Symbiosis, 21 (1996) 41–48
Balaban, Philadelphia/Rehovot

Field Inoculation of Common Bean (*Phaseolus vulgaris* L.) and Chick Pea (*Cicer arietinum* L.) with *Azospirillum brasilense* Strain Cd

SAUL BURDMAN, SHLOMO SARIG, JAIME KIGEL and YAACOV OKON*
Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot 76100, Israel, Tel. +972-8-9481216, Fax. +972-8-466794

Received January 7, 1996; Accepted March 27, 1996

Abstract

Field inoculation with *Azospirillum brasilense* strain Cd increased nodule dry weight (90%), plant-growth parameters and seed yield (99%) of naturally nodulated *Cicer arietinum* L. (chick pea). In *Phaseolus vulgaris* L. (common bean), inoculation with *Rhizobium etli* TAL182 and *R. tropici* CIAT899 increased seed yield (13%), and combined inoculation with *Rhizobium* and *Azospirillum* resulted in a further increase (23%), while plants inoculated with *Azospirillum* alone did not differ in yield from uninoculated controls, despite a relative increase in shoot dry weight.

Keywords: *Phaseolus vulgaris* L., *Cicer arietinum* L., *Azospirillum*, *Rhizobium*, nodulation

1. Introduction

Data accumulated throughout the world over the past 20 years indicate that free-living rhizobacteria of the genus *Azospirillum* are capable of increasing the yield of important crops grown in various soils and climatic regions (Okon and Labandera-Gonzales, 1994). The plant growth-promoting effects of *Azospirillum* inoculation are attributed mainly to improved root development

*The author to whom correspondence should be sent.

0334-5114/96/$05.50

and to the subsequent increase in the rate of water and mineral uptake. There is some evidence that the excretion of phytohormones by the bacteria may be responsible for the observed positive effects on root morphology and activity (Fallik et al., 1994).

Combined inoculation of legumes with *Rhizobium* and *Azospirillum* has received increasing attention in recent years, and positive effects on nodulation and yield have been reported for several legumes (Iruthayathas et al., 1983; Sarig et al., 1986; Yahalom et al., 1988; Itzigsohn et al., 1993). The increase in dry-matter and nitrogen content of coinoculated plants may be attributed to early and increased nodulation, higher N_2-fixation rates and a general improvement of root development (Volpin and Kapulnik, 1994).

In the present study, attempts were made to evaluate the contribution of inoculation with *Azospirillum* on nodulation, growth and yield of field-grown common bean and chick pea.

2. Materials and Methods

Bacteria and inocula preparation

Azospirillum brasilense strain Cd was grown for 24 h on liquid malate minimal medium (Okon et al., 1977) at 30°C. Cells were harvested by centrifugation (1000 g, 10 min, twice) and resuspended in water for inoculation of common bean. Chick peas were inoculated with a peat carrier containing *Azospirillum* cells, as described previously (Sarig et al., 1986).

Rhizobium tropici CIAT 899 (UMR1899) and *Rhizobium etli* TAL182 (SEMIA4021) were used for the experiment with common bean. Cultures were grown for 72 h on solid yeast mannitol agar, pH 6.8 (Vincent, 1970), at 30°C in Roux flasks. Cells were extracted by rolling sterile glass beads on the agar and suspending them in water. *Rhizobium* inoculum was composed of a mixture of equal amounts of these bacteria.

Field experiments

Phaseolus vulgaris L. (common bean), cv Bulgarian (Gedera Seeds Co., Gedera, Israel), was grown in the experiment station of the Faculty of Agriculture, Rehovot, on a Haploxeralfs, Brown-Red Sandy Soil (3.8% clay, 0.0% silt, 96.2% sand), pH 6.9, with 0.4% organic matter. Seeds were sown in early May with 0.5 m spacing between rows and a final stand of 20 plants/m row. A randomized block design with four plots per treatment was used. Each plot consisted of four rows, 4 m in length. Treatments were applied 3 days after sowing and they included inoculation with *Rhizobium* at 5×10^5 cfu/ml, *Azospirillum* at 2×10^7 cfu/ml, coinoculation with both bacteria and uninoculated control. Inocula were applied at 1 l per m of row, with control plots receiving the same quantity of water without bacteria. Water supply was by drip irrigation, and no fertilizers were applied. Nodule number was determined 20 days after sowing by counting nodules of 16 randomly chosen plants per treatment. Dry weight accumulation in shoots and number of pods were recorded during growth by randomized harvesting of four 0.5 m samples (9–10 plants per sample) from each plot (16 samples per treatment). Yield components were determined at the end of the growth period, 3 months after sowing, using a similar sampling method.

Cicer arietinum L. (chick pea), cv Ayelet (Hazera Co., Haifa, Israel), was grown near Kibbutz Sdeh Yoav in the Northern Negev (Lachish area), on a Calcixerolls, Brown Steppe Soil (50% clay, 26% silt, 24% sand), pH 7.9, with 0.5% organic matter. Seeds were sown in early January with 0.5 m spacing between rows to give a final stand of 380,000 plants per ha. Field plots (16 m^2 each) were set out in a completely randomized block design with four replicates. Treatments were inoculation with *Azospirillum* and uninoculated controls. No *Rhizobium* inoculum was applied since a natural *Rhizobium* population is present in this soil. Inoculation was performed at time of planting using a peat carrier containing *Azospirillum* at 10^9 cfu/g peat, as described by Sarig et al. (1986). One g of *Azospirillum* inoculant (in 200 ml of water) was injected per 1 m of row, with control plots receiving the same quantity of bacteria-free medium. P_2O_5 was applied as superphosphate at 80 kg/ha. Irrigation was according to the standard practice for chick pea, and plant growth was also supported by 600 mm of winter rains (November–April). Plants were harvested 6 and 16 weeks after sowing by taking 10 soil cores (6 cm in diameter, 20 cm in deep), each containing the shoot and roots of one plant. Shoot, root and nodule dry weight were determined. Twenty two weeks after sowing, plants were harvested for shoot dry weight determination and measurement of yield components.

Dry weight was determined after drying the plant material at 70°C for 48 h, in an air-forced oven.

3. Results

Common bean

Low levels of nodulation observed in controls not inoculated with *Rhizobium* were probably due to the local native population of *Rhizobium*. *Azospirillum* increased nodulation, and a further increase was observed after coinoculation with *Rhizobium* (Table 1). Despite a trend of increased nodulation in the presence of *Azospirillum*, significant ($p=0.05$) differences were obtained only between the coinoculation treatment and uninoculated controls.

Shoot dry weight was determined 1 and 2 months after sowing. In the first harvest, the three inoculation treatments showed higher values of shoot biomass than uninoculated controls (by 18%, on average), but differences among inoculation treatments were not significant (results not shown). Two months after sowing, coinoculated plants showed a significantly ($p=0.05$) higher shoot dry weight (including pods) than uninoculated controls (Table 1). This increase was on the order of 31%, while separate inoculation with *Rhizobium* and *Azospirillum* increased shoot dry weight by 24 and 16%, respectively. These differences, however were not significant at $p=0.05$.

A final harvest was carried out at physiological maturity, 3 months after sowing, and yield components were determined. Combined inoculation with *Rhizobium* and *Azospirillum*, and inoculation with *Rhizobium* alone, increased seed yields by 23 and 13% respectively, compared to uninoculated controls (Table 1), but differences were significant ($p=0.05$) only for the coinoculation treatment. These higher yields were caused by enhanced pod formation; no differences in the average seed-weight or the average number of seeds per pod were observed among treatments (Table 1). Inoculation with *Azospirillum* alone did not lead to an increase in seed yield at the final harvest, despite the increase in shoot biomass accumulation observed 2 months after sowing.

Table 1. Effects of separate and combined inoculation with *Azospirillum* and *Rhizobium* on nodulation, shoot biomass accumulation and parameters of yield in field-grown common bean. Values indicate means of 16 plants for nodule number, or 16 random 0.5 m row samples for the other parameters. Different letters indicate significant differences at $p=0.05$.

Treatment	Nodules per plant[1]	Shoot dry weight (g)[2]	Pods per sample[3]	Seeds per pod[3]	Average weight of 1 seed (mg)[3]	Seed yield (g)[3]
Rhi + Azo	18.3 a	155 a	75 a	3.9 a	256 a	75 a
Rhizobium	14.6 ab	146 ab	70 ab	4.0 a	253 a	69 ab
Azospirillum	13.4 ab	137 ab	62 b	4.0 a	254 a	61 b
Control	5.5 b	118 b	61 b	4.0 a	250 a	61 b

[1] 20 days after sowing; [2] 2 months after sowing; including stems, leaves and pods; and [3] at maturity, 3 months after sowing.

Chick pea

Inoculation with *Azospirillum* significantly (p=0.05) increased shoot and nodule dry weight (by 44 and 90%, respectively) in comparison with the uninoculated control at the first harvest date (Table 2). Sixteen weeks after sowing, *Azospirillum* increased shoot dry weight (49%), dry weight of pods (44%) and number of pods per plant (56%) above controls.

The experiment was terminated 22 weeks after sowing, when plants reached maturity. Seed yield components are shown in Table 3. *Azospirillum* significantly (p=0.05) increased seed yield (99%), number of mature pods per plant (81%) and number of seeds per plant (95%), compared to the control.

Table 2. Effect of *Azospirillum* on nodulation, growth and pod production of field-grown chick pea plants, at two harvest dates. Values indicate means of 4 replicates, each consisting of 10 plants. Different letters indicate significant differences at p=0.05.

	Harvest 6 weeks after sowing			Harvest 16 weeks after sowing		
Treatment	Shoot dry weight (g/plant)[1]	Root dry weight (g/plant)	Nodule dry weight (mg/plant)	Shoot dry weight (g/plant)[2]	Pod dry weight (g/plant)	Pods per plant
Azospirillum	2.23 a	0.29 a	95 a	29.6 a	10.8 a	39 a
Control	1.55 b	0.24 a	50 b	19.8 b	7.5 b	25 b

[1] including stems and leaves; [2] including stems, leaves and pods.

Table 3. Effect of *Azospirillum* on number of pods and seeds per plant and on seed yield per plant of field-grown chick pea plants at physiological maturity, 22 weeks after sowing. Values indicate means of 4 replicates, each consisting of 10 plants. Different letters indicate significant differences at p=0.05.

Treatment	Pods per plant	Seeds per plant	Seed yield (g/plant)
Azospirillum	38 a	43 a	16.9 a
Control	21 b	22 b	8.5 b

4. Discussion

Inoculation with *Azospirillum* clearly increased the number of nodules and favoured growth and yield of field-grown chick peas. These findings are in accordance with previous results from field inoculation of chick pea with *Azospirillum* (Sarig et al., 1986). Under greenhouse conditions, *Azospirillum* also increased nodulation, N_2 fixation, and other plant parameters in chick peas inoculated with *Rhizobium* (Fabbri and Del Gallo, 1995). A trend towards increased nodulation was also observed following inoculation of common bean with *Azospirillum* in the field, although this effect was smaller and not significant. Plant growth of common bean was positively affected by *Azospirillum*, as shown by increased shoot biomass accumulation, however the positive effects on yield of common bean caused by the bacteria were not as pronounced as those observed in chick peas. It is important to note that the field experiment with common bean was conducted with an optimal water supply. It is conceivable that the positive effects of *Azospirillum* could be relatively greater under conditions of lower water availability that limit plant development, particularly in the case of common bean which is regarded as a crop very sensitive to drought conditions.

Yield enhancement in both chick pea and common bean was due to an increase in pod production. Inoculation treatments did not affect the number of seeds per pod, or the average weight of individual seeds in either crop. Since pod production is related to total biomass accumulation (plant size) it is possible that the beneficial effects on yield were due to a general improvement of plant growth and not to specific effects on seed development or seed size.

In experiments with potted common bean, we observed that coinoculation with *Rhizobium* and *Azospirillum* consistently increased nodulation and N_2 fixation in comparison to inoculation with *Rhizobium* alone. Moreover, the number of upper nodules (those formed earlier) was found to be greater following inoculation with *Azospirillum* (Burdman et al., 1996a). This suggests that *Azospirillum* may lead to earlier nodulation and/or to an increased susceptibility of roots to nodulation by *Rhizobium* (Volpin and Kapulnik, 1994). In laboratory experiments with common bean it was found that *Azospirillum* clearly promotes root hair formation and secretion of *nod*-gene inducing flavonoids by roots (Burdman et al., 1996b). These effects may, at least partially, explain the consistent enhancement of nodulation by *Azospirillum*. Promotion of root hair formation is a well demonstrated effect of *Azospirillum* on several cereals and grasses (Tien et al., 1979; Okon and Kapulnik, 1986). The mechanism by which *Azospirillum* affects flavonoid secretion is not clear. There is evidence that *Azospirillum* affects plants by altering the balance among growth-promoting substances. Flavonoid content of the roots is developmentally regulated (Tiller et al., 1994), but it is not known how changes in hormonal balance may regulate synthesis and secretion of these compounds.

Increased nodulation has been shown in other studies in which *Azospirillum* and *Rhizobium* inoculum combinations were used (Iruthayathas et al., 1983; Sarig et al., 1986; Yahalom et al., 1988; Itzigsohn et al, 1993). Moreover, greater nodulation was associated with yield increases in several legumes grown in the greenhouse and field. Some strains of *Pseudomonas putida* also increased number of nodules per plant in common bean, apparently by improving phosphorous uptake (Grimes and Mount, 1984; De Freitas et al., 1993). Inoculation of grasses with *Azospirillum* increases mineral and water uptake by the roots, and a greater accumulation of dry matter in plant parts can be observed (Okon and Kapulnik, 1986). Similar trends can be expected for *Azospirillum*-inoculated legumes, with the additional benefit of increased nodulation and enhanced N_2 fixation.

Results from this work suggest that inoculation with *Azospirillum* could potentially increase nodulation, N_2 fixation and yield of field-grown legumes. This is particularly important for common bean which is generally considered a poor nitrogen fixer requiring relatively large quantities of nitrogen fertilization to produce good yields (Schroder, 1992; Chaverra and Graham, 1992). Appropriate concentrations and timely application of *Azospirillum* and efficient strains of *Rhizobium* should increase bean yields under conditions of limited water and nitrogen, and may reduce the need for fertilization, lowering production costs and reducing negative effects on the environment.

Acknowledgements

This research was supported by the Wolfson Foundation for Scientific Research and by a grant from the Council for Vegetable Production and Marketing, Israel.

REFERENCES

Burdman, S., Kigel, J., and Okon, Y. 1996a. Effects of *Azospirillum brasilense* on nodulation and growth of common bean (*Phaseolus vulgaris* L.). *Soil Biology and Biochemistry*, in press.

Burdman, S., Volpin, H., Kigel, J., Kapulnik, Y., and Okon, Y. 1996b. Promotion of *nod*-gene inducers and nodulation in common bean (*Phaseolus vulgaris* L.) roots inoculated with *Azospirillum brasilense* Cd. *Applied and Environmental Microbiology*, in press.

Chaverra, M.H. and Graham, P.H. 1992. Cultivar variation in traits affecting early nodulation of common bean. *Crop Science* **32**: 1432–1436.

De Freitas, J.R., Gupta, V.V.S.R., and Germida, J.J. 1993. Influence of *Pseudomonas syringae* R25 and *Pseudomonas putida* R105 on the growth and nitrogen fixation (acetylene reduction activity) of pea (*Pisum sativum* L.) and field bean (*Phaseolus vulgaris* L.). *Biology and Fertility of Soils* **16**: 215–220.

Fabbri, P. and Del Gallo, M. 1995. Specific interaction between chick pea (*Cicer arietinum* L.) and three chick pea-*Rhizobium* strains inoculated singularly and in combination with *Azospirillum brasilense* Cd. In: *Azospirillum VI and Related Microorganisms*, I. Fendrik, M. Del Gallo, J. Vanderleyden, and M. De Zamaroczy, eds. Springer-Verlag, Berlin, Heidelberg, pp. 257–267.

Fallik, E., Sarig, S., and Okon, Y. 1994. Morphology and physiology of plant roots associated with *Azospirillum*. In: *Azospirillum/Plant Associations*, Y. Okon, ed. CRC Press, Boca Raton, FL, pp. 87–110.

Grimes, H.D. and Mount, M.S. 1984. Influence of *Pseudomonas putida* on nodulation of *Phaseolus vulgaris*. *Soil Biology and Biochemistry* **16**: 27–30.

Iruthayathas, E.E., Gunasekaran, S., and Vlassak, K. 1983. Effect of combined inoculation of *Azospirillum* and *Rhizobium* on nodulation and N_2 fixation of winged bean and soybean. *Scientia Horticulturae* **20**: 231–240.

Itzigsohn, R., Kapulnik, Y., Okon, Y., and Dovrat, A. 1993. Physiological and morphological aspects of interactions between *Rhizobium meliloti* and alfalfa (*Medicago sativa*) in association with *Azospirillum brasilense*. *Canadian Journal of Microbiology* **39**: 610–615.

Okon, Y., Albrecht, S.L., and Burris, R.H. 1977. Methods for growing *Spirillum lipoferum* and for counting it in pure culture and in association with plants. *Applied and Environmental Microbiology* **33**: 85–88.

Okon, Y. and Kapulnik, Y. 1986. Development and function of *Azospirillum*-inoculated roots. *Plant and Soil* **90**: 3–16.

Okon, Y. and Labandera-Gonzales, C.A. 1994. Agronomic applications of *Azospirillum*: an evaluation of 20 years worldwide field inoculation. *Soil Biology and Biochemistry* **26**: 1591–1601.

Sarig, S., Kapulnik, Y., and Okon, Y. 1986. Effect of *Azospirillum* inoculation on nitrogen fixation and growth of several winter legumes. *Plant and Soil* **90**: 335–342.

Schroder, E.C. 1992. Improvement of the *Phaseolus/Rhizobium* symbiosis, with particular reference to the Caribbean region. In: *Biological Nitrogen Fixation and Sustainability of Tropical Agriculture*, K. Mulongoy, M. Gueye and D.S.C. Spencer, eds. J. Wiley & Sons, Chichester, UK, pp. 79–95.

Vincent, J. 1970. A manual for the practical study of the root-nodule bacteria. I.B.P. Handbook No. 15. Blackwell, Oxford. 164 pp.

Tien, T.M., Gaskins, M.H., and Hubbell, D.H. 1979. Plant growth substances produced by *Azospirillum brasilense* and their effects on the growth of pearl millet (*Pennisetum americanum* L.). *Applied and Environmental Microbiology* **37**: 1016–1024.

Tiller, S.A., Parry, A.D., and Edwards, R. 1994. Changes in accumulation of flavonoid and isoflavonoid conjugates associated with plant age and nodulation in alfalfa (*Medicago sativa* L.). *Physiologia Plantarum* **91**: 27–36.

Volpin, H. and Kapulnik, Y. 1994. Interaction of *Azospirillum* with beneficial soil microorganisms. In: *Azospirillum/Plant Associations*, Y. Okon, ed. CRC Press, Boca Raton, FL, pp. 111–118.

Yahalom, E., Okon, Y., and Dovrat, A. 1988. Early nodulation in legumes inoculated with *Azospirillum* and *Rhizobium*. *Symbiosis* **6**: 69–80.