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ORIGINAL PAPER

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Increase in auxiliary photoprotective photosynthetic pigments in wheat seedlings induced by *Azospirillum brasilense*

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Abstract Inoculation of wheat seedlings with the plant growth-promoting bacterium *Azospirillum brasilense* Cd was immobilized in alginate microbeads and, without applying any stress, significantly increased the quantity of several photosynthetic pigments, such as chlorophyll *a*, chlorophyll *b*, and the auxiliary photoprotective pigments violaxanthin, zeaxanthin, antheroxanthin, lutein, neoxanthin, and β-carotene. This resulted in greener plants with no apparent visible stress. After monitoring the quantity of photosynthetic pigments for 4 weeks, we observed that inoculated plants had higher quantities of pigments in shoot and stem. The greatest difference in the quantity of all pigments between inoculated and noninoculated plants occurred in the first week of growth. Regardless of treatment, the quantity of pigments in stems was three to four times less than the quantity of these pigments in shoots. Application of *Azospirillum*, either as liquid inoculant or as alginate microbeads, did not alter the positive effect of the bacteria on pigment production or the positive response of the plants towards *A. brasilense* Cd inoculation.

Keywords Antheroxanthin · *Azospirillum* · Bacterial inoculants · β-carotene · Lutein · Neoxanthin · Plant growth-promoting bacteria · PGPR · Plant pigments · Photosynthesis · Violaxanthin · Xanthophyll · Zeaxanthin

Introduction

Inoculation of many plant species, either crop, forest, ornamental, or environmental (desert) vegetation, with the plant growth-promoting bacterium (PGPB) *Azospirillum* sp. frequently results in healthier and larger (Bashan et al. 2004) and sometimes greener (Swedrzynska and Sawicka 2000) plants, suggesting enhanced photosynthesis (Amir et al. 2001). This is usually attributed to the growth-promoting effects that this genus of bacteria exerts on plants (Bashan et al. 2004). No significant negative effect of *Azospirillum* inoculation on plant growth is known, and the bacterium is considered harmless to plants (Bashan 1998). Several mechanisms by which *Azospirillum* affects plant growth are proposed. Hypothetical mechanisms include hormonal influence, increased water and mineral uptake, changes in membrane function, or a combination of small mechanisms affecting the plant in concert (Bashan et al. 2004).

The effect of inoculation of *Azospirillum* on photosynthetic pigments in inoculated plants is solely recorded as enhancement of total chlorophyll content of the inoculated plants, whether in higher plants (Bambal et al. 1998; Omar et al. 2000; Panwar and Singh 2000; Singh and Panwar 1997) or single-cell plants (de-Bashan et al. 2002; Gonzalez and Bashan 2000). However, the effect of inoculation on the auxiliary photoprotective pigments in wheat was not studied.

Carotenoids act as light-harvesting molecules inside the cell, allowing the efficient utilization of the light spectrum (Porra et al. 1997). Besides this, carotenoids protect the pigment–protein complexes and the chloroplast against photooxidation (Demmig-Adams 1990). In wheat, the production of these auxiliary photoprotective pigments is mainly enhanced under stress conditions, such as brief water stress or longer drought (Choudhury et al. 1994; Loggini et al. 1999; Panwar 1992; Tambussi et al. 2002; Xu et al. 1999), cold (Lidon et al. 2001; Xu et al. 2000), leaf senescence (Lu et al. 2001); and aging of chloroplast (Choudhury and Choe 1996; Choudhury et al. 1996) as a possible compensation for reduction in the general photosynthetic activity of the plant (Falbel et al. 1994; Pfuldel and Bilder 1994).

This study assessed (1) the quantity and the kinetics of auxiliary photoprotective pigments and general photosynthetic pigments within the wheat plant incurred by inoculation with *Azospirillum brasilense* Cd, (2) whether

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unstressed wheat plants react to *Azospirillum* inoculation in the same manner as they react to stressors by increasing cellular quantities of the photoprotective pigments, and (3) whether the new microbead inoculant technology produces results similar to that of the common, experimentally tested direct liquid inoculation.

Materials and methods

Bacteria, bacterial growth conditions, and inoculant production

A. brasilense Cd (DSM 1843, Braunschweig, Germany) served as a model bacterium. Bacterial growth conditions (Bashan et al. 1993), liquid inoculum (Bashan 1986), and microbead alginate inoculant were prepared as described previously (Bashan et al. 2002).

Inoculation of wheat plants with alginate microbead or liquid inoculants containing *A. brasilense Cd*

We used local desert soil. This was done to replicate a near-natural arid environment to evaluate the role of *Azospirillum* inoculation on pigment production under realistic soil conditions under arid zone agriculture, and at the same time avoid masking the effect of *Azospirillum* sp. inoculation by using overly fertile desert soil, where PGPB are less effective (Carrillo-Gracia et al. 2000), and avoid any drought, salt, or strong light stresses (known to affect pigment production in wheat; see Introduction).

All pot experiments were conducted in a mixture (1:1, v/v) of sieved (500 mesh), poor desert soil obtained from barren areas where perennial plants usually do not grow and rich, sieved desert soil found under the canopy of old mesquite trees in the same area and supporting exuberant perennial growth (Carrillo-Garcia et al. 1999). The detailed soil analysis was previously published (Bashan et al. 2000; Carrillo-Gracia et al. 2000).

Wheat seeds (*Triticum aestivum* cv. Rayon), known to respond positively to *Azospirillum* inoculation (Bashan et al. 2002), were inoculated with bacteria as follows. The growth substrate was placed in round, opaque small pots (6 cm diameter, 100 ml of substrate). The substrate was saturated with 50% Hoagland's nutrient solution before sowing. Pots were immediately sown with ten microbead-inoculated seeds, as previously described (Bashan et al. 2002), and thinned to five seedlings after germination. Alternatively, plants were inoculated with liquid inoculant, employing the standard seed's vacuum procedure (Puente and Bashan 1993), but using 0.006 M phosphate buffer pH 7.0 instead of the commonly used 0.06 M phosphate saline buffer. This modification avoids adding NaCl to the soil, a factor that affects photoprotective pigment production in wheat (see Introduction). Distilled water was added only when needed to keep the pots at the water-holding capacity of the soil. At no point during the trials did the plants suffer from lack of water. To avoid salt stress, the conductivity of the soil was measured before and after the trials, an average of 158 and 143 μS cm^{-1}, respectively. Pots containing wheat seedlings were incubated in a growth chamber at 24±2°C, 14 h illumination at 200 μmol m^{-2} s^{-1} (Conviron TC 16, Controlled Environments, Winnipeg, Canada). Equal light intensity was assured by replacing all light sources in the growth chamber with new bulbs and fluorescent tubes and placing the plants at the same distance from the light source, and keeping all pots randomly distributed within the growth chamber. No variation in light intensity was detected in separate sections of the growth chamber. To reduce the level of possible microbial contamination from the air, pots were sown under a laminar flow hood and immediately covered with sterile aluminum foil. Three days after germination, when coleoptiles emerged, small holes were aseptically pierced in the foil to allow the leaves to grow under normal light condition, while restricting contaminants from reaching the soil surface.

No quantitative counts of *A. brasilense* Cd colonizing roots were performed; countless experiments with this plant–bacteria combination had always shown sufficient root colonization in controlled, short-term experiments (Bacilio et al. 2004). Qualitative evaluation for the presence of *A. brasilense* Cd was routinely done (Bashan et al. 2002).

Pigment extraction and determination from leaves

Pigments were extracted from leaves of microbead-inoculated and noninoculated plants at the end of the trials, which lasted 28 days. Additionally, plants growing from seeds inoculated with liquid inoculant by vacuum were sampled. Three plants were sampled for pigment analysis at weekly intervals, and the others were used for comparing fresh weight and dry weight ratios of plants in each pot. This was an essential technical procedure because pigments must be extracted from fresh tissue, yet fresh weight determination can vary unpredictably among the pots and measuring procedures (Bashan and de-Bashan 2005), and a dry weight must be calculated. Therefore, after calculation, results were finally expressed on a dry weight basis.

Pigments were extracted separately from the shoots and stems. This avoided dilution of pigments, since pigments are significantly lower in quantity in stems, compared to shoots, but stems have a relatively higher dry weight. As a further precaution to avoid known degradation of pigments during cold storage at 4°C, plant samples were taken and pigments were immediately extracted by HPLC-grade acetone overnight at −40°C, and immediately thereafter, analyzed without storage. Pigments were detected with the diode array absorbance signal at 440 nm. Identification was made by comparing retention time and spectral characteristics with commercial pigment standards supplied by DHI (International Agency for ^{14}C Determinations, Denmark, http://www.c14.dhi.dk/index.htm). For quantification of results, we used the pigment response factor (HPLC peak area/pigment mass), which was obtained from the

commercial pigment standards according to the method described by Mantoura and Repeta (1997). Results are expressed as μg pigment g^{-1} dw plant or ng mg^{-1} plant dw. Kinetics of pigment production over time was determined at 7-day intervals starting 7 days after sowing and terminating 28 days later. Dry weight of plants was measured after desiccation in a forced-draft oven at 75±2°C for 16 h, as previously described in detail (Bashan et al. 2002). Data on photoprotective pigments involved in the violaxanthin cycle (violaxanthin, antheroxanthin, and zeaxanthin or VAZ) were grouped, because epoxidation of zeaxanthin and deepoxidation of violaxanthin occur with half-times of about 1 and 10 min, respectively. These half-times were shorter than the processing time for extraction of these pigments from the samples.

Experimental design and statistical analysis

Two treatments (inoculated and noninoculated) were performed. In experiments measuring growth parameter response of the plants, heat-killed bacteria controls were used. The design of the experiments, in five replicates, was completely randomized. Each replicate contained 22 pots; three pots (5 seedlings pot^{-1}) were sacrificed for pigment analysis at each of the four sampling times and the remaining ten pots were used for dry weight determination (a total of 110 experimental units per treatment for 550 plants). Each experiment was repeated twice, and a total of four experiments were performed. Pigment analysis was done in triplicate runs of HPLC of independent samples from the experiment. For statistical analysis we used one-way ANOVA or Student's *t* test at $P \leq 0.05$, and results were analyzed with Statistica v. 6 software (2001) (StatSoft, Inc., Tulsa, OK, USA). All statistical data in graphs are accompanied by standard error bars.

Results

Change in pigments induced by inoculation with *A. brasilense* Cd

In general, following inoculation of seed with liquid *A. brasilense* Cd, there was a significant increase in all recorded pigments in the shoot over noninoculated plants (Fig. 1). The most notable effect appeared 1 week after germination (Fig. 1a–f). Differences in pigment quantities between inoculated and noninoculated plants declined after this time, but remained statistically significant, except for β-carotene and neoxanthin (Fig. 1d–e). The same trend, although with significantly lower pigment content, was recorded in stems (Fig. 1h–m). In contrast to the amounts present in the shoots, the quantity of β-carotene in inoculated plants was always higher than that in noninoculated plants (Fig. 1l).

The VAZ photoprotective pigments followed the same pattern as the other pigments, that is, higher concentration in leaves, but not in stems, of inoculated plants (Fig. 2f,m). However, the chlorophyll *a*/VAZ ratio was similar in both inoculated and noninoculated plants and in leaves and stems (Fig. 2g,n).

When plants were inoculated with alginate microbeads containing *A. brasilense* Cd, similar increases in content of all pigments were recorded 20 days after inoculation (Fig. 2a–d).

Effect of inoculation with microbead inoculants containing *A. brasilense Cd*

Alginate microbeads without *A. brasilense* Cd did not have any positive effect on plant growth compared to non-inoculated and heat-killed bacteria controls. Microbeads without *A. brasilense* Cd appeared to reduce root development somewhat. Inoculation with microbead with or without *A. brasilense* Cd or with live or heat-killed bacteria did not affect germination of wheat seeds (data not shown). However, inoculation of wheat seeds with *A. brasilense* Cd encapsulated in alginate microbeads significantly increased plant height (Fig. 2e), dry weight of shoots (Fig. 2f), and dry weight of roots (Fig. 2g).

Discussion

Increased chlorophyll content and, consequently, enhanced photosynthesis, is a known response of plant to inoculation with several PGPBs (Alam et al. 2001; Deka and Dileep 2002; Malek 1996; Sharma et al. 2003), including *Azospirillum* spp. (Tsimilli-Michael et al. 2000). It was assumed that increased production of photosynthates enhanced plant growth and yield (Alam et al. 2001; Panwar 1991). Additionally, many of the best-known effects of *Azospirillum* inoculation occurs when the plant grows under stress conditions, such as water shortage, salinity, and toxic substances applied in agriculture practices (for reviews, see Bashan and Holguin 1997; Bashan et al. 2004).

Our hypothesis was that inoculation with *Azospirillum*, under normal growth conditions, enhanced production of auxiliary photoprotective pigments that are advantageous for plant growth. To grow plants under optimal growth conditions and avoid any possible stress to the plant, usually yielding production of these photoprotective pigments unrelated to *Azospirillum* inoculation, special precautions were taken to avoid salt, water stress, and intense light that enhance auxiliary photosynthetic pigment production (listed in Introduction). These potential stressors were avoided.

Liquid and microbead inoculation similarly enhanced the production of all pigments, including chlorophyll *a* and *b*, known from other studies, six auxiliary photoprotective pigments as determined in this study, and growth parameters. Generally, enhancement of chlorophyll content is considered a parameter that coincides with enhancement in photosynthesis (Amir et al. 2001; Elanchezhian and Panwar 1997; Panwar 1992). However, in the absent

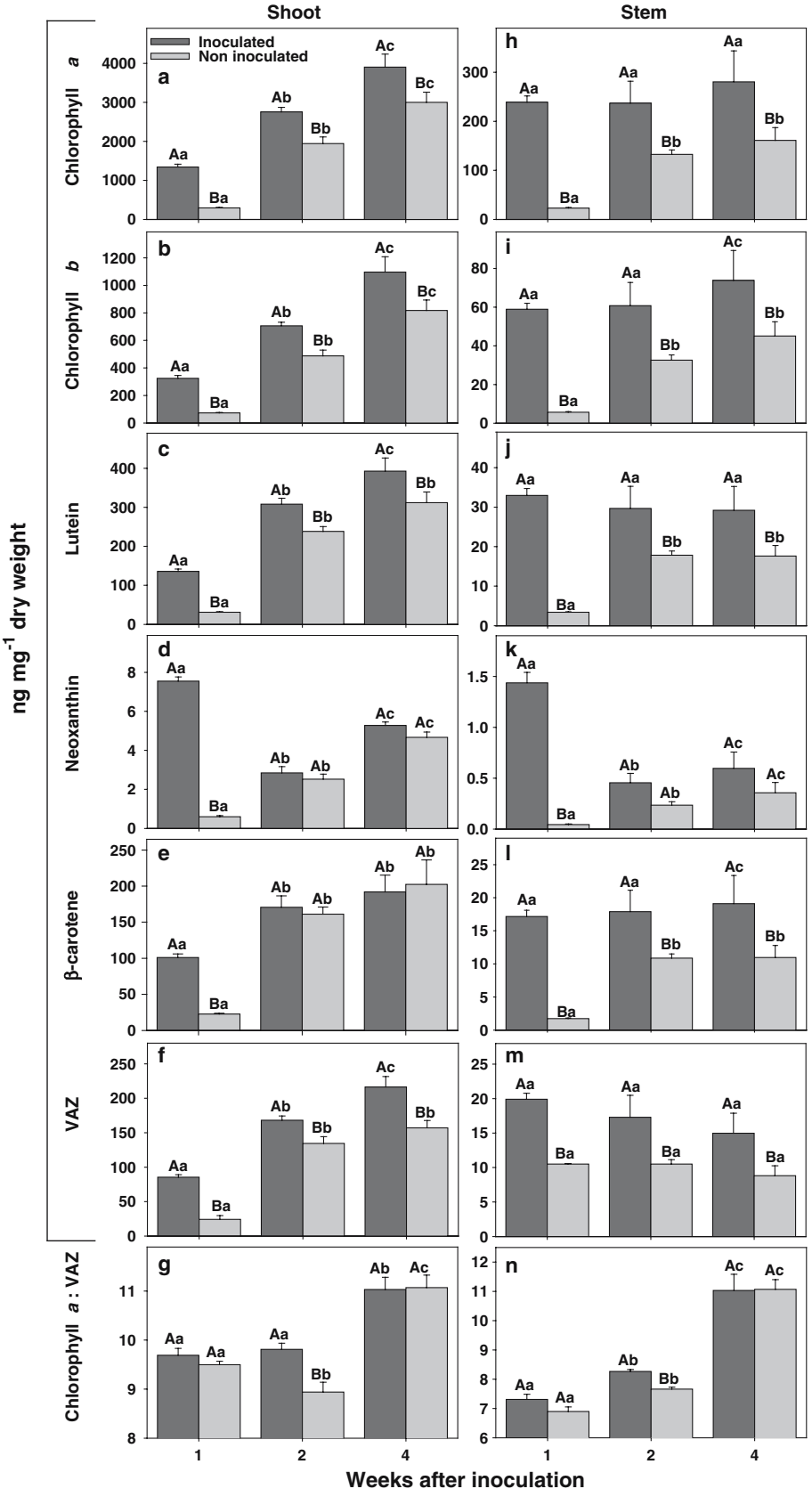

Fig. 1 Changes in the quantities of chlorophyll *a*, chlorophyll *b*, leutin, neoxanthin, β-carotene, and photoprotective pigments (VAZ) in the shoots (**a**–**f**) and stems (**h**–**m**) of wheat inoculated with a liquid suspension of *A. brasilense* Cd and chlorophyll *a*/VAZ ratio (expressed in mol) in shoots (**g**) and stems (**n**). (VAZ is the total content of violaxanthin, antheroxanthin, and zeaxanthin.) Values in each subfigure, for inoculated and noninoculated plants, denoted separately by a different lowercase letter differ significantly at $P \leq 0.05$ (one-way ANOVA). *Pairs of columns* denoted by a different capital letter differ significantly at $P \leq 0.05$ (Student's *t* test). *Bars* represent standard error (SE). Absence of a *bar* indicates negligible SE

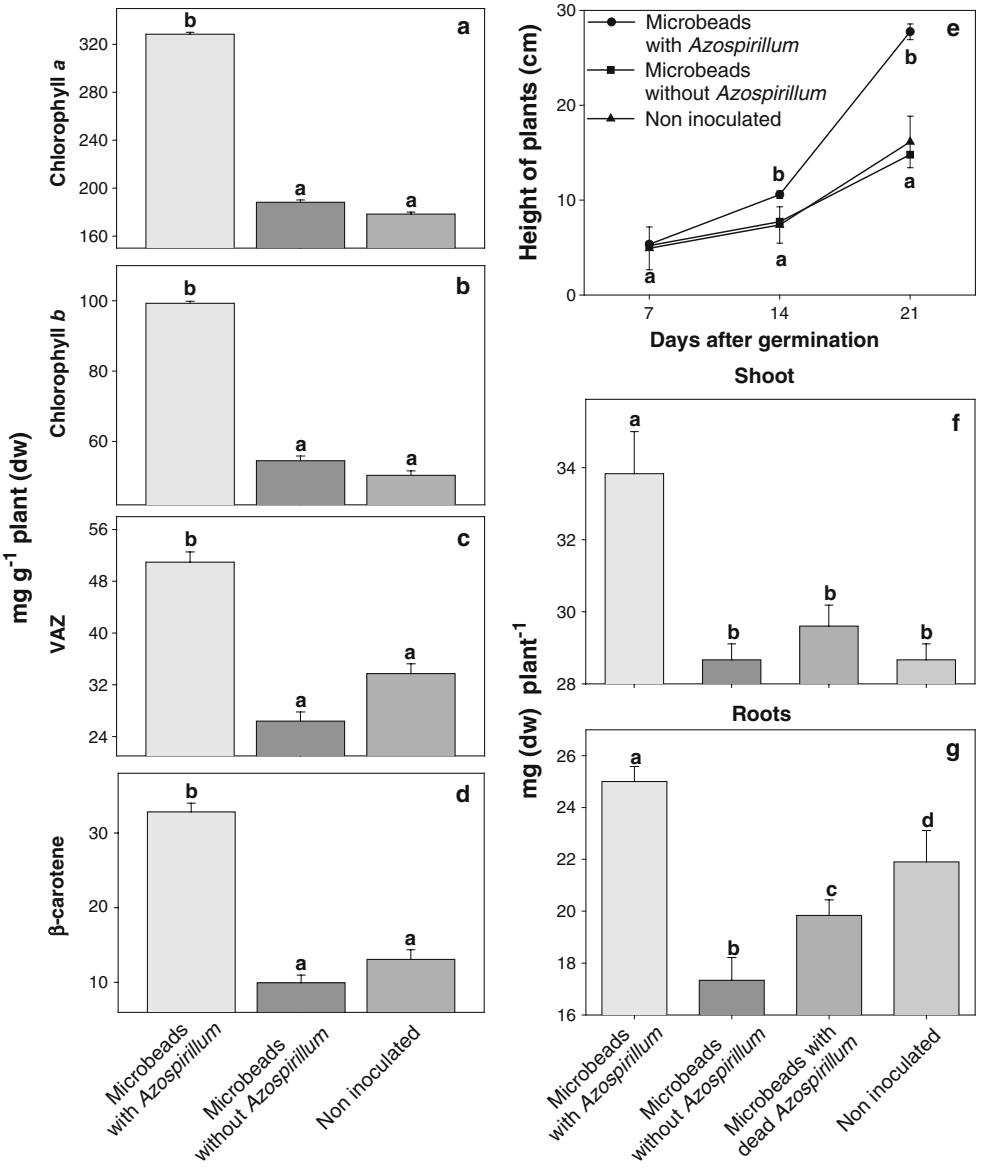

Fig. 2 Changes in pigment production of entire wheat seedling inoculated with *A. brasilense* Cd immobilized in alginate microbeads (**a**–**d**). (VAZ is the total content of the photoprotective pigments violaxanthin+antheroxanthin +zeaxanthin.) Development of wheat for 3 weeks after inoculation with *A. brasilense* Cd (**e**–**g**). *Points on curves* or *columns* in each subfigure, denoted by a different letter, differ significantly at $P \leq 0.05$ (one-way ANOVA). *Bar* represents standard error (SE)

of stress, enhanced production of auxiliary photoprotective pigments by inoculation with PGPB was not documented.

In wheat, under various stress conditions, there are more photoprotective pigments in relation to chlorophyll content (Loggini et al. 1999). The physiological mechanism helps maintain photosynthesis for growth. We did not observe any change of the chlorophyll *a*/VAZ ratio. This suggests that inoculation of *A. brasilense* Cd does not stress the plant, and also supports an earlier study indicating the harmless nature of *Azospirillum* to plants (Bashan 1998).

Studies of the mode of action of *Azospirillum* on plants commonly focus on a single mechanism, such as hormonal effect, N_2 fixation, proton extrusion, and mineral uptake (for review, see Bashan et al. 2004). While every proposed mechanism, by itself, has ample supporting experimental data, other proposals points to the "Additive hypothesis" proposal, which states that multiple mechanisms—rather than one mechanism—are involved in the association. These mechanisms may operate simultaneously or in succession, the contribution of one mechanism being less significant if evaluated separately. The sum of their activities, under appropriate environmental conditions, results in the observed changes in plant growth (Bashan and Levanony 1990). The evidence presented here (that root inoculation enhanced photosynthetic pigment production in shoots) lends support to the hypothesis that inoculation affects the whole plant, as suggested in a previous work (Bashan and Dubrovsky 1996), and not as a point or zone mechanism. Indirectly, in this study, the evidence for enhancement of pigment production suggests that the mode of action of *Azospirillum* sp. is probably composed of multiple mechanisms.

In summary, wheat plants, grown under optimal conditions with the inoculant *A. brasilense* Cd, enhanced the production of auxiliary photoprotective photosynthetic pigments that usually increase in wheat grown under stress conditions. At the same time, plant growth increased.

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