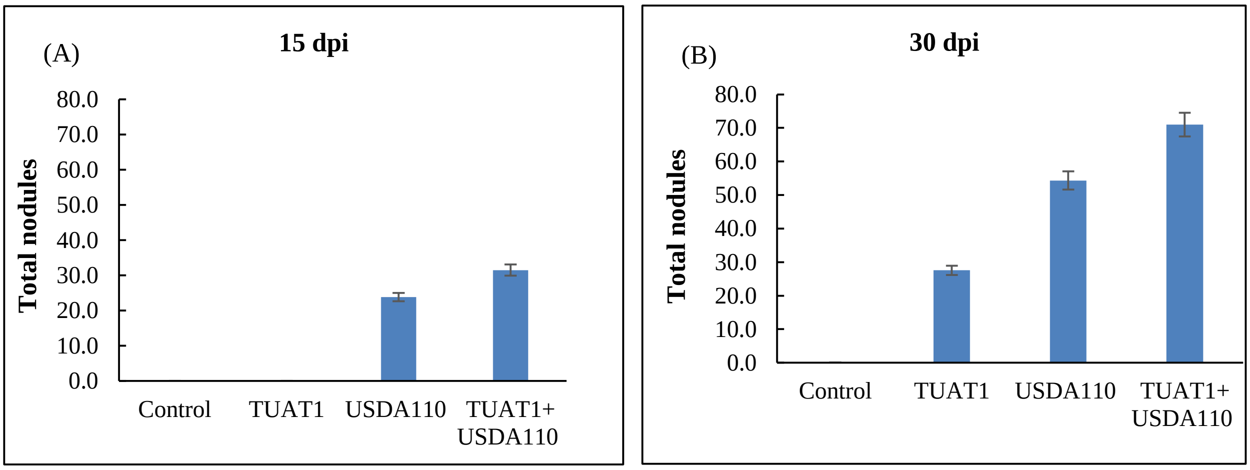

Figure 5 Number of nodules on principal, secondary root, and total of soybean seedling at (A) 15 days and (B) 30 days after sowing. The histograms in co-inoculation treatment are significantly different at 30 days, $P > 0.05$ (t-test). The bar on each histogram indicates standard deviation (SD).

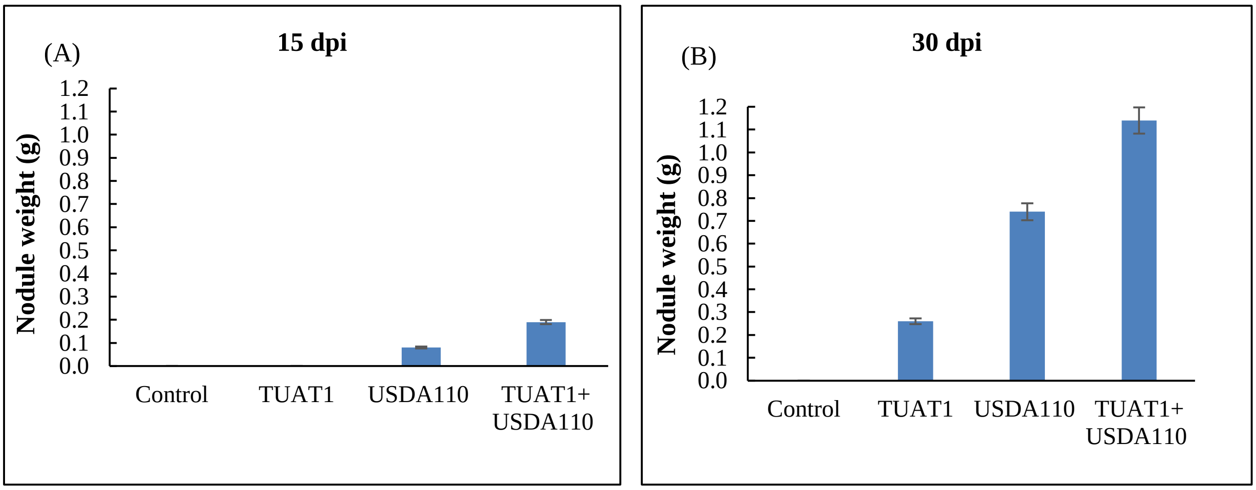

Figure 6 Nodule weight of soybean seedling at (A) 15 days and (B) 30 days after sowing. The histograms at each treatment are not significantly different at $P < 0.05$ (t-test). The bar on each histogram indicates standard deviation (SD).

Discussion

The dual inoculation of non-rhizobial bacterial strains improves the nodulation and N-fixing when combined with rhizobial strains. Plant growth promoting rhizobacteria (PGPR) plays an interesting role in the agricultural sector because it can improve soil fertility and increase crop productivity and nutrients. The beneficial effect of rhizobial inoculation in legumes has been the focus of biological nitrogen fixation, and it plays an important role in sustaining the fertility of a cropping system (Deshwal *et al*., 2003). In this research work, the results showed that *B. japonicum* USDA 110 with *B. pumilus* TUAT1 can increase plant weight, nodule number, nodule weight, and root weight by inoculation alone or in combination.

Soybean productivity can be determined by nodulation and subsequent nitrogen fixation, which is an important factor. According to the study of Wang and Martinez-Romero (2000), the nitrogen nutrition can be determined based on the number of nodules occupied by effective bacteria, and the symbiotic relationship of legume crops to achieve maximum nitrogen fixation. The current results indicated the ability to promote early soybean seedling growth and nodulation when the two strains are together in the plant box. The research conducted by Vessey and Buss (2002) showed that the co-inoculation of rhizobia with PGPR can improve nodules and nitrogen fixation. Reported by Tilak *et al.,* (2006) and Wani *et al.,* (2007) demonstrated that synergism between *Bacillus* and *Bradyrhizobium* improve forming nodules and plant biomass in the rhizosphere. Similarly, the increased nodulation was obtained in the current study at 30 days after sowing by the dual inoculation of TUAT1 and USDA110. A significant increase in the number of nodules by co-inoculation can be considered as an improvement in nitrogen fixation.

Although one PGPR strain increases the efficacy of the *Rhizobium* species in one legume, it does not perform the same in other legumes. Study by Camacho *et al*., (2001) clearly revealed that the dual inoculation of *Bacillus* sp. CECT 450 with *Rhizobium tropici* CIAT 899 improved nodules in common bean, although when co-inoculated with *B. japonicum* USDA 110 strain, it performed poorly in nodule formation in soybeans. However, the present study demonstrated that *B. pumilus* TUAT1 increases significantly nodulation in soybean when co-inoculated with *B. japonicum* USDA110.

The extensive reports of inoculating soybeans with suitable rhizobium strains have shown positive effects. Previous reported by Sajid *et al*., (2010) and Solomon *et al*., (2012) revealed that the significant increase of nodule number per plant was achieved by inoculation with Rhizobium or *B. japonicum* strains alone, although in the present study was not achieved the similar outcome by single inoculation. In addition, Rajendran *et al*., (2008) proved that the plant growth promoting bacterium *Bacillus* can promote plant weight. However, in the current study, TUAT1 alone did not support promoting plant weight.

Compared with *Bradyrhizobium* alone, the dual inoculation of *Bradyrhizobium* and PGPR microbes significantly increased the growth performance and nodulation improvement in soybean (Dubey, 1996, Wasule *et al*., 2007, and Abbasi *et al*., 2011). Interestingly, the present results recorded the high branches of leaf in the co-inoculated and TUAT1 inoculated plants after 15 days as well as the highest plant weight was obtained in co-inoculation than the USDA110 alone at 30 days post inoculation. The present investigation indicated the potential of non-rhizobial strain when combined with rhizobial strain enhance the growth and nodulation of cultivated legumes such as soybean. This approach meets the needs of modern agriculture, economy, and environmental sustainability.

Conclusion

The present study demonstrated that *Bacillus pumilus* TUAT1 was co-inoculated with *Bradyrhizobium japonicum* USDA110, enhanced the growth and nodulation of soybean cv. Enrei, under laboratory conditions. All treatments have a positive effect on soybeans, and co-inoculation with USDA110 has the largest increase at 30 days post-inoculation compared with single inoculation of TUAT1 and USDA110 alone. Therefore, it can be concluded that the combined use of TUAT1 and USDA110 will be helpful for soybean production by providing plant growth and nodulation. This combination can be used as an alternative to expensive inorganic fertilizers to provide farmers with product formulations, while reducing the excessive use of inorganic fertilizers and helping to alleviate environmental problems. Further studies under field conditions with a great number of soybean cultivars will be needed to corroborate the findings of this study.

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