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Research Paper

EFFECTS OF MOLYBDENUM ON BIOLOGICAL NITROGEN FIXATION BY COMBINATION OF RHIZOBIUM AND AZOSPIRILLUM IN SOYBEAN UNDER DRIP IRRIGATION SYSTEM

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A field experiment was conducted on Serdang soil, to study the effect of both molybdenum application and combination of *Rhizobium* and *Azospirillum* on the growth and yield of soybean crop under drip irrigation system. Factorial experiment was used with randomize complete block design (RCBD) for molybdenum and dual inoculation factors, the molybdenum levels were 0, 1, 1.5 and 10 Kg of Mo /ha, while dual inoculation contained two treatments (dual inoculation and control) with three replicates. The following parameters were measured: number of nodules, weight of nodules, plant dry weight, total nitrogen in plant, molybdenum content in plant, phosphorus content in plant, potassium content in plant, yield, weight of 100 seeds, number of pods, weight of pods and oil in seeds. The results showed that the inoculation treatment was significantly higher than non-inoculation treatment, also results showed that increasing in all parameters at 1 Kg of Mo /ha. We can conclude that the dual inoculation with molybdenum application for soybean crops caused increasing in both yield and its components.

Keywords: Molybdenum, *Rhizobium*, *Azospirillum*, Soybean, Drip irrigation system

INTRODUCTION

The co-inoculation of *Rhizobium* with other plant-promoting bacteria has received a great attention in legume growth promotion for a long time (Anandham et al., 2007). The co-inoculation of *Rhizobium* and *Azospirillum* is one of these important dual inoculations, which has proved its effect on many legumes. The inoculation of *Azospirillum* and *Rhizobium* can stimulate or inhibit nodule formation and growth, depending on the concentration and timing of inoculation (Yahalom et al., 1991). The increase in plant dry matter and nitrogen content, as a result of co-inoculation of *Rhizobium* and *Azospirillum,* may be related to early nodulation, higher N_2 fixation and improvement of root development (Burdman et al., 1997). According to Iruthayathas et al., (1983), the shoot yield of soybean increased as a result of dual inoculation of *R. japonicum* and *A. brasilense,* compared with *Rhizobium* alone. Rai (1983) reported similar effect on chickpea by

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finding that co-inoculation increased yield, nodule dry weight and nitrogen fixation. During an experiment under laboratory conditions, Molla et al., (2001) observed significant root growth and nodulation stimulated during co-inoculation with *Bradyrhizobium* and *Azospirillum.* This co-inoculation also increased total root length, plant dry matter, root number and root dry matter. Groppa et al., (1998) recorded an increase in nitrogen content of dual inoculation (23% over *B. japonicum* - single inoculation) as well as in a number of most active nodules. Accordingly, this study aimed to investigate the beneficial effect of co-inoculation of *A.brasilense* and *R. japonicum* with molybdenum to determine its effect on soybean growth and production. The experiment was carried out under economic amount of water in drip irrigation system.

MATERIALS AND METHODS

Rhizobium strain UPMR020 was obtained from soil microbiology lab (UPM). The liquid yeast mannitol agar extract was inoculated with strain and put in shaking incubator (100rpm) at 28ºC for five days. The living cells of root bacteria were counted by dilution and plate count, the number of which was 45×10^6 cfu ml^{-1}. *A. brasilense* isolated from root of corn, was grown on slant at 30ºC for 24 hour. The number of its living cells was 1.5×10^6 cfu ml^{-1}. Then, the flasks containing sterilized Nfb were inoculated by 1ml of inoculum and incubated at 30ºC for 72 hrs. The seeds AGS190 were obtained from Department of Crop Science in Faculty of Agriculture (UPM) and inoculated with *Azospirillum* and *Rhizobium* together (Yahalom et al., 1987). The pots were filled with 20 kg of Serdang soil with the properties shown in (Table 1). Calcium carbonate was added two weeks before planting to raise soil pH. Six seeds were planted in each pot and after two weeks only three plants were left. Drip irrigation system was used for all the pots with a discharge 2 L of water /hr. The added fertilizers contained 40 kg of N/ ha as ammonium sulfate (added one time before planting), as well as 120 kg of P/ ha as triple super phosphate and 100 kg of K/ ha as potassium sulfate (both added in twice, once before planting and the second time before flowering period). Molybdenum was added at four levels 0, 1, 1.5 and 10 kg of Mo/ ha as ammonium molybdate. After 16 weeks, plants were harvested and plants parts were digested to determine plant content of total N, Mo, P, K and oil content in seeds. Other parameters like number of nodules, weight of nodules, yield, number of pods, weight of pods, weight of 100 seeds and plant dry weight were taken to show the effect of molybdenum and bacterial inoculation by *Azospirillum* and *Rhizobium* under drip irrigation system on soybean plants.

Experimental Design and Determination of Growth Parameters

The experiment was laid out in a Randomized Complete Block Design (RCBD) with three replicates. The treatments were: 1- Control, 2- 1 kg of Mo /ha, 3-1.5 kg of Mo /ha, 4- 10 kg of Mo /ha, 5- *A.brasilense*+*Rhizobium*+1 kg of Mo /ha, 6- *A.brasilense*+*Rhizobium*+1.5 kg of Mo /ha, 7- *A.brasilense*+*Rhizobium*+10 kg of Mo /ha, and 8- *A.brasilense*+*Rhizobium*. The measurements taken in this experiment were: number of nodules /plant, weight of nodules mg /plant, plant dry weight g /plant, number of pods /plant, weight of pods g / plant, yield g /plant, weight of 100 seeds g/ plant, %N in plant, phosphorus content in plant µg /g, potassium content in plant µg /g, molybdenum content in plant µg /g and oil content in seeds. The measurements are made during the growth and also at the harvest after 16 weeks of growth.

Table 1: Chemical Properties of Serdang Series Soil (0-15 cm Depth)

Analyses	Results
pH	4.2
N%	0.13
Available P (ppm)	2.50
Available K (ppm)	12.30
Available Mo (ppm)	0.03

RESULTS

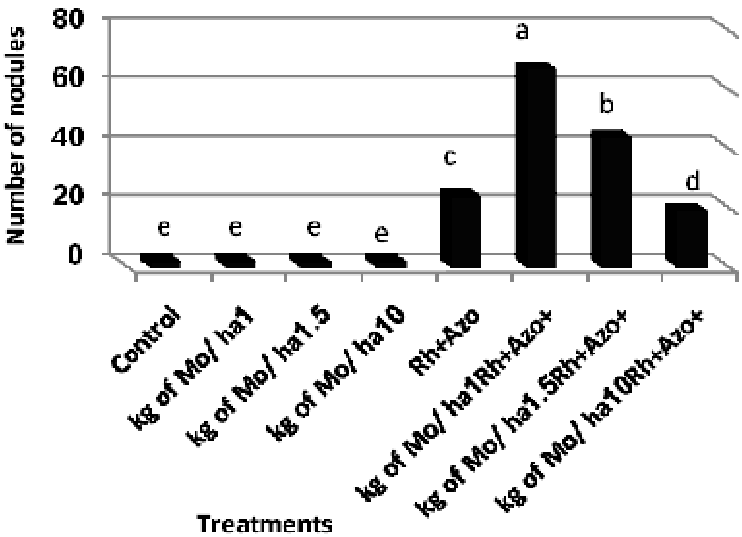
Number of Nodules

Bacterial inoculation revealed a significant increase in the number of nodules, compared with the control (Pd”0.01) Figure 1. Adding molybdenum at different levels did not show any effect on the number of nodules; adding 1, 1.5 and 10 kg of Mo /ha all gave 2 nodules. Among all the interactions between molybdenum and dual inoculation by *Azospirillum* and *Rhizobium,* the highest result was observed in the dual inoculation with 1 kg of Mo /ha. Under this treatment, the number of nodules was 67 nodules, followed by 1.5 kg of Mo /ha which gave 44 nodules. The lowest number of nodules under interaction treatments was observed when 10 kg of Mo /ha was added to co-inoculation between *Azospirillum* and *Rhizobium*.

Weight of Nodules

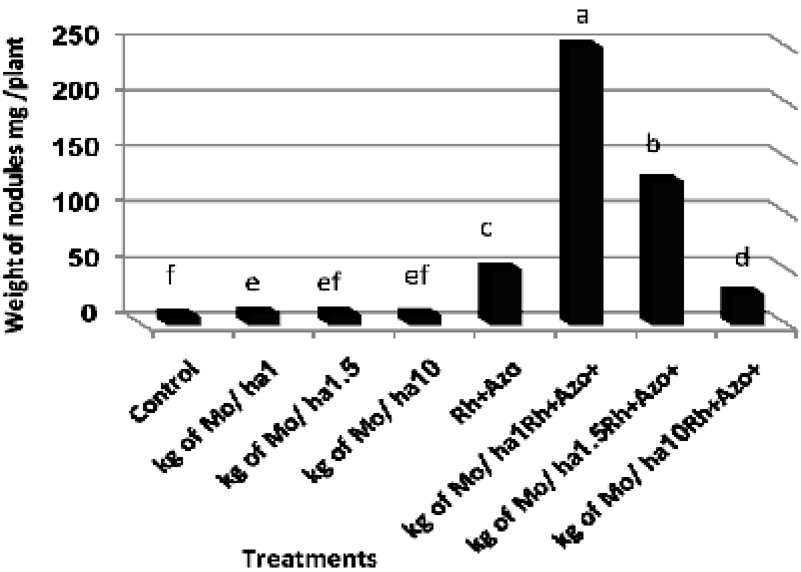
Under dual inoculation, the weight of nodules increased to 50 mg/plant, compared with the control which registered only 8 mg /plant. Regarding the treatments with different levels of molybdenum, 1 kg of Mo /ha increased the weight of nodules, whereas 1.5 and 10 kg of Mo /ha did not show any difference. Accordingly, the interaction showed the highest result for dual inoculation with 1 kg of Mo /ha, where the weight of nodules was 250 mg /plant. This can be compared with 1.5 and 10 kg of Mo /ha, which respectively registered 129 and 28 mg /plant (Figure 2).

Figure 1: Effect of Molybdenum and Co-Inoculation By *Rhizobium* and *Azospirillum* on Number of Nodules in Soybean Under Drip Irrigation System



Note: The letters above the bars represents significant differences at 0.01 level of significance.

Figure 2: Effect of Molybdenum and Co-Inoculation by *Rhizobium* and *Azospirillum* on Weight of Nodules in Soybean Under Drip Irrigation System



Note: The letters above the bars represents significant differences at 0.01 level of significance.

Plant Dry Weight

Significant differences between plants which received dual inoculation and molybdenum, compared with plants which only received molybdenum or did not receive anything (Pd"0.01) (Figure 3). The control registered the lowest plant dry weight during the experiment (16g/ plant), compared with co-inoculation treatment which gave a high weight of 72 g /plant. The treatment of 1 and 1.5 kg of Mo /ha gave a much higher weight than 10 kg of Mo /ha. The results revealed that the significant differences between co-inoculation interactions with molybdenum, where co-inoculation with 1 kg of Mo /ha gave 97.56 g / plant. This can be compared with 1.5 and 10 kg of Mo /ha, which registered 74.30 and 64 g /plant ($P \geq 0.01$).

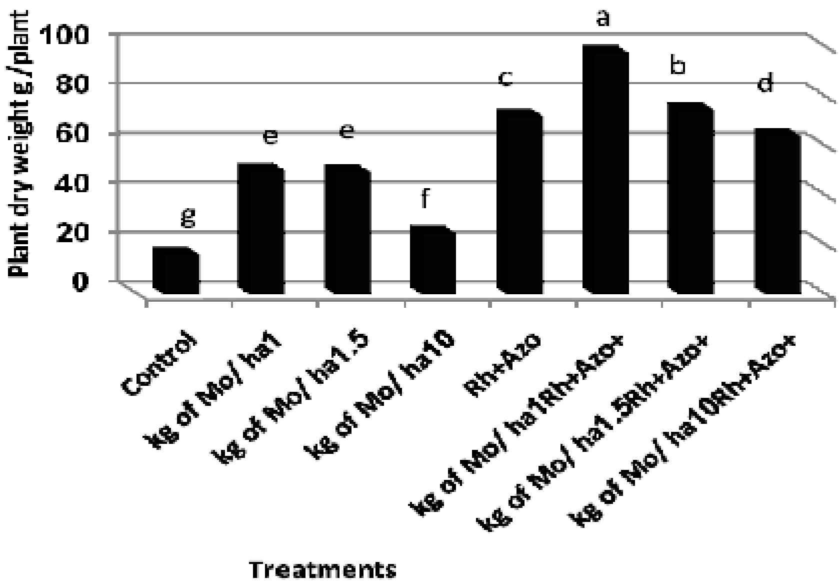
Total Nitrogen in Plant

As noticed in Figure 4, there is a significant increase in total nitrogen under treatment with the co-inoculation of *Azospirillum* and *Rhizobium,* compared with the control (Pd"0.01). Accordingly, total nitrogen under co-inoculation treatment gave 5.77%, compared with 1.02% for the control. Adding molybdenum at different concentration led to an increase of total nitrogen, the highest percentage of which was recorded under 1 kg of Mo /ha (4.18%), compared with 2.19% and 2.10% for 1.5 and 10 kg of Mo /ha. The interaction between molybdenum and co-inoculation gave the best results during the experiment as the interaction between 1 kg of Mo /ha and co-inoculation recorded the highest total nitrogen (7.56%). This percentage was significant (Pd"0.01), compared with other interaction which recorded 6% and 5.40% without significant difference between them.

Molybdenum Content in Plant

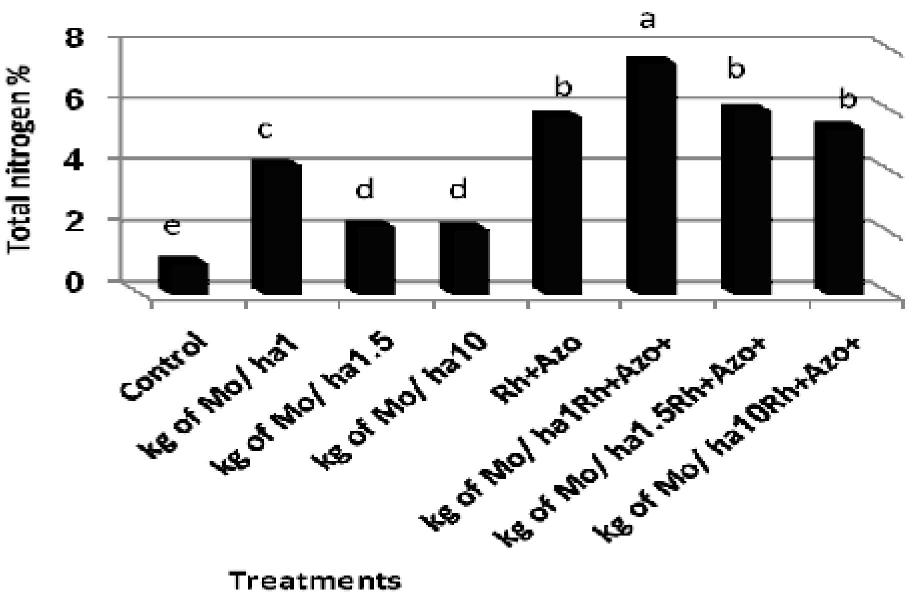
Molybdenum content in plant was affected by co-inoculation which registered significant increase

Figure 3: Effect of Molybdenum and Co-Inoculation by *Rhizobium* and *Azospirillum* on Plant Dry Weight in Soybean Under Drip Irrigation System



Note: The letters above the bars represents significant differences at 0.01 level of significance.

Figure 4: Effect of Molybdenum and Co-Inoculation by Rhizobium and Azospirillum on Total Nitrogen in Soybean Under Drip Irrigation System



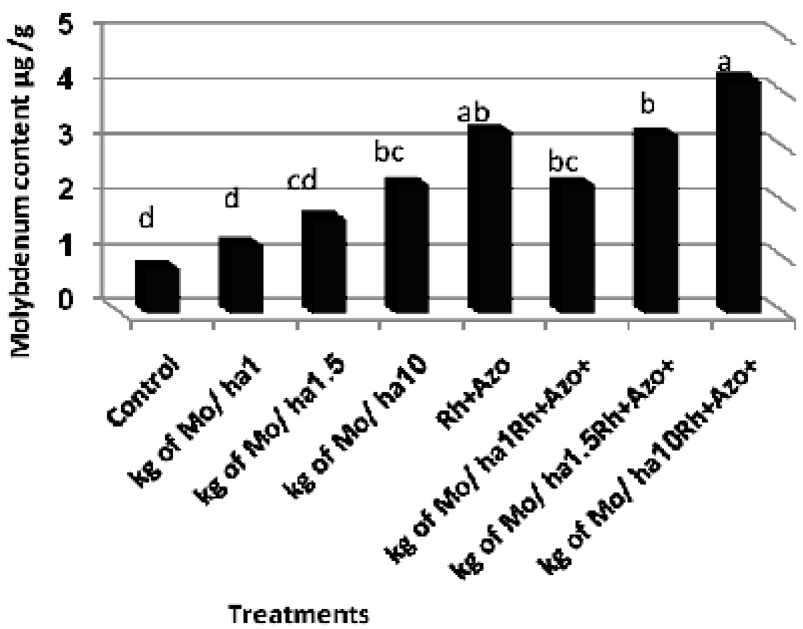
Note: The letters above the bars represents significant differences at 0.01 level of significance.

(Pd”0.01), compared with the control which gave the lowest molybdenum content during the experiment. While the molybdenum content under co-inoculation alone was 3.29 µg /g, it was 0.81 µg/g. under the control treatment. Molybdenum application revealed significant increase (Pd”0.01) for 10 kg of Mo /ha, compared with other treatments where molybdenum content raised with the increase of molybdenum levels. Adding 10 kg of Mo /ha gave 2.33 µg /g, whereas 1.5 and 1 kg of Mo /ha respectively gave 1.70 and 1.23 µg /g. Adding 10 kg of Mo /ha with co-inoculation proved the significance of this treatment, compared with other interactions. Molybdenum content under this treatment was 4.21 µg /g, and it was 3.22, 2.33 µg/g for the interactions between 1.5 and 1 kg of Mo /ha with co-inoculation (Figure 5).

Phosphorus Content in Plant

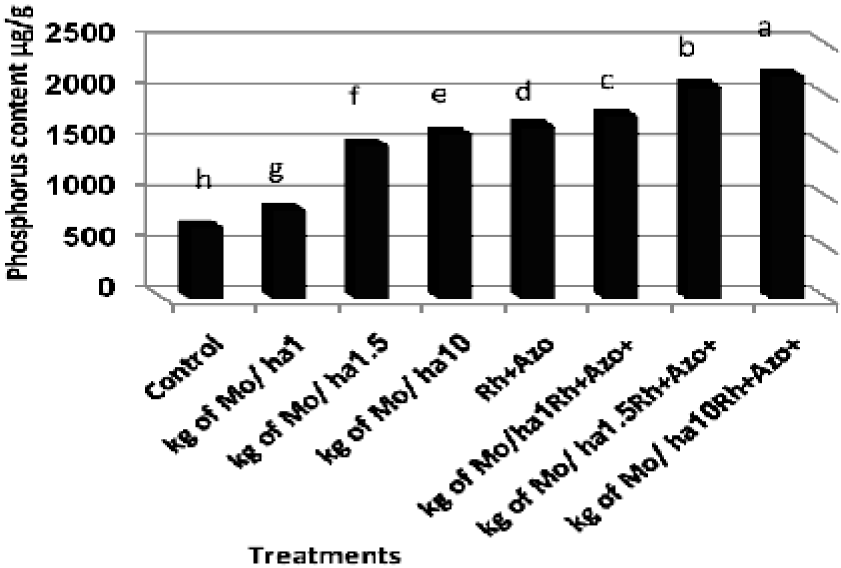
Co-inoculation produced the highest results, with or without adding molybdenum. The plants treated with *Azospirillum* and *Rhizobium* gave 1696.33 µg /g, compared with 702 µg /g for the control. Significant increase (Pd”0.01) was noticed between molybdenum levels when 1, 1.5 and 10 kg of Mo /ha were added. Accordingly, phosphorus content was 1623 µg /g for 10 kg of Mo /ha, compared with 1506 and 875 µg /g for 1.5 and 1 kg of Mo /ha. The interaction between molybdenum and co-inoculation significantly increased (Pd”0.01) phosphorus content under interaction between 10 kg of Mo /ha and co-inoculation, compared with the co-inoculations with 1.5 and 1 kg of Mo /ha (Figure 6).

Figure 5: Effect of Molybdenum and Co-Inoculation by Rhizobium and Azospirillum on Molybdenum Content in Soybean Under Drip Irrigation System



Note: The letters above the bars represents significant differences at 0.01 level of significance.

Figure 6: Effect of Molybdenum and Co-Inoculation by *Rhizobium* and *Azospirillum* on Phosphorus Content in Soybean Under Drip Irrigation System



Note: The letters above the bars represents significant differences at 0.01 level of significance.

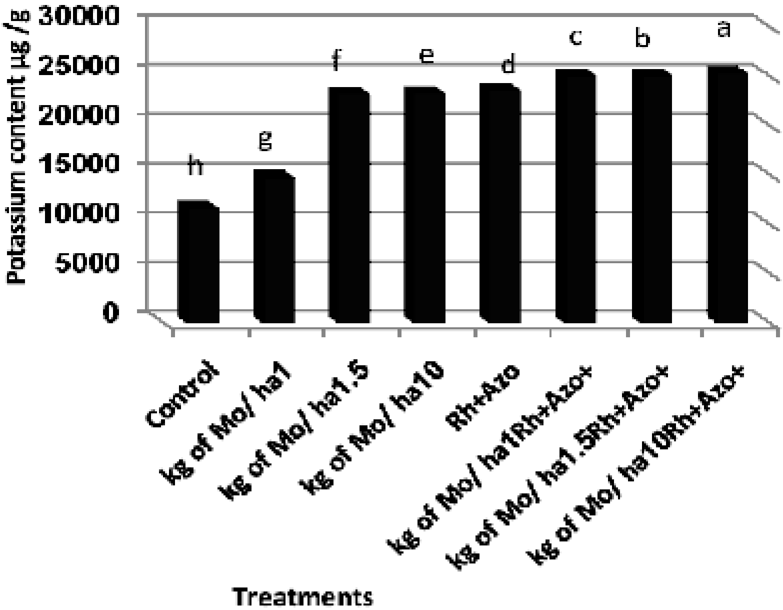
Potassium Content in Plant

The potassium content in plant was different in different treatments, and the lowest amount was recorded under the control. In comparison with the control, co-inoculation significantly increased potassium content (Pd"0.01), where potassium content was 23595.66 µg /g. Adding molybdenum alone proved the significance of 10 kg of Mo /ha (Pd"0.01), in which potassium content increased until it reached 23295 µg /g, followed by 23250 and 14796 µg /g for respectively 1.5 and 1 kg of Mo /ha. The highest reading was registered at the end of the experiment under the interaction between co-inoculation and 10 kg of Mo /ha, where potassium content in plant was 25295 µg /g and at a significant difference (Pd"0.01) with other interactions (Figure 7).

Yield

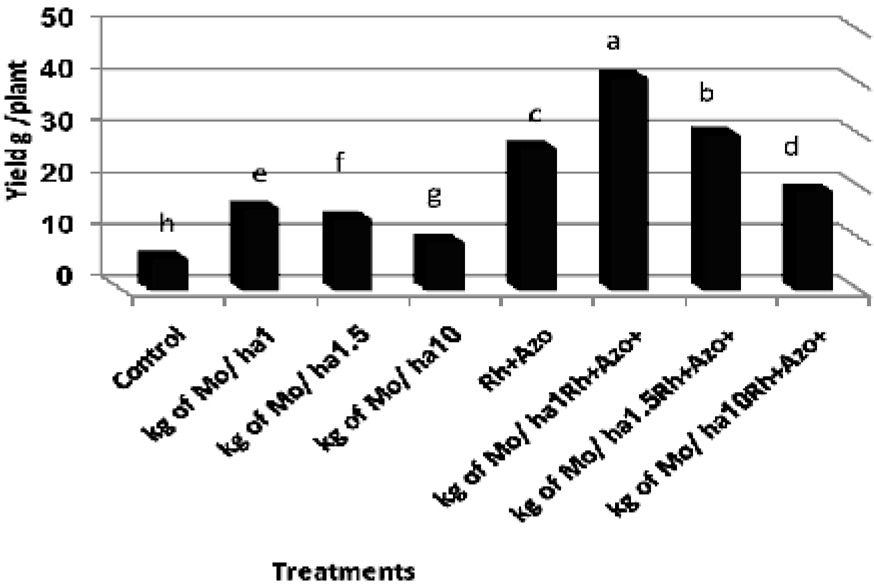
Co-inoculation between *Rhizobium* and *Azospirillum* showed a significant increase in the yield (Pd"0.01), compared with the control (Figure 8). Accordingly, the yield was 27.26 g /plant in the co-inoculation treatment, compared with 6 g /plant for the control. The interaction between 1 kg of Mo /ha and co-inoculation seemed the best treatment for plants as the yield under this treatment registered 40.76 g /plant and was significant differently with all treatments (Pd"0.01). Other interactions registered 29.80 and 19 g /plant for 1.5 and 10 kg of Mo /ha with co-inoculation. Adding molybdenum alone gave a lower yield than the interactions in which 1, 1.5 and 10 kg of Mo /ha respectively gave 15, 13.66 and 9.15 g / plant.

Figure 7: Effect of Molybdenum and Co-Inoculation by *Rhizobium* and *Azospirillum* on Potassium Content in Soybean Under Drip Irrigation System



Note: The letters above the bars represents significant differences at 0.01 level of significance.

Figure 8: Effect of Molybdenum and Co-Inoculation by *Rhizobium* and *Azospirillum* on Yield of Soybean Under Drip Irrigation System



Note: The letters above the bars represents significant differences at 0.01 level of significance.

Weight of 100 Seeds

The results in Figure 9 showed significant increase (Pd"0.01) in the weight of 100 seeds between the co-inoculation treatment and the control. While the control gave only 6 g, co-inoculation gave 12.92 g. Adding molybdenum had obvious effect on this feature as 1, 1.5 and 10 kg of Mo /ha gave 11.80, 10.83 and 7 g for un-inoculated plants and 19.76, 13.50 and 12.60 g for inoculated plants. Obviously, 1 and 1.5 kg of Mo /ha contributed significantly to the increase in the weight of seeds, with or without adding molybdenum. This can be compared with the treatment which added 10 kg of Mo /ha.

Number of Pods

For un-inoculated soybean plants (without molybdenum) a lower number of pods was registered (5 pods /plant), whereas a significantly higher number was recorded for inoculated plants (43 pods /plant). All the inoculated plants gave higher number of pods than un-inoculated plants. Accordingly, adding molybdenum had a significant effect on the number of pods which increased especially when 1 and 1.5 kg of Mo /ha was added. The numbers were 86 and 49 pods for inoculated plants, whereas 31 and 19 pods were recorded for un-inoculated plants (Figure 10). As mentioned earlier, these numbers emphasized the important role of molybdenum on biological functions of plant and reactivated nitrogen fixation process reflected in many features of plant.

Weight of Pods

The effect of co-inoculation continued under the feature of the weight of pods as co-inoculation registered 50 g /plant, compared with 2 g /plant for the control. Adding molybdenum obviously increased the weight of pods. Under 1 and 1.5 kg of Mo /ha, the weight of pods was 26.26 and 19.82 g /plant for un-inoculated plant. Meanwhile, the co-inoculated plant significantly increased the weight of pods (Pd"0.01) to reach 86.65 and 63.37 g /plant. Adding 10 kg of Mo /ha gave a lower weight than the two other levels, with or without co-inoculations (Figure 11).

Oil in Seeds

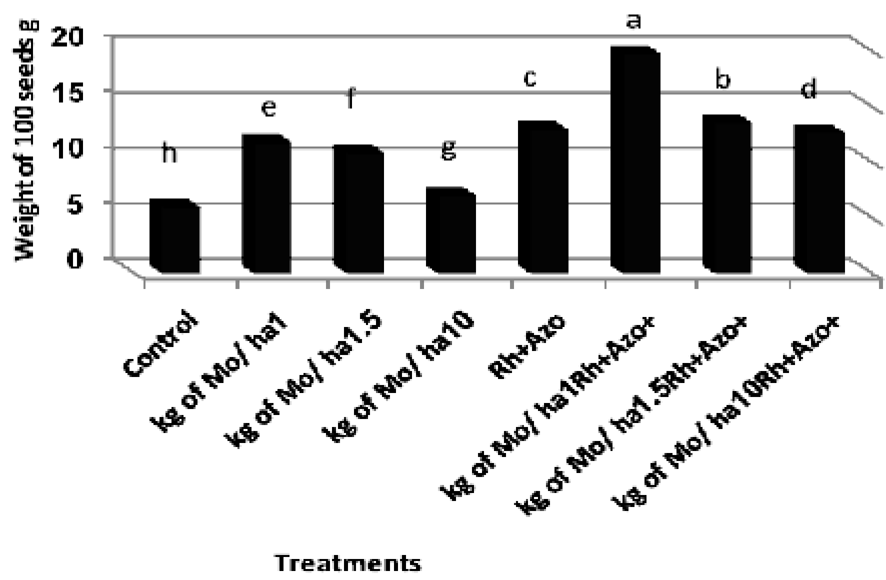
Figure 12 reveals that co-inoculation had a significant effect (Pd"0.01) in decreasing oil content in seeds. Accordingly, the oil content was 16.46%, compared with the control plants which accumulated 17.1%. The difference in the oil content between 1 and 1.5 kg of Mo /ha was a slight one, but the oil content under 10 kg of Mo /ha was significantly higher than the other two. Interaction between co-inoculation and molybdenum showed significant increase (Pd"0.01) in the oil content under interaction between co-inoculation and 10 kg of Mo /ha.

DISCUSSION

Combined inoculation of soybean plants with *Rhizobium* and *Azospirillum* significantly increased number of nodules, nodules dry weight and dry matter production. The increase may be related to early nodulation, which can encourage root development. Also, the concentration of bacteria and timing of inoculation contributed to the increase in plant features (Volpin and Kapulnik, 1994).

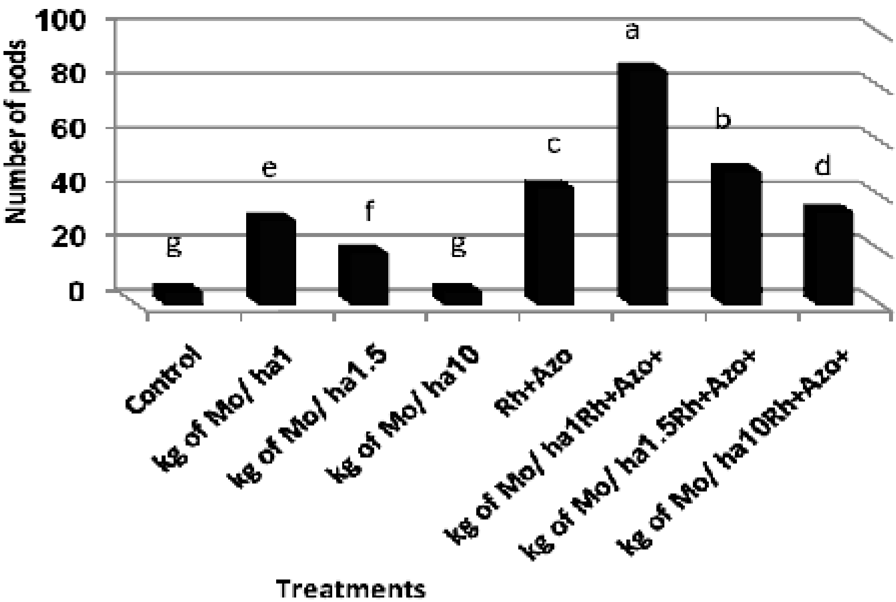
Stimulation of nodulation following inoculation with *Azospirillum* can be associated with an increase in the production of lateral roots in root hair density and in root hair branching (Okon and Kapulnik, 1986). In experiments carried out in a hydroponic system, Burdman et al. (1997) found that co-inoculation by *Rhizobium* and *Azospirillum* increased the excretion of nod-gene by seedling roots of bean. It was also noticed that root hair

Figure 9: Effect of Molybdenum and Co-inoculation by *Rhizobium* and *Azospirillum* on Weight of 100 Seeds in Soybean Under Drip Irrigation System



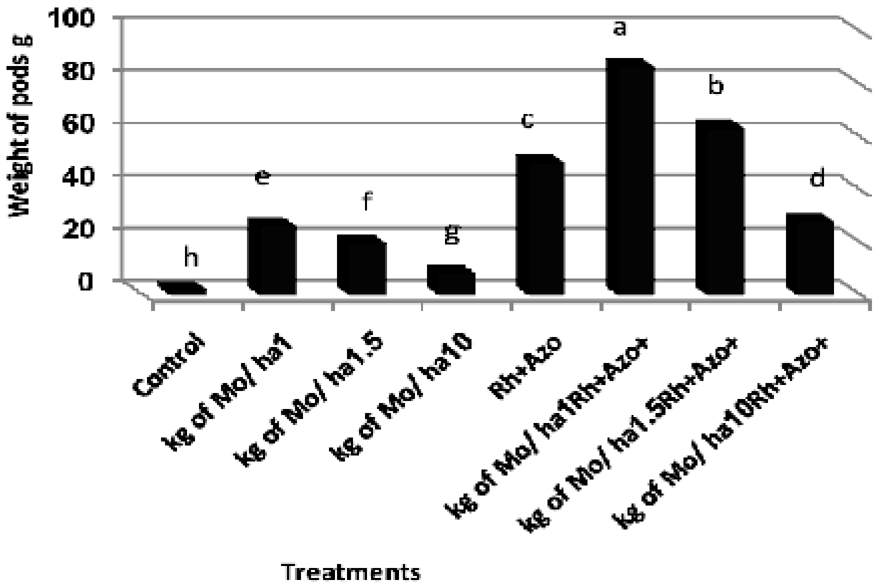
Note: The letters above the bars represents significant differences at 0.01 level of significance.

Figure 10: Effect of Molybdenum and Co-inoculation by Rhizobium and Azospirillum on Number of Pods in Soybean Under Drip Irrigation System



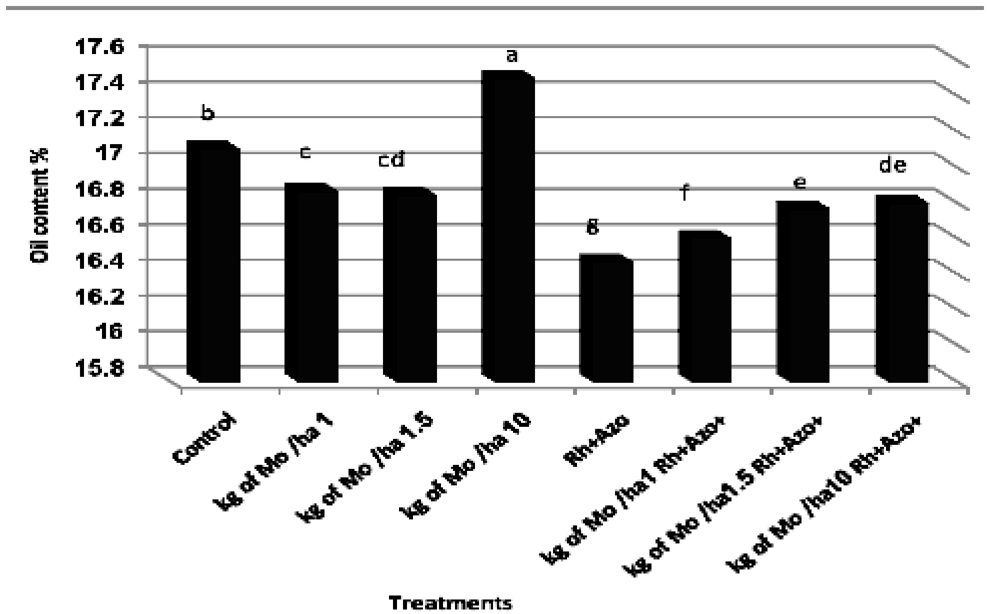
Note: The letters above the bars represents significant differences at 0.01 level of significance.

Figure 11: Effect of Molybdenum and Co-Inoculation by *Rhizobium* and *Azospirillum* on Weight of Pods in Soybean Under Drip Irrigation System



Note: The letters above the bars represents significant differences at 0.01 level of significance.

Figure 12: Effect of Molybdenum and Co-Inoculation By *Rhizobium* and *Azospirillum* on Oil Content in Soybean Under Drip Irrigation System



Note: The letters above the bars represents significant differences at 0.01 level of significance.

formation was improved by *Azospirillum.* Significant increase in nodule number, shoot dry weight and yield after combined inoculation with *Rhizobium* and *Azospirillum* were observed as well. This may suggest that *Azospirillum* bacteria have some compounds which encourage new hair formation and then infection (Iruthayathas et al., 1983).

Due to the stimulation effect of adding *Azospirillum* to co-inoculation with *Rhizobium, Azospirillum* can colonize roots and nodules of *R. japonicum.* According to Singh and Subba Rao (1979), soybean plants were first inoculated by *Rhizobium* and then stimulated by *Azospirillum,* a known isolate inoculated in unsterilized soil.

The increase in mineral uptake (N, P, and K) and microelements may be due to the increase in root length rate which improves after dual inoculation (Dobbelaere and Okon, 2007). This leads to a better mineral nutrition of the plant molybdenum, phosphorus and iron, beneficial for *Rhizobium* nodule formation and nitrogen fixation activities (Burdman et al., 1998).

The essential effect of co-inoculation on increasing susceptibility to *Rhizobium* infection may be that *Azospirillum* stimulates the formation of a large number of epidermal cells that differentiate into infectable root hairs (Yahalom et al., 1987). In a similar manner, Okon (1985) indicated that the improvement of root proliferation, water status and mineral uptake of plant was caused by *Azospirillum*. The increase in plant growth and nitrogen uptake was observed due to the mixed inoculation of *Rhizobium* and *Azospirillum.* Such an increase in nodulation and plant growth in soybean crop was attributed to the production of growth promoting substances by *Azospirillum* (Tien et al., 1979).

The inoculation of mixed cultures of *Azospirillum* and *Rhizobium* obviously increased nodule number and grain yield in soybean. This was in agreement with the result of a study conducted by Iruthayathas et al., (1983) on wingbean and soybean. The increase in nodulation may be related to protein or enzymatic product which is available in the cells of bacteria (Burns et al., 1981).

The beneficial effect of *Azospirillum* added to a combination with *Rhizobium* was *Azospirillum* ability to convert tryptophan into Indole Acetic Acid, a substance that promoted the infection process of *Rhizobium* in root hair (Marre, 1976). On the other hand, cells of *Azospirillum* might have contained some compounds that could induce new root hair formation and subsequent infection.

The increase in plant parameters was due to co-inoculation stimulated by adding molybdeunm because maximum production from leguminous crops could be attained only when the plants were effectively nodulated and well supplied with molybdenum (Burton and Curley, 1966). The responses to molybdenum were obvious in the increases of both yield and nitrogen concentrations in soybean plants. Molybdenum increase in plant tissues could be due to high pH which increased by adding lime. The increase in pods yield under co-inoculation and molybdenum was caused by the effect of co-inoculation which was related to accumulative effects of N, P and other insoluble nutrients in the rhizosphere of the plants (Anandham et al., 2007). Early nodulation, increase in most active nodules, early development of seedlings, fresh weight of nodules and high nitrogen percentage have been reported for several crops inoculated with co-inoculation (Cassan et al., 2009; Molla et al., 2001; Groppa et al., 1998). Molybdenum application affected the growth and plant N yield with inoculation, which could be related to the increase in metabolic pools for the synthesis of saccharides. In a similar vein, adding molybdenum led to an increase in potassium content in the plant because molybdenum increased potassium absorption from soil to roots. This was due to higher accumulation of protein and the increase in absorption zone as a result of improvements in roots branches and hairs (Abd El- Samad et al., 2005). The increase in plant dry matter and other plant parameters as a result of co-inoculation and molybdenum was accompanied by a decrease in oil content under these treatments and an increase under un-inoculated treatments. This seemed to be caused by an increase in protein content as a result of co-inoculation. It could also result from a decrease in protein under un-inoculated treatments, in which it did not receive enough nitrogen supply as a result of un-inoculation (Wilcox and Guodong, 1997). The increase in molybdenum content was accompanied with an increase in phosphorus content in the plants, especially the co-inoculated plants. This could be due to synergism between the nutrients, where phosphate (PO_4^{-3}) was released by molybdate (MoO_4^{-2}) from the soil complex. Accordingly, phosphorus availability increased to be absorbed by the roots (Basak et al., 1982).

CONCLUSION

The positive effect of *Azospirillum* co-inoculation with *Rhizobium* was very obvious in increasing growth, yield and N concentration in soybean plants. Therefore, combined inoculation could substantially improve symbiotic effectiveness. Molybdenum was very important in increasing all plant parameters, and it was observed that 1kg of Mo /ha interacted with combined inoculation and gave the best growth and yield, compared with other treatments.

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