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Nodulation, Symbiotic Growth and Yield of Vegetable Soybean Inoculated with *Photorhizobium* and *Bradyrhizobium*

Zulkifli H. Shamsuddin and S. B. Ang
Department of Land Management Faculty of Agriculture University Putra Malaysia

Abstract

A pot experiment with vegetable soybean (*Glycine max* L.) AGS190 inoculated with *Photorhizobium* was conducted with the following five treatments: three inoculation treatments with *Photorhizobium* (-N + Br) and a combination of both inocula (-N + Pr + Br), and two uninoculated nitrogen treatments, with and without inorganic nitrogen (+N-I, -N-I). The plants were harvested for symbiotic growth and yield after 42 and 65 days, respectively. The results showed that *Photorhizobium* was able to nodulate vegetable soybean; the nodules formed were larger (74mg/nodule) than the other two inoculation treatments involving *Bradyrhizobium* (32 and 35mg/nodule). The analysis N-solutes in xylem exudates indicated that nodules formed by *Photorhizobium* have high N_2 fixing activity (98.3 %). However, a low concentration (0.19 %) of reducing sugar in the nodule tissues compared to the *Bradyrhizobium* and combined inoculum treatments (1.72 and 1.92 % respectively) lowered the total amount of N_2 fixed; consequently, the relatively lower leaf N concentration (2.73 %) compared to control treatment with inorganic nitrogen (+N-I) (3.12%). A mixed inoculum of *Photorhizobium* and *Bradyrhizobium* showed a synergistic effect on the leaf N concentration (3.64 %) and pod yield (4.21 g/plant) compared to a single inoculation with *Bradyrhizobium* (3.31 %, 4.05 g/plant). *Photorhizobium* inoculation alone produced a significant reduction in pod yield (3.42g/plant) which corresponded directly to the lower leaf N concentration (2.73 %). This stud showed that *Photorhizobium* can nodulate vegetable soybean but have no positive effect in supplying photosynthate to the nodules and produced lower leaf N concentration and pod yield compared to *Bradyrhizobium* inoculation. co-inoculation with *Photorhizobium* and *Bradyrhizobium* produced a synergistic effect on the leaf N concentration and pod yield.

Introduction

Legume root nodules depend on the supply of photosynthate as energy source and carbon skeleton for nodule growth and maintenance, bacteroid respiration, N_2 fixation, and N assimilation (Mahon, 1983; Minchin *et al.*, 1981). The interdependence of photosynthesis and N_2 fixation in nodulated legumes is clearly evident in plant growth, carbon assimilation, and N assimilation (Heichel, 1987; Heichel and Vance, 1983). A reduction in rates of photosynthesis would reduce nodule mass, N_2 fixation and N accumulation (Mahon, 1983; Minchin *et al.*, 1981).

Photosynthate is also important for the growth of rhizobia. The introduction of Photorhizboium, a bacterium claimed to fix N_2 symbiotically while being photosynthetic, could reduce the energy demand by the microsymbiont on the plant host (Eaglesham *et al.*, 1990).

In symbiotic N_2 fixation, legume root nodules are dependent on the supply of carbohydrates from the host plant Photosynthetic products manufactured by the plant supply energy required by the Rhizobium to reduce atmospheric nitrogen (Bergersen, 1982). In the operation of legume nodules the carbon cost is estimated to be about 12 g carbohydrate per g N_2 fixed (Rainbird *et al.*, 1984). The addition of NO_3 can cause a reduction in sugar and starch contents in nodules of peas (Nelson and Edie, 1988; Taylor *et al.*, 1988; Streeter, 1983) and field beans (Wasfi and Prioul, 1986; Streeter, 1983). A reduction in the rate of N_2 fixed was also observed indicating a possible direct role of carbohydrate in N_2 fixation of legumes. Pea nodules with lower carbohydrate reserves seemed to show greater inhibition in N_2 fixation, suggesting the significant role of photosynthate (Nelson and Edite, 1991). Plant photosynthates are also translocated to nodules to supply energy for N_2 fixation.

In symbiotic N_2 fixing systems, a blockage in the import of photosynthate to the nodules would drastically affect the rate of N_2 fixation (Luthra *et al.*, 1985) as measured by acetylene reduction assay (Hardy *et al.*, 1986). The N_2 fixing activity in soybean nodules decreased by 56 per cent in the first hour and 79 per cent in two hours after the transport of photosynthate is blocked (Sloger, 1985).

In soybean, the effect of 50 per cent shading at the end of flowering decreased the amount of N_2 fixed from 125 to 91 kg N/ha/season. The presence of supplemental light increased the rate of N_2 fixed to 165 kg N/ha/season. These observations showed a direct relationship between light intensity and rate of N_2 fixation and are interpreted as an expression of the amount of photosynthate. In another study, partial defoliation by removal of two leaflets from each soybean leaf after flowering reduced the N_2 fixed from 125 to 100 kg/N/ha (Ham *et al.*, 1976; Brun, 1972).

In a study to examine the diural carbon fixation, storage and export characteristics of white clover leaves, it was estimated that during the photoperiod, 60 per cent of carbon exported from the leaf was directed towards the nodulated root; 45 per cent to nodules and 15 per cent to roots (Gordon *et al.*, 1987).

Carbon dioxide assimilation is the major function to provide carbohydrate to bacteroid in ureide-exporting nodules such as soybean (Glycine max L.) and adzuki bean (Phaseolus angularis). It was demonstrated that about 70 to 87 per cent of the ^{14}C in xylem exudate following labelling of these nodules with $^{14}CO_2$ was in organic acids (Vance *et al.*, 1985). Photosynthate limitation in N_2 fixation can be overcome by CO_2 enrichment. In the study on effects of carbon dioxide enrichment on soybean plants, the nitrogen input is 80 per cent N_2 for carbon dioxide enriched plants compared with 25 per cent for control. The observation indicates the beneficial effects of photosynthate on N_2 fixation (Hardy and Havelka, 1976).

Photorhizboum *thompsonum* is the first known symbiotic bacterium that is both photosynthetic (Ladha and So, 1994; Fleischman *et al.*, 1991; Evans *et al.*, 1990) and N_2 fixing, even as free living cultures (Evans *et al.*, 1990). The new bacterium, *Photorhizobium* strain BTAil, was isolated from stem nodules of *Aeschynomene* in flooded condition (Eaglesham *et al.*, 1990). It grows aerobically on a malate-yeast extract medium in a dark-light cycle and contains bacteriochlorophyll a, an essential pigment for photosynthesis. *Photorhizobium* forms stem nodules on *Aeschynomene* (Eaglesham *et al.*, 1990) but has not been reported to nodulate roots of other legumes. This study was undertaken to a) observe the ability of *Photorhizobium* to nodulate roots of vegetable soybean, b) test the ability of Photorhizboum to produce photosynthate, increase the N_2 fixation rate, symbiotic growth and yield of vegetable soybean.

Materials and Methods

Treatments: A pot study was conducted using vegetable soybean AGS190 grown on 3kg Serdang sandy loam soil (Typic Paleudult) in undrained plastic pots with the following five inoculation treatments.

(I) -N + Pr	-inoculated with *Photorhizobium* (Pr) strain MKAa2
(ii) -N + Br	-inoculated with *Bradyrhizobium* (Br) strain UPMR48
(iii)-N + Pr + Br	-combined inoculation with Photorhizboum strain MKAa2 and *Bradyrhizobium* strain UPMR48
(iv)-N-I (Inoculum)	- no inorganic nitrogen was applied (Uninoculated Control)
(v) + N-I	-inorganic nitrogen was applied at a rate equivalent to 35 kg N/ha (+ N Control)

Photorhizobium MKAa2 was from Dr. Fleichman, Department of Biochemistry, Wright State University, Dayton, Ohio, U.S.A., and *Bradyrhizobium* UPMR48, a local soybean isolate obtained from soybean, were obtained from the culture collection at the senior author's Soil Microbiology Laboratory, Department of Soil Science, University Putra Malaysia.

The experiment was laid out in a completely randomised design with five replications and two harvests (D_{42}, D_{65}). A total of 50 undrained pots internally lined with plastic, each containing 3.0 kg soil pot^{-1}, were used.

Basal Nutrients: Basal nutrients (Shamsuddin, 1987) containing all the essential elements, except N, were added to all pots a week before planting using micropipettes. Nitrogen was added only to the +N treatment. The soil was allowed to dry for two days and the basal nutrients were then mixed thoroughly with the soil before planting.

Seed Inoculation: *Photorhizobium* spp. was grown on Ye Broth and incubated at 28°C under incandescent light (6 680 lux) o a 16h/8h light-dark cycle on a rotary shake 110 rpm rom 3-4 days to achieve an estimated popula of 5 x 10^9 cells mL^{-1} (O.D.$_{6000}$) before being used as inoculum. Vegetable soybean AGS190 seeds, visu selected to be approximately of the same size (1.7g seeds), were surface sterilized by immersing them in per cent ethanol for 1 minute and 15 per cent sodi hypochlorite for 5 minutes followed by seven washing w sterile distilled water.

In the inoculated treatment, the seeds were soaked in br culture with the respective photorhizobial and bradyrhizo strains for 1 hour. In the combines *Photorhizobium* *Bradyrhizobium* treatment, both strains were inocula simultaneously. Six seeds/pot were planted and ag inoculated with the respective strains at the rate of 1 per seed.

Maintenance: The plants were thinned to two plants/ one week after planting. The plants were watered daily field capacity (16.5 % moisture) and harvested after (D_{42}) 65 (D_{65}) days of growth.

Harvesting

First Harvest (D_{42}): The youngest expanded leaves (Y from both plants (2 plants/pot) were picked before stems were cut 1 cm from soil surface and Tygon tu were connected to the remaining root section. Xy exudate were collected in the tube and transferred to a mL micro-centrifuge tube using a Pasteur pipette. T micro-centrifuge tubes were placed inside an ice box du sampling to prevent denaturing of enzyme activity and k kept in a freezer at -20°C before being analysed for solutes.

Fresh weights of the YEL and plant tops were recorded the plant tissues were oven dried at 60°C for 72 h before determining their dry weights.

Root nodules were separated after washing the roots w tap water. The nodules were counted and weighted.

Second Harvest (D_{65}): The number of green pods per p were counted, weighed and oven dried at 60°C for days before recording their dry weights.

Plant Analysis: Concentration of reducing sugar in nod tissue were determined using the Somogyi-Nelson met (Somogyi, 1945). The nitrogen concentration in the Y were determined by a modified micro-Kjeldahl method u an autoanalyzer (Singleton and Stockinger, 1983; Lad *al.*, 1981; Woomer *et al.*, 1981). Analysis of N-solute xylem exudates were done using xylem-solute meth (Herridge, 1984; Yong and Conway, 1942).

Statistical Analysis: All data were subjected to analysi variance and means were compared by Least Signific Difference test.

Table1: Effect of N supply and inoculation with *Photorhizobium* and *Bradyrhizobium* on nodulation of vegetable soybean at D_{42}.

Treatment	Nodule Number Per plant	Nodule Weight (mg/plant)	Specific Nodule Weight (mg/nodule)
-N-I	3c	238b	78a
+N-I	3c	146b	57ab
-N+Pr	12b	858a	74a
-N+Br	31a	1032a	35bc
-N+Pr+Br	33a	1060a	32c

Means with the same letter in the same column are not significantly different at P<0.05 (LSD)

Table 2: Effect of N supply and inoculation with *Photorhizobium* and *Bradyrhizobium* on reducing sugar concentration in nodule, relative ureide-N in xylem exudates and N concentration in the youngst expanded leaves (YEL) of vegetable soybean at D_{42}.

Treatments	Reducing Sugar Conc. in Nodules (%)	Relative Ureide-N Xylem Exudates (%)	N Conc. in YEL (%)
-N-I	1.27bc	59.4b	1.70c
+N-I	0.57c	39.2b	3.12ab
-N+Pr	0.19b	98.3a	2.73b
-N+Br	1.72ab	96.6a	3.31ab
-N+Pr+Br	1.92a	97.0a	3.64a

Means with the same letter in the same column are not significantly different at P<0.05 (LSD)

Table 3: Effect of N supply and inoculation with *Photorhizobium* and *Bradyrhizobium* on top dry weight (D_{42}) and pod dry weight (D_{42}) of vegetable soybean

Treatments	Top Dry Weight g/(plant)	Pod Dry Weight g/(plant)
-N-I	4.45a	2.71c
+N-I	4.76a	3.67ab
-N+Pr	4.58a	3.42bc
-N+Br	4.07a	4.05b
-N+Pr+Br	4.18a	4.21a

Means with the same letter in the same column are not significantly different at P<0.05 (LSD)

Results and Discussion

Effect of *Photorhizobium* Inoculation on Nodulation: Results obtained showed that *Photorhizobium* can nodulate vegetable soybean (Table 1). *Photorhizobium* inoculation (-N + Pr) gave higher nodule numbers compared to uninoculated control (-N -I). *Bradyrhizobium* treatment (-N +Br) produced much higher nodule number than the *Photorhizobium* treatment. However, co-inoculation with *Photorhizobium* and *Bradyrhizobium* (-N + Pr + Br) did not produce any further increase in nodule numbers. The presence of nodules in the -N-I and +N-I treatments indicated the existence of indigenous *Bradyrhizobium* in the original soil, albeit at a relatively very low number.

All three inoculation treatments increased the nodule weights from about 0.9 to 1.1 g/plant (Table 1). Again, the co-inoculation treatment did not produce an increase in nodule weight compared to the *Bradyrhizobium* treatment. These results showed that *Photorhizobium* was able to nodulate vegetable soybean but did not contribute effectively when used as a mixed inoculum with *Bradyrhizobium*.

The specific nodule weights showed that inoculation with *Photorhizobium* produced larger nodules compared to the other inoculation treatments (Table 2). The larger nodules indicated that they are richer in carbohydrates, not necessarily reducing sugars, but not with the N_2-fixing bacteroides. Other studies have also shown that starchy tissues appeared in nodules which are less effective (Taylor *et al.*, 1988).

Effect of *Photorhizobium* Inoculation on Reducing Sugar Concentration in Nodules, Ureide -N Concentration in Xylem Exudates and N Concentration in YEL: Generally, nodules formed by *Photorhizobium* showed the ability to fix N_2 (Table II). This was clearly shown in the relative ureide-N concentration. The analysis of N-solutes in xylem exudates provides an index of N_2 fixation activity, rather than a quantitative estimate of N_2 fixed (Neves *et al.*, 1985).

The N_2 fixing activity in the *Photorhizobium* (-N +Pr) treatment was equally high as the *Bradyrhizobium* (-N +Br) and co-inoculated (-N+Pr+Br) treatments (Table II). However, N concentration in YEL of *Photorhizobium* treatment was significantly lower than the co-inoculated treatment. The results demonstrated that although the respective *Photorhizobium* and *Bradyrhizobium* treatments showed no significant difference in N concentration, a synergistic effect was observed when combined inoculation of *Photorhizobium* and *Bradyrhizobium* were used.

A lower N concentration in YEL in the *Photorhizobium* treatment can be related to the lower concentration of reducing sugar in the nodule tissue. In this treatment, the concentration reducing sugar was one-tenth that of the other inoculated treatments (Table 2). The results indicated, that the ability of *Photorhizobium* to fix N_2 is limited by the availability of reducing sugar in the nodules. A similar phenomenon has also been observed by Walsh *et al.* (1987).

Photorhizobium cells have two functions. As a rhizobial cell, Photorhizboum forms nodules and fixes N_2. Concomitantly, the photosynthetic activity of *Photorhizobium* can supply the fixed carbon required for N_2 fixation and nodule growth. In he experiment, the low concentration of reducing sugar in nodule clearly showed that the photosynthetic function did not operate in the root nodules. Presumably, *Photorhizobium* could not fix carbon in the dark since *Photorhizobium thompsonum* was originally isolated from stem nodules of *Aeschynomene*, whereby sunlight energy was available (Eaglesham *et al.*, 1990).

A lack of reducing sugar in the *Photorhizobium*-inoculated nodules was a major limiting factor to N_2 fixation in this treatment. This indicated that photosynthate supply by plant tops to the nodules was adequate for nodule growth as expressed in a higher specific nodule weight but inadequate for N_2 fixation.

Effect of *Photorhizobium* Inoculation on Top Dry Weight and Yield: There was no significant difference in top dry weight due to the treatments imposed (Table III). However, *Photorhizobium* inoculation produced a significant reduction in pod yield which corresponded directly to the lower N concentration in the YEL compared to the co-inoculation with *Photorhizobium* and *Bradyrhizobium*. The latter treatment seemed to produce a synergistic effect on pod dry weight when compared to the individual effect of *Photorhizobium* or *Bradyrhizobium*. The cause of this synergistic effect warrants further investigation.

Bradyrhizobium inoculation formed more nodules and produced higher leaf N concentration and consequently higher pod yield of vegetable soybean than *Photorhizobium* treatment. *Photorhizobium* inoculation formed relatively bigger nodules but have no effect in enhancing the supply of photosynthates to the nodules and produced no effect on leaf N concentration and pod yield.

A mixed inoculation of *Photorhizobium* and *Bradyrhizobium* did not produce any increase in nodulation. However, a synergistic effect was observed in the leaf N concentration and pod yield.

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