

## Root Colonization vs. Seedling Growth, in two *Azospirillum*-inoculated Wheat Species

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(Received 4 October 2006; accepted 11 June 2007)

Data evaluating the growth promoting effects of *Azospirillum* on wheat seedlings according to the inoculum level/root colonization effectiveness (number of bacterial cells), is scarce. Uniform 1-cm size, 72-h old wheat seedlings grown in the dark at 22 °C were inoculated with: i)  $10^3$ ,  $10^5$ ,  $10^7$  and  $10^8$  *A. brasilense* cells per *T. aestivum* cv. ProINTA Federal seedling; ii)  $10^2$ ,  $10^5$  and  $10^8$  *A. brasilense* cells per *T. durum* cv. Buck Topacio seedling; iii)  $10^6$  HKB cells per cultivar seedling; iv) phosphate buffer pH 6.8 (NI) as control seedlings for both cultivars. Afterwards, seedling growth proceeded in water in the dark at 22 °C for another 48 h. Alive or dead *Azospirillum* cells were suspended in phosphate buffer pH 6.8. Root and shoot growth were determined measuring the length and projected area of their digitalized images. When treated with inocula concentrations ranging from  $10^2$  to  $10^5$  cells per seedling, both *Triticum* species reached a maximum level of colonization harboring  $10^6$  to  $10^7$  cells per seedling. No differences could be detected between NI and HKB treated seedlings for both *Triticum* species. *Triticum aestivum* cv. ProINTA Federal seedlings reached the maximum growth promotion when roots were colonized with a number of bacterial cells ranging from  $5 \cdot 10^6$  to  $1.5 \cdot 10^8$  per seedling. *Triticum durum* cv. Buck Topacio seedlings showed maximum growth promotion when  $3.3 \cdot 10^7$  cells were present in their roots. Higher values of colonization showed no growth promoting effects with respect to the controls. It may be concluded that in these experimental conditions the optimum inoculum concentration is  $5 \cdot 10^5$  cells per seedling for both *T. aestivum* cv. ProINTA Federal and *T. durum* cv. Buck Topacio.

**Keywords:** *Azospirillum* colonization, wheat, seedlings growth, growth promotion

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## Introduction

*Azospirillum* spp. is a free-living plant-growth-promoting bacterium capable of affecting growth and yield of numerous plant species, many of them of agronomic and ecological significance (Bashan et al. 2004). In general, a successful colonization either of the rhizosphere, the surface and/or the interior of the root is determinant to enhance plant growth and crop yield in rhizobacteria-based biotechnologies (Arunakumari et al. 1992; Okon and Itzigsohn 1995). However, most of the works published in relation to plant growth promotion by *Azospirillum* only mention inoculum size and not the effective root colonization reached.

To survive on the root surface, the bacteria must have some permanent anchoring mechanism (Bashan 1993). The mechanism of azospirilla attachment to plant roots was determined to be a two-step process (Bashan et al. 2004). Heat killing of *Azospirillum* cells eliminates attachment, indicating that active bacterial metabolism is necessary in the process (de Troch and Vanderleyden 1996). However, even though most of the field trials used non inoculated plants as controls (Okon and Labandera-González 1994), the possibility that metabolites released by *Azospirillum* could be influencing wheat seedling growth beyond the initial attachment process, should not be discarded. In fact, inoculation methods that do not depend on the usual root protrusion-*Azospirillum* motility-bacterial attachment sequence were also successful in promoting growth in wheat and maize seedlings (Creus et al. 1997; Casanovas et al. 2002). Therefore, we were convinced that experiments concerning *Azospirillum*-wheat interaction studies should include HKB as controls (Creus et al. 1997).

*Triticum aestivum* and *T. durum* species are considered to be of utmost importance in Argentina. One of the most marked *Azospirillum*-inoculation effects on wheat seedlings grown at the lab in hydroponics is the development of a larger root system at an inoculation level of  $10^6$ – $10^7$  bacterial cells  $\text{ml}^{-1}$  (Dobbelaere et al. 1999). However, a significant decrease in the size of the elongation zone of the roots was observed at a higher inoculum level ( $10^8$  bacterial cells  $\text{ml}^{-1}$ ) (Dobbelaere et al. 1999). This fact shows that not only low but also high inoculum level could be inadequate to achieve growth promotion. On the other hand, previous work in our laboratory has shown different growth and physiological parameters in *T. aestivum* and *T. durum* species in response to the same amount of *A. brasilense* Sp245 cells detected into their roots (Creus et al. 1997).

According to the considerations mentioned above, our objectives in relation to wheat seedling inoculation with *Azospirillum* were: a) to compare inoculum concentration vs. effective root colonization; b) to detect possible growth differences between non-inoculated seedlings (NI) and those inoculated with heat-

killed bacteria (HKB); c) to evaluate growth promoting effects according to the level/effectiveness (number of bacterial cells) of root colonization.

### Materials and Methods

*Azospirillum brasilense* Sp245 and wheat seedlings of *T. aestivum* cv. ProINTA Federal and *T. durum* cv. Buck Topacio were used as models. *Azospirillum brasilense* Sp245 was cultured on agar media containing Congo Red and prepared for inoculation as previously described (Creus et al. 1997). The concentration of each cell suspension was calculated from Most Probable Number (MPN) determined in NFb media (Postgate 1969) and related to OD determined at 540 nm.

Wheat seeds were disinfected in 1% NaClO for 3 min, thoroughly rinsed in tap water and germinated on wet filter paper, in sealed trays stored in the dark and at  $22 \pm 1$  °C for 72 h. Uniform 1-cm coleoptile size seedlings were selected from each germinated pool and roots submerged in the inoculum for 3 h, as previously described (Creus et al. 1997). All *A. brasilense* suspensions were made in phosphate buffer (pH 6.8). Treatments were as follows: i)  $10^3$ ,  $10^5$ ,  $10^7$  and  $10^8$  bacterial cells seedling<sup>-1</sup> in *T. aestivum* cv. ProINTA Federal; ii)  $10^2$ ,  $10^5$  and  $10^8$  bacterial cells seedling<sup>-1</sup> in *T. durum* cv. Buck Topacio; iii)  $10^6$  autoclaved bacterial cells seedling<sup>-1</sup> (HKB controls) in both *Triticum* species; iv) phosphate buffer pH 6.8 (NI controls) in both *Triticum* species. After inoculation, seedlings were grown in water for another 48 h in the dark at 22 °C.

Seedlings were dissected in three main tissues: shoot, root and remaining seed. Roots were washed twice with distilled water and then submerged for 1 min in 0.5% bromothymol blue in 0.2 M NaOH. After eliminating the excess solution on an absorbent paper, shoot and root images were digitalized on a flat bed scanner. Both shoot and root length, and their respective projected areas were determined on the digitalized images by means of GSRoot 5.00 software (Guddanti and Chambers 1993). However, since root hairs are not detected by this software, root projected area corresponds only to main and lateral roots.

Bacterial colonization of roots was quantified through MPN determination (Postgate 1969).

The experiments were a factorial combination of inoculation levels in complete randomized blocks. Twenty seedlings were analyzed in each treatment. Experiments were repeated three times. The results were analysed through PROC GLM procedure using SAS statistical package (SAS Institute 2000). Significance levels ( $P < 0.05$ ) were determined through ANOVA and Duncan's multiple range tests.

## Results and Discussion

### *Inoculum concentration and effective plant root colonization*

Wheat growth promotion by *A. brasilense* Sp245 has been extensively studied in our laboratory (Alvarez et al. 1996; Creus et al. 1997; Creus et al. 1998; Creus et al. 2004). A significant plant growth enhancement was demonstrated in *T. aestivum* cvs. ProINTA Oasis and Buck Pucará. However, such enhancement did not occur in *T. durum* cv. Balcarceño INTA 48 hours after being colonized by  $10^6$ – $10^7$  bacterial cells seedling<sup>-1</sup> (Creus et al. 1997). These colonization levels were routinely achieved after exposing seedling roots to  $10^8$  bacterial cells seedling<sup>-1</sup> inoculum suspensions.

However, in the present work both *T. aestivum* cv. Pro INTA Federal and *T. durum* cv. Buck Topacio seedlings were colonized by ca.  $10^6$ – $10^7$  bacterial cells seedling<sup>-1</sup> 48 h after roots were inoculated with bacterial concentrations ranging from  $10^2$  to  $10^5$  cells seedling<sup>-1</sup> (Table 1). More concentrated inoculum led to colonization levels over  $10^8$  cells seedling<sup>-1</sup> (Table 1). Multiplication of endophytic bacteria in plants in vivo is difficult to demonstrate, although there is some evidence suggesting a compatible and dynamic association of the bacteria with the host (Hallmann et al. 1997). It has been shown that several endophytes approach and maintain certain plateau population densities, regardless of the inoculum concentration (Chen et al. 1995).

Table 1. Bacterial colonization (Most Probable Number of cells seedling<sup>-1</sup>) of *T. aestivum* cv. Pro INTA Federal, and *T. durum* cv. Buck Topacio roots inoculated with different *A. brasilense* Sp245 inocula concentrations and grown for 48 h in hydroponics in the dark, at 22 °C

Inoculum concentration ( <i>A. brasilense</i> Sp245 cells seedling <sup>-1</sup> ) <sup>a</sup>	Colonization	
	<i>T. aestivum</i> cv. Pro INTA Federal	<i>T. durum</i> cv. Buck Topacio
0 (Buffer control)	<100	<100
$5 \cdot 10^2$	ND	$3.3 \cdot 10^6 \pm 1.1 \cdot 10^6$
$5 \cdot 10^3$	$5.0 \cdot 10^6 \pm 1.1 \cdot 10^6$	ND
$5 \cdot 10^5$	$2.5 \cdot 10^7 \pm 1.2 \cdot 10^7$	$3.3 \cdot 10^7 \pm 2.1 \cdot 10^7$
$5 \cdot 10^7$	$1.5 \cdot 10^8 \pm 9.0 \cdot 10^7$	ND
$5 \cdot 10^8$	$2.5 \cdot 10^8 \pm 1.0 \cdot 10^8$	$3.3 \cdot 10^8 \pm 2.0 \cdot 10^8$

<sup>a</sup> Determined by OD at 540 nm. Results are the average of three replications.

ND: not determined

*Plant growth responses to root colonization level*

Most probable number of bacteria detected in the roots of both *Triticum* species treated with HKB or NI controls was less than 100 (Table 1). Moreover, no differences in growth parameters in seedlings of both *Triticum* species were found in either treatment (Fig. 1). These results would validate the use of either control in this type of experiments, and provide more evidence that metabolically active bacteria are needed to induce the well-known growth promotion effect (Dufrêne et al. 1996; Dobbelaere et al. 1999).

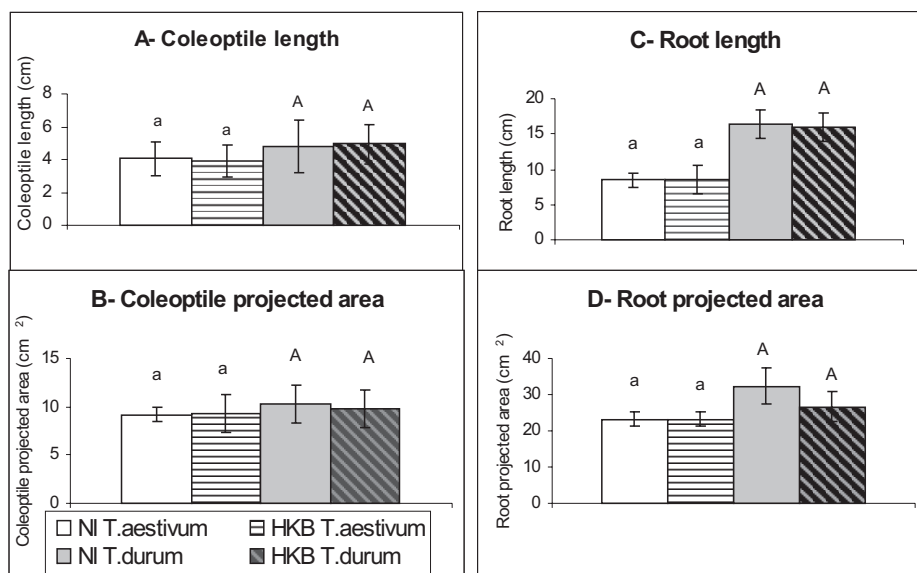


Figure 1. Coleoptile and root growth, in either non-inoculated (NI) wheat seedlings, or inoculated with  $10^6$  heat-killed *A. brasilense* Sp245 cells seedling<sup>-1</sup> (HKB). White and gray bars represent *T. aestivum* cv. Pro INTA Federal and *T. durum* cv. Buck Topacio, respectively. Striped bars correspond to wheat inoculated with  $10^6$  heat-killed bacteria. Lines on top of bars represent SD. Different letters on top of bars mean significant differences between treatments ( $P < 0.05$ ). Lowercase letters and capital letters correspond to ProINTA Federal and Buck Topacio, respectively

According to the length and projected areas of both coleoptile and roots, *T. aestivum* cv. ProINTA Federal seedlings reached a maximum growth when roots were colonized by a number of bacteria ranging from  $5 \cdot 10^6$  to  $1.5 \cdot 10^8$  cells seedling<sup>-1</sup> (Fig. 2). Root area has been described as the most reliable criterion to measure and evaluate *Azospirillum* effects on root growth (Jacoud et al. 1998). However, while no differences were observed both in coleoptile and root lengths or in root projected area within the  $2.5 \cdot 10^7$  and  $1.5$  to  $10^8$  bacterial cells seedling<sup>-1</sup>

colonization range, a progressively higher projected area of the coleoptile was evident (Fig. 2). An underlying principle is that successful colonization of plant roots must take place before subsequent effects of the bacteria on plant growth can occur (Arunakumari et al. 1992). No significant ( $P < 0.05$ ) increase with respect to the controls was detected in coleoptile and root projected areas when seedlings were colonized by  $2.5 \cdot 10^8$  bacterial cells seedling<sup>-1</sup>. Furthermore, a decrease in the length of both organs was also evident at this bacterial concentration, which could have reached a high, however non-optimal, colonization level (Fig. 2). Decreases in root length and density were observed upon inoculation of wheat seeds with high concentrations of *A. brasilense* Sp245 and Sp7 strains (Dobbelaere et al. 1999).

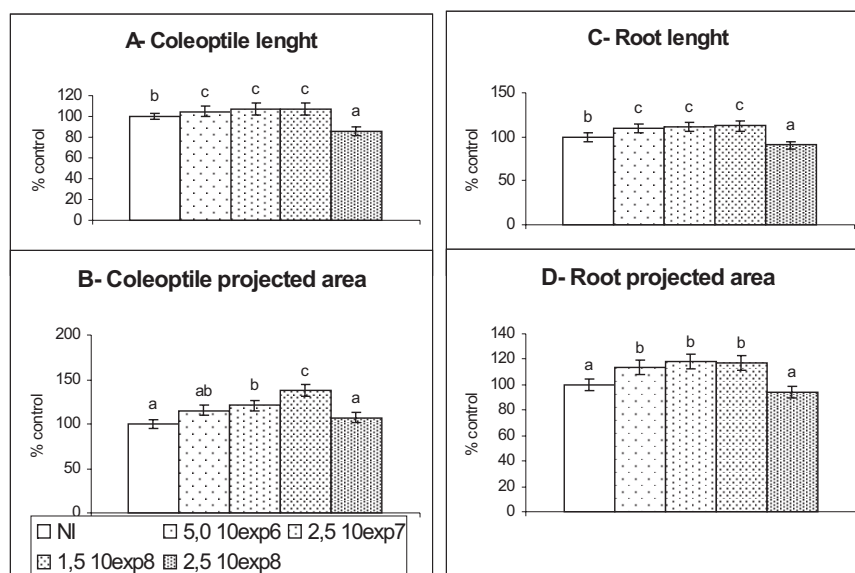


Figure 2. Coleoptile and root growth vs. colonization level, in *T. aestivum* cv. Pro INTA Federal seedlings inoculated with *A. brasilense* Sp245. Lines on top of bars represent SD. Different letters on top of bars mean significant differences among treatments ( $P < 0.05$ )

According to these results, an inoculum concentration in the range of  $5 \cdot 10^5$  to  $5 \cdot 10^7$  *A. brasilense* cells seedling<sup>-1</sup>g would be adequate to pursue the optimum  $5 \cdot 10^6$  to  $1.5 \cdot 10^8$  cells seedling<sup>-1</sup> colonization range in *T. aestivum* cv. ProINTA Federal seedlings (Table 1). In short, to diminish the risks of overloading the roots and losing the beneficial growth promoting effects exerted by *A. brasilense* Sp245 on *T. aestivum* cv. ProINTA Federal seedlings, it would be advisable to use an inoculum containing less than  $5 \cdot 10^7$  bacterial cells seedling<sup>-1</sup>g.

Both coleoptile length and projected area parameters were maximum in *T. durum* cv. Buck Topacio seedlings when roots were colonized by  $3.3 \cdot 10^7$  bacterial cells seedling<sup>-1</sup> (Fig. 3). Moreover, a progressively higher projected area of the coleoptile at both  $3.3 \cdot 10^6$  and  $3.3 \cdot 10^7$  bacterial cells seedling<sup>-1</sup> levels was evident (Fig. 3). On the other hand, while root length did not change at these colonization levels, its projected area significantly increased in the range  $3.3 \cdot 10^6$  to  $3.3 \cdot 10^7$  bacterial cells seedling<sup>-1</sup>. At  $3.3 \cdot 10^8$  bacterial cells seedling<sup>-1</sup> colonization level, length and projected area parameters remained unchanged in both plant tissues (Fig. 3). As in *T. aestivum* cv. ProINTA Federal, the results show the disadvantage of inoculating *T. durum* cv. Buck Topacio seedlings with  $5 \cdot 10^8$  *Azospirillum* cells seedling<sup>-1</sup>, if growth promotion is required.

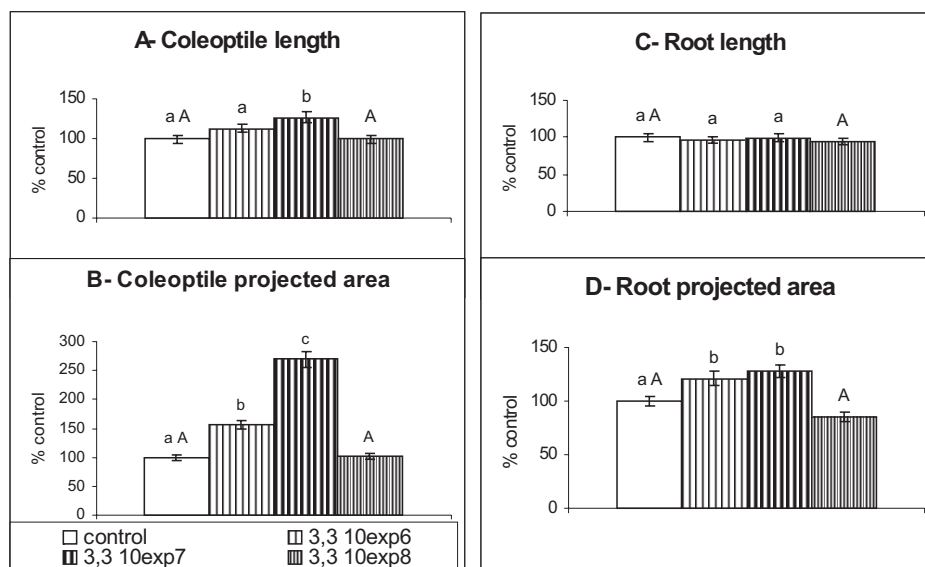


Figure 3. Colonization level vs. coleoptile and root growth, in *T. durum* cv. Buck Topacio seedlings inoculated with *A. brasilense* Sp245. Lines on top of bars represent SD. Different letters on top of bars mean significant differences among treatments ( $P < 0.05$ )

According to these results, an inoculum concentration in the range of  $5 \cdot 10^2$  to  $5 \cdot 10^5$  *A. brasilense* cells seedling<sup>-1</sup> would be adequate to achieve the optimum  $3.3 \cdot 10^6$  to  $3.3 \cdot 10^7$  bacterial cells seedling<sup>-1</sup> colonization range in *T. durum* cv. Buck Topacio seedlings (Table 1).

Seedling establishment occurs at a phenological stage at which drought and other abiotic stresses could be particularly harmful to annual plants. In wheat, successful seedling establishment is highly dependent on proper coleoptile develop-

ment. This specialized tissue could have an important role not only in seedling survival when drought strikes shortly after seeding but also in much later stages as physiological maturity, influencing grain yield at harvest (Gan et al. 1992). In this regard, *A. brasilense* Sp245 could minimize drought negative effects on both wheat and maize seedlings growth (Creus et al. 1998; Casanovas et al. 2002). *Azospirillum*-inoculated plants of both cereals exposed to water stress during anthesis had better crop yields than those non-inoculated controls (Casanovas et al. 2003; Creus et al. 2004). As it is well known that conclusions drawn from laboratory experiments often do not apply to different field situations the optimal level of soil inoculation would have to be determined to make sure that the soil is not overloaded with inocula, to achieve the desired plant growth promotion. Experiments at the field are necessary to determine if variations in soil type, management practices and weather conditions affect *Azospirillum* efficacy in improving crop yield at harvest.

### Conclusions

In studies concerning plant growth promotion by *A. brasilense* Sp245: a) NI or HKB-inoculated seedlings could be equally valid controls; b) since the bacteria could have been multiplying inside the plant, root colonization should be taken into account instead of inoculum concentration; c) bacterial MPN inside roots should not exceed  $2.5 \cdot 10^8$  cells seedling<sup>-1</sup>.

### Acknowledgements

This work was supported by Consejo Nacional de Investigaciones Científicas y Técnicas, Agencia Nacional de Promoción Científica y Tecnológica y Universidad Nacional de Mar del Plata, Argentina. We wish to thank Silvia Alicia and Cristina Larraburu for their help at the lab, Agr. Eng. H. Bariffi for kindly providing the seeds, and Prof. Ivone C. Barassi for editing the English text.

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