

Enhancement of Nodulation, N₂-Fixation and Growth of Faba Bean (*Vicia faba* L.) by Combined Inoculation with *Rhizobium leguminosarum* bv. *viceae* and *Azospirillum brasilense*

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Abstract

Azospirillum brasilense strains M1, O2 and Sp7 were assayed with *Rhizobium leguminosarum* bv. *viceae* strain Z25 for combined inoculation of faba bean (*Vicia faba*) under gnotobiotic conditions. All *A. brasilense* strains tested increased significantly the total N content of the coinoculated plants at the flowering stage, when compared with plants inoculated with strain Z25 alone. *A. brasilense* strain Sp7 (ATCC 29145) also promoted significant increase (over 30%) in shoot and root dry matter accumulation and acetylene-reduction activity of nodulated plants. Strain M1 did not enhance plant dry matter accumulation but induced an increment of N concentration (%) in shoots and roots. Magnitude and direction of plant responses are strongly related to the cell density of *Azospirillum* applied as inoculum.

Keywords: *Rhizobium*, *Azospirillum*, *Vicia faba*, legume inoculation

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1. Introduction

Many reports have described positive effects of the free-living, diazotrophic bacteria of the genus *Azospirillum* on the rhizobial-legume symbiosis. These effects are related to increases in the number of nodules per plant, dry matter accumulation in nodules, N₂-fixation, N content or yield of several forage and grain legumes, as a result of mixed inoculation with *Rhizobium* and *Azospirillum* (Sarig et al., 1986; Yahalom et al., 1987; del Gallo and Fabbri, 1991; Hassouna et al., 1994). Nevertheless, no response or even negative effects have also been reported (Iruthayathas et al., 1983; Plazinsky and Rolfe, 1985). In addition, the legume response to mixed inoculation may be affected by some external factors such as the presence of certain levels of combined N (del Gallo and Fabbri, 1991).

Faba bean (*Vicia faba* L.) is a grain legume widely cultivated in Mediterranean countries and is rated 6th on a world basis (Buttery et al., 1992). *V. faba* can nodulate profusely, showing little or no response to soil N (Sprent et al., 1993), and inoculation with appropriate strains succeeds even in areas where a native rhizobial population is well established (Buttery et al., 1992). The introduction of faba bean in soils of low fertility has been recently recommended by the European Union (Carroue, 1993); however, few attempts were made to improve inoculation programs for this crop (Knaak et al., 1992; Caba et al., 1993).

The main purpose of this work was to study the effects of combined inoculation of *Rhizobium leguminosarum* bv. *viciae* and *Azospirillum* on growth and symbiotic performance of faba bean under gnotobiotic conditions. The influence of different levels of cell concentration of *Azospirillum* strains applied as inocula was also evaluated.

2. Materials and Methods

Bacterial strains

Azospirillum brasilense Sp7 (ATCC 29145) is a reference strain originally isolated in Brazil (Krieg^o and Döbereiner, 1984). *Rhizobium leguminosarum* bv. *viciae* strain Z25 (Spanish Type Culture Collection, CECT 4585) is a Nod⁺ Fix⁺ wild type isolate from nodules of faba bean, obtained from a clay agricultural soil (Calcixerollic Xerochrepts) collected in La Zubia (Granada, Spain) (Rodelas et al., 1994).

Soil sampling, isolation and characterization of native Azospirillum strains

Soil samples were collected from the top 15 cm of fields periodically cropped with faba beans, in two agricultural locations (Monachil and Otura) near Granada (Spain). The soil collected in Monachil was a Fluventic Xerochrepts with silty clay texture, pH(H₂O) 7.98 and 0.15% total N. The soil collected in Otura was a Calcixerollic Xerochrepts with silty loam texture, pH(H₂O) 7.73 and 0.12% total N. *Azospirillum brasilense* strains were enriched and isolated from soil samples according to previously described methods (Döbereiner and Day, 1976; Rodríguez-Cáceres, 1982). Identification and biochemical characterization of isolates were achieved according to Krieg and Döbereiner (1984), Bergey's Manual of Determinative Bacteriology (1994) and Penot et al. (1992).

Tests for in vitro antagonism

In vitro antagonism between *Rhizobium* and *Azospirillum* strains used in the study for combined inoculation of faba bean plants was tested on solid media. Assays were carried out on tryptone yeast-extract agar plates (Beringer, 1974) following a slight modification of the deferred antagonism procedure previously described by Gross and Vidaver (1978).

Plant assays

Test plants (*Vicia faba* cv. Alborea) were purchased from Semillas Pacífico, S.A., Sevilla, Spain. This commercial cultivar was chosen because it is commonly used in Mediterranean agricultural areas of Spain due to its good adaptation and high yielding. The seeds were surface disinfected in 90° ethanol for 2 min, rinsed in six changes of sterile distilled water and then left to imbibe in the dark at 25°C for 5 h. Swollen seeds were pregerminated aseptically on sterile moist vermiculite in an incubator at 25°C for 48 h. Selected seedlings (2 cm radicle) were planted in modified Leonard jars filled with sterile vermiculite and amended with the N-free nutrient solution of Rigaud and Puppo (1975), according to Caba et al. (1993).

Culture conditions and inocula preparation

Each *Azospirillum* strain was grown for 24 h in malate liquid medium amended with 0.2% NH₄Cl (Döbereiner and Day, 1976) at 28°C on a rotatory shaker (110 rpm). Late log-phase cells were harvested by centrifugation (10,000 g) and washed twice with 0.9% sterile saline. *Rhizobium*

leguminosarum bv. *viceae* strain Z25 was cultured for 24 h in yeast-extract mannitol broth (Jordan, 1984) at 28°C with gentle agitation (110 rpm). Cell growth of all strains was monitored by spectrophotometer measurements of culture samples at 550 nm (A_{550}), and cell densities were related to viable cell numbers, measured as colony forming units per ml (CFU ml⁻¹) by standard plate counts. *Azospirillum* numbers were adjusted to 10⁸ CFU ml⁻¹ and ten-fold diluted to obtain suspensions with 10⁷ and 10⁶ CFU ml⁻¹. Cell numbers of log-phase cultures of *Rhizobium* were adjusted to 5 × 10⁶ CFU ml⁻¹. Plate counts were also carried out in every experiment as control.

Combined inoculation

Seedlings in Leonard jars were inoculated with 1 ml of *Azospirillum* cell suspension, covered with sterile aluminium foil and placed in a plant culture chamber. After 24 h, 1 ml of *R. leguminosarum* bv. *viceae* strain Z25 cell suspension was pipetted directly to each seedling. The controls received only a single inoculation with strain Z25. After inoculation, the jars were covered with a 2 cm-layer of sterile perlite before being placed again in the culture chamber. Plants were raised in a controlled environment under a combination of fluorescent and incandescent light with an intensity of 16,000 lux, a 16-8 h light-dark cycle, 23-17°C day-night temperature and 55-75% relative humidity. After 39 days, plants were harvested at the beginning of flowering stage.

Determination of nodulation, N₂-fixation and plant growth parameters

At the time of plant harvest, the following parameters were determined: specific ARA of nodule tissue ($\mu\text{mol C}_2\text{H}_4 \times \text{g nodule dry weight}^{-1} \times \text{h}^{-1}$); total ARA of nodulated roots ($\mu\text{mol C}_2\text{H}_4 \times \text{plant}^{-1} \times \text{h}^{-1}$); nodule dry matter accumulation ($\text{mg} \times \text{plant}^{-1}$); number of nodules per plant and dry matter accumulation in roots and shoots (g). ARA was estimated in nodulated root segments of the plants by gas chromatography (Caba et al., 1993). Plant parts were dried at 70°C in an oven for 48 h, weighed and ground. Total N in ground samples of shoots and roots was determined by Kjeldahl analysis.

Statistical analysis

The results of plant assays were analyzed by computer-assisted two-factor ANOVA with interaction, using the software package STATGRAPHICS version 5.0 (STSC Inc., Rockville, MD, USA). Least significant differences

(LSD) were calculated at 95% confidence intervals. Pearson's lineal correlation coefficients among studied variables were also computed.

3. Results

Local *A. brasilense* isolates M1 and O2, along with the reference strain Sp7, were selected for combined inoculation of faba bean. No inhibition zones were observed in the tests carried out to detect *in vitro* antagonism on solid media between these three strains and *R. leguminosarum* bv. *viceae* strain Z25.

Effects on nodulation, N₂-fixation and growth parameters

Nodulation parameters (accumulation of dry matter in nodules and nodule number) of faba bean plants remained unaffected by combined inoculation (Fig. 1). The highest values in nodule dry matter accumulation were obtained for mixed inoculations with strain Sp7 (at the three cell densities applied), increasing nodule dry weight up to 18% over control, and inoculation with strain M1 at 10^8 CFU ml⁻¹ decreased nodule dry weight up to 27%; however,

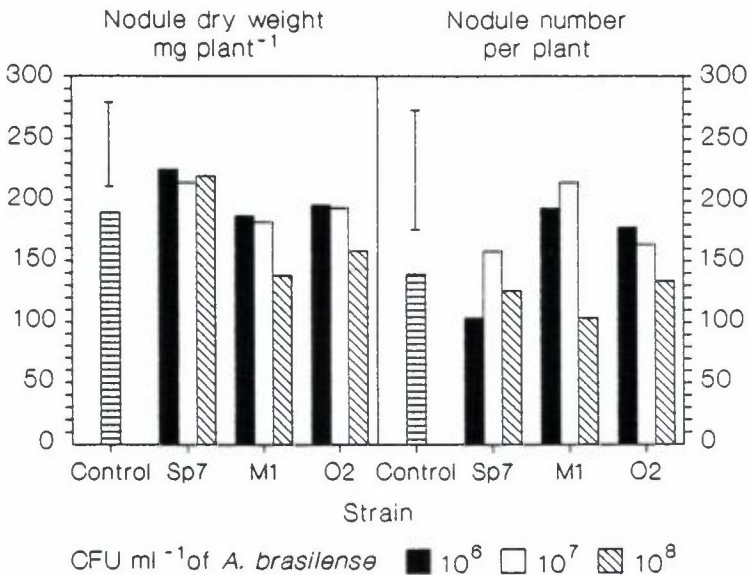


Figure 1. Effect of combined inoculation on nodule dry weight (g plant⁻¹) and nodule number per plant. Values are means of seven plants per treatment. I-bars: LSD ($P < 0.05$). Bars marked with * are significant versus control.

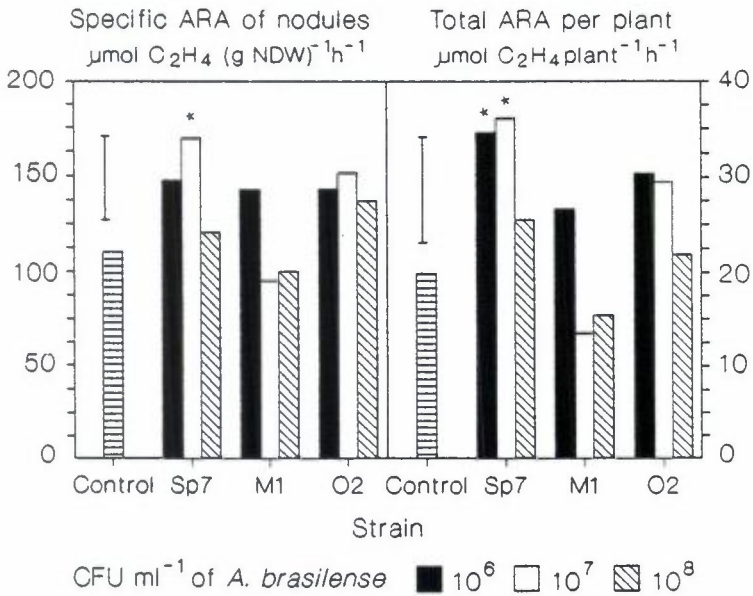


Figure 2. Effect of combined inoculation on specific ARA of nodules ($\mu\text{mol C}_2\text{H}_4 \times \text{g nodule dry weight}^{-1} \times \text{h}^{-1}$), and total ARA per plant ($\mu\text{mol C}_2\text{H}_4 \times \text{plant}^{-1} \times \text{h}^{-1}$). Values are means of seven plants per treatment. I-bars: LSD ($P < 0.05$). Bars marked with * are significant versus control.

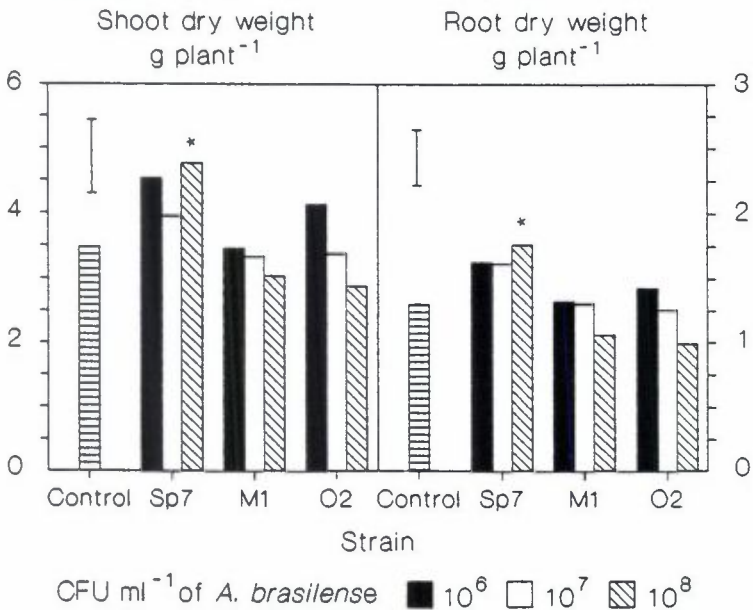


Figure 3. Effect of combined inoculation on dry matter accumulation in shoots and roots ($\text{g} \times \text{plant}^{-1}$). Values are means of seven plants per treatment. I-bars: LSD ($P < 0.05$). Bars marked with * are significant versus control.

these differences were not statistically significant at the 5% level. There was a great variation in nodule number due to the different inoculation treatments, but again there were no significant differences versus plants that received a single inoculation with *Rhizobium*.

Specific ARA of nodule tissue and total ARA per plant for all the combinations examined are shown in Fig. 2. Both traits were enhanced by mixed inoculation with *A. brasilense* strains Sp7 and O2, and the differences were significant for strain Sp7 applied at a cell density of 10^7 CFU ml⁻¹. We also observed differences between *A. brasilense* strains in their ability to exert positive effects on growth parameters of inoculated plants (Fig. 3). *Vicia faba* showed similar accumulation of dry matter in roots of plants coinoculated with strains M1 and O2 or control plants, whereas strain Sp7 exhibited growth-promoting activity. Combined treatment with strain Sp7 applied at 10^8 CFU ml⁻¹ significantly increased dry weight of aerial parts (37%) and roots (35%) of plants. Accumulation of dry matter in nodules and whole plants were positively and significantly correlated ($r^2 = 0.90$). Root/shoot dry weight ratios remained unaffected for all treatments including controls.

Effects on N composition of plants

The effect of combined inoculation on N concentration (%) and content (mg N) of faba bean plants is shown in Table 1. Total N content per plant was enhanced by mixed inoculation with all *A. brasilense* strains and there was a significant interaction with the cell density applied, especially for strain O2. Total N content was positively and significantly correlated to dry matter accumulation in nodules and whole plants ($r^2 = 0.86$ and 0.96 , respectively).

Plants treated with strain Sp7 showed a significantly lower N concentration in shoots compared to control, especially when applied at 10^8 CFU ml⁻¹ (9% decrease). However, these plants showed the highest values of total N per plant as a consequence of their larger accumulation of dry matter. Roots generally exhibited increase in N concentration due to combined inoculation, with the only exception of strain O2 at 10^6 CFU ml⁻¹. Strain M1 was the only one that significantly improved N concentration in aerial parts of plants up to 14% over control when applied at 10^7 or 10^8 CFU ml⁻¹. Treatment with this strain at 10^7 CFU ml⁻¹ improved total N content of whole plants about 10%, a portion of N which is not attributable to the increase on plant dry matter accumulation (Fig. 2).

Table 1. Effect on combined inoculation on N concentration (% N) and total N content (mg N) of shoots, roots and whole plants of *Vicia faba* cv. Alborea

Strain	CFU ml ⁻¹	Shoot		Root		Whole plant	
		% N	mgN	% N	mgN	% N	mgN
Control		3.60	125.16	2.72	35.40	3.36	160.57
Sp7	10 ⁶	3.32*	152.36*	2.87	46.84*	3.23	198.76*
	10 ⁷	3.59	147.08*	2.85	46.12*	3.36	193.46*
	10 ⁸	3.26*	155.17*	2.88	50.75*	3.16*	205.92*
M1	10 ⁶	3.61	125.27	2.93*	38.97	3.42	164.24
	10 ⁷	4.10*	136.83*	3.04*	39.78*	3.80*	176.61*
	10 ⁸	3.87*	117.65	3.04*	32.19	3.66*	149.84
O2	10 ⁶	3.72	153.87*	2.44*	34.89	3.39	188.76*
	10 ⁷	3.67	124.21	2.85	35.91	3.45	160.22
	10 ⁸	3.61	103.87*	2.99*	29.57*	3.46	133.44*
LSD	(P<0.05)	0.23	9.78	0.20	3.89	0.19	10.88

Values are means of three determinations representatives of seven plants per treatment. Means marked with * are significant versus control (P<0.05).

4. Discussion

Azospirillum inoculation can play an important role on *Rhizobium-Vicia faba* symbiosis, which involves effects on N₂-fixation, dry matter accumulation and N composition of plants. The results of this work agree with earlier ones (Iruthayathas et al., 1983; Plazinsky and Rolfe, 1985; Yahalom et al., 1987) indicating that the effects observed with a mixed inoculation of the legumes with *Rhizobium* and *Azospirillum* spp. are mainly dependent upon the chosen strains of these organisms, as well as the existence of a clear relationship between plant responses and cell numbers of azospirilla applied as inocula. Similar conclusions are also reached in many studies concerning the inoculation of non-leguminous plants with *Azospirillum* spp. (Okon and Labandera-González, 1994). The lack of beneficial effects reported for some legumes may be determined by the inappropriate selection of compatible bacterial

combinations and optimal cell ratios for each strain. Careful determination and control of these two variables appear essential for the success of mixed inoculation of faba bean under field conditions, despite of the possible involvement of other factors affecting the symbiosis. The involvement of antibiosis in antagonism between strains used for combined inoculation may also be checked, since production of bacteriocin-like inhibitory substances (BLIS) is common between rhizobia and azospirilla (Gross and Vidaver, 1978; Bashan and Levanony, 1990). Although we previously reported BLIS production by strain Z25 (Rodelas et al., 1994), we verified the lack of *in vitro* antagonism between the strains used through this study prior to combined inoculation assays.

Strain Sp7, applied at 10^8 CFU ml⁻¹, affects the distribution of N between plant organs, lowering the percent of N in shoots and enhancing it in roots. Similar responses regarding to N distribution have been reported for various maize cultivars inoculated with strain Sp7 (Jain and Patriquin, 1984) and also for digitgrass and sorghum inoculated with other growth-promoting *Azospirillum* strains (Schank et al., 1981; Pacovsky et al., 1985). Inoculation with strain Sp7 at 10^8 CFU ml⁻¹ clearly elicited a faster growth rate in faba bean plants which resulted in a higher dry matter yielding, but concomitantly diluted N concentration in plant tops. In this sense, it is worth noting that dry matter accumulation in whole plants and nodules, as well as total ARA, are significantly and negatively correlated to the percent of N found in plant tissues ($r^2 = -0.73, -0.72$ and -0.71 , respectively). Specific or total ARA of faba bean plants were also significantly enhanced after inoculation with strain Sp7 applied at 10^6 or 10^7 CFU ml⁻¹, although the results of the experiments suggest that the benefits brought about by this strain are not only due to enhancement of N₂-fixation activity of nodules. Strain Sp7 is an effective producer of phytohormones and B-group vitamins (Bashan and Levanony, 1990; Rodelas et al., 1993) and the role of these biologically active substances is well established as the main mechanism of plant growth promotion by azospirilla. The effects of *Azospirillum* strains on *Rhizobium*-legume symbiosis can be mimicked treating plants with low concentrations of phytohormones, at similar levels as those naturally produced by these bacteria (Iruthayathas et al., 1983).

Strain M1 applied at 10^7 CFU ml⁻¹ did not enhance faba bean growth but induced a significant increment of the N concentration of plants, suggesting that the mechanisms of *Azospirillum* interaction with the same legume vary between strains. Stimulation of specific ARA of nodules cannot be explained as a result of hormonal effects and may also be involved in plant growth responses. The ability of certain strains of *Azospirillum* to colonize internally the nodules of several legumes is well known (Iruthayathas et al., 1983;

Plazinsky and Rolfe, 1985) and it cannot be discarded a direct involvement of N_2 -fixation by azospirilla in the enhancement of % N of legumes, as proposed by del Gallo and Fabbri (1990). Alternative explanations to these effects may require further research.

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