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Genetic Variation in Wheat for *Azospirillum brasilense* to Adhere to the Seedling Root

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Wheat improves some traits when inoculated with *Azospirillum*. However, inconsistent results have been observed in field experiments. The ability of *Azospirillum brasilense* Cd to adhere to wheat was tested in a germplasm panel consisting of a number of bread wheat cultivars, synthetic hexaploids, synthetic derivatives, and a partial set of single chromosome substitution lines to determine the plant genetic role. Seeds of genotypes were grown in modified Hoagland's medium at seedling stage and then roots were inoculated with the bacteria and adhered cells were counted. The majority of the bread wheat cultivars and synthetic derivatives and some of the synthetic hexaploids were able to support bacterial adhesion. Neither the age of the seedling nor the imposition of either salinity stress or nitrogen starvation had any effect on the extent of adhesion. A pedigree analysis revealed that the root-adhered *A. brasilense* cultivars shared common ancestor(s), and the substitution line analysis suggested that the genes underlying the trait were located on chromosomes 5D and 6D. The present results are consistent with the notion that the D genome is the source of genetic variation for the capacity of *A. brasilense* to adhere to the seedling roots.

Keywords: *Triticum, Azospirillum,* adhesion, polymorphism, salinity, substitution lines

Introduction

Azospirillum from α-proteobacteria is one of the most studied genera of free-living bacteria due to its plant growth promotion and its association with important crops as corn, wheat, rice, and other non-cereal plants as tomato, pepper, cotton and soybean (Bashan et al. 1991). It has been established that the attachment of *Azospirillum* is essential for a long-term association with the host plant root in order to promote the plant growth. Several studies have revealed that *Azospirillum* mainly colonizes root hairs and elongation zones, and in some cases the root interior such as *Azospirillum* Sp-245 in wheat (Assmus

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et al. 1995). The attachment to wheat roots was reported to be in two stages, the first one adsorption, probably mediated by proteins and the second anchoring, based probably on bacterial polysaccharides. Hydrophobic studies show that *Azospirillum* cell wall possesses mildly hydrophobic proteins in the first step of attachment (Dufrene and Rouxhet 1996; Castellanos et al. 1997). Also, outer membrane proteins exhibited lectin activity probably related with root adsorption (Castellanos et al. 1998; Burdman et al. 2001) cell to cell adhesion and subsequent aggregation (Mora et al. 2008). However, there is no definite agreement on how exactly the bacteria can effect plant growth. It has been suggested that the promotion of plant growth by *Azospirillum* could be by combination of a few or many mechanisms in each case of inoculation. These may vary according to the plant species, the *Azospirillum* strain, and environmental conditions when the interaction occurred (Bashan and de-Bashan 2010). Root colonization, survival, and role of root exudates have been documented (Vancura and Hanzlikova 1972; Van Bastelaere et al. 1993; Pothier et al. 2007), but little is known about the location and role of plant genes involved in the interaction with *Azospirillum* spp. A general positive growth response in numerous plant species is evident in the majority of cases of inoculation, but the effect is not always apparent in terms of economic productivity (Okon and Labandera-Gonzalez 1994; Díaz-Zorita and Fernández-Canigia 2009) due to observed inconsistencies of beneficial effects on wheat cultivars under field conditions (Okon and Labandera-Gonzalez 1994; Dubini et al. 2008). It has been observed that a mix of *Azospirillum* spp. affected differently the biochemistry and physiology of maize (García de Salomone and Döbereiner 1996). Similarly *Azospirillum* inoculation distinctly affected also two wheat genotypes; *Triticum durum* dry weight, nitrogen accumulation, and yield always benefited from *Azospirillum*, but the bread wheat genotype did not (Rodriguez-Sala et al. 2007).

Saubidet and Barneix (1998) suggested that a potential exists for *A. brasilense* to supply considerable nitrogen to wheat plants, probably dependent on specific bacteria–cultivar interaction. With this idea, we used synthetic hexaploids which have been promoted as providing a potential source of novel genetic variation useful for bread wheat improvement (Mujeeb-Kazi 2003). A number of such materials have been introduced as breeding parents for programmes concerned with biotic and abiotic stresses, e.g. salinity tolerance (Zavala-Fonseca et al. 1998; Pritchard et al. 2001, 2002; Mujeeb-Kazi and Díaz De León 2002; Colmer et al. 2006). It has been suggested that much of the genetic basis for this potential resides in the wild progenitor D genome (Mujeeb-Kazi et al. 1996; Mujeeb-Kazi and Díaz De Leon 2002). We focus in the adhesion of *Azospirillum* to a collection of wheat roots to test the hypothesis that there is a role of the genotype of the plant possible due to a preference or to molecular recognition of the wheat plant by bacteria rather than chemoattraction to root exudates. We present results on the capacity of a collection of wheat genotypes to influence the adhesion of *A. brasilense* to its roots. The level of intervarietal variation suggested that bacterial adhesion was heavily influenced by the allelic state at various host gene(s), implying that the inconsistency experienced with respect to the benefit of *Azospirillum* inoculation in bread wheat reflected the unwitting choice of cultivars to which the bacteria could only adhere poorly.

Materials and Methods

Plant materials and germination

The host was represented by a selection of bread wheat cultivars (BW), derivatives of synthetic and bread wheat crosses (BW-SHd) and synthetic hexaploid (SH) stocks held at the Plant Biotechnology Laboratory Seed Bank of the Universidad Autónoma de Baja California Sur, along with a set of 17 of the possible 21 Chinese Spring/Synthetic 6x single chromosome substitution lines (SLs), the origin of which has been described by Pestsova et al. (2006) (Table 1). Grains were initially surface-sterilized by a 10 min vacuum infiltration of 6% (v/v) NaOCl, rinsed three times in sterile distilled water, then germinated at 25°C under 24 h illumination for three days in closed sterile Petri dishes (ten grains per 9 cm dish), each containing 10 mL sterile nutrient medium half strength Hoagland's solution (HSH), composed of 1*M* $Ca(NO_3)_2.4H_2O$; 1*M* KNO_3, 1*M* $NH_4H_2PO_4$, 1*M* $MgSO_4.7H_2O$, 0.047*M* H_3BO_3 0.009*M* $MnCl_2.4H_2O$, 0.0007*M* $ZnSO_4.7H_2O$, 0.0003*M*

Table 1. Wheat materials tested for their ability to adhere *Azospirillum brasilense*

Entry	Pedigree	Source
BW set		
Opata M 85	BJY "S"/JUP	CIMMYT
Ciano T79	BY/MAYA "S"/4/BB/HD832.5/ON/3/CON/PJ	CIMMYT
Flycatcher	JUP/EMU"S"//GJO"S"	CIMMYT
Bacanora T88	JUP / BJY /2/ Ures T 81	
Pilón	HAHN/PRL	CIMMYT
Oasis	AGATHA/3*YR	CIMMYT
SH set		
WSHCPI208	CPI/GEDIZ/3/GOO//J069/CRA/4/*Ae. squarrosa* (208)	CIMMYT
WSHYAV498	YAV_3/SCO//J069/CRA/3/YAV79/4/*Ae. squarrosa* (498)	CIMMYT
WSHD67.2-257	D67.2/P66.270//*Ae. squarrosa* (257)	CIMMYT
WSHDOY458	DOY1/*Ae. squarrosa* (458)	CIMMYT
WSHCHEN205	CHEN/*Ae. squarrosa* (205)	CIMMYT
WSHSCOTT314	SCOTT/MEX//*Ae. squarrosa* (314)	CIMMYT
BW.SHd set		
WSHdSABUF895	SABUF/3/BCN//CETA/*Ae. squarrosa* (895).	CIMMYT
WSHdMAYOOR222	MAYOOR//TKSN1081/*Ae. squarrosa* (222).	CIMMYT
WSHdTUR/BUC205	TURACO/5/CHIR3/4/SIREN/Altar 84/ *Ae. squarrosa* (205)/3/3*BUC-Chen/ *Ae. squarrosa* (205)	CIMMYT
SL set		
Chinese spring	BW Landrace	CIMMYT
WSHS6x	Synthetic 6x	IPK
17 SLs	1A, 1B, 1D, 2B, 2D, 3A, 3B, 3D, 4B, 4D, 5A, 5B, 5D, 6A, 6D, 7B, 7D	IPK

Abbreviations: BW: bread wheat; SH: synthetic hexaploid wheat; BW.SHd: synthetic hexaploid derivative; SL: Substitution lines; CIMMYT: Centro Internacional para el Mejoramiento del Maíz y Trigo; IPK: Institut für PflanzenGenetik und KulturPflanzenForschung

$CuSO_4.5H_2O$, 0.0001*M* $H_2MoO_4.H_2O$, 0.001*M* EDTA, 0.001*M* $FeSO_4.7H_2O$. From the fourth day onwards, the HSH was replaced every 12 h until the seedlings were ready for inoculation with *Azospirillum brasilense* Cd.

Preparation of inoculum

Azospirillum brasilense Cd. (DMS 7030, Braunschweig, Germany) was grown on Nutrient Broth medium (Difco) at 30°C without agitation for 24 h until the population had reached the stationary growth phase. The cells were then pelleted by centrifugation (10 min, × 950 g), the pellet was suspended in 1mL 0.34*M* NaCl, re-centrifuged (10 min, × 950 g) and rinsed twice in the same saline solution. The pellet was finally re-suspended and diluted to a concentration of 10^6 colony forming units (CFU) per mL in a range of different media. To optimize this medium, the rinsed cells were re-suspended in HSH, HSH plus 150*mM* NaCl or HSH containing wheat root exudate, and cultured at 30°C without agitation. The wheat root exudate was recovered from the remaining HSH left in the dish after the *in vitro* germination (four, seven and eight days old) of bread wheat cv. Opata M85 seedlings, and sterilized by filtration (0.25 μ). Cell densities were monitored by the plate count method (Postgate 1969) on solid nutrient broth medium (Difco) at intervals between 0 and 120 h.

Bacterial adhesion to wheat seedling roots

Four days old wheat seedlings were rinsed three times in sterile distilled water, and the roots were blotted dry. They were then submerged in a 9 cm Petri dish containing 10 mL HSH and a population of 10^7 *A. brasilense* cells. Modified HSH solutions were used to test adhesion under conditions of either salinity stress or nitrogen starvation. The former comprised 1*M* $Ca(NO_3)_2.4H_2O$, 1*M* KCl, 1*M* $NaH_2PO_4.H_2O$, 1*M* $MgSO_4.7H_2O$, 0.047*M* H_3BO_3, 0.009*M* $MnCl_2.4H_2O$, 0.0007*M* $ZnSO_4.7H_2O$, 0.0003*M* $CuSO_4.5H_2O$, 0.0001*M* $H_2MoO_4.H_2O$, 0.001*M* EDTA, 0.001*M* $FeSO_4.7H_2O$ and 0.106*M* NaCl, while the latter was identical except for the absence of NaCl and having 1*M* $CaCl_2$ replacing 1*M* $Ca(NO_3)_2.4H_2O$. The seedlings were maintained in a sterile environment at room temperature for 2 h without agitation, after which the roots were rinsed three times in sterile distilled water and blotted dry. The central 2 cm portion of the primary root of each seedling was removed to 2 mL saline solution and homogenized for 15 s (Polytron PT1200C). A 100 μL aliquot of the homogenate was seeded onto a solid nutrient broth medium, and incubated at 30°C for two days, after which colony numbers were obtained as above.

Statistical analysis

Each experiment comprised three replicates of ten seedlings. Variation among germplasm entries was analyzed by a one-way analysis of variance (Statistica software, StatSoft, Tulsa, OK, USA), applying a significance threshold of $P < 0.05$.

Results

The cultured population of *A. brasilense* Cd reached its stationary growth phase after 36 h. All of the incubation media were effective, producing a mean of 8.93 $\log_{10}$ CFU. mL^{-1} after 48 h of culture. Longer periods of culturing were associated with a reduced level of cell viability (Table S1*).

Among the bread wheat entries, cv. Opata M85 was identified as being an efficient host for bacterial adherence while the synthetic hexaploids WSHCPI208, WSHYAV498, WSHDOY458 and WSHD67.2-257 were poor hosts. These entries were therefore used to study the response of seedling age and abiotic stress on root extension and bacterial adhesion. While salinity stress and nitrogen starvation both compromised root extension in Opata M85 (Table S2), they did not significantly reduce or enhance adhesion. The three synthetic hexaploids remained unable to influence the adhesion of the bacteria throughout (Table 2). The mean level of adhesion among the bread wheat cvs. Opata M85, Ciano 179, Bacanora T88 and Flycatcher, and the derivatives of synthetic hexaploid × bread wheat crosses WSHdSABUF895, WSHdMAYOR222, WSHdTUR/BUC205 and the synthetic hexaploid WSH6x was 54.6% of the $\log_{10}$ CFU of the total inoculated *A. brasilense* cells (Table 3). The SLs varied with respect to adhesion, presumably reflecting the inheritance of genes from either the donor synthetic hexaploid (WHSS6x, able to adhere) or the recipient bread wheat cv. Chinese Spring (unable to adhere). The only SLs which showed any significant level of adherence were those which had inherited their chromosomes 5D or 6D from WHSS6x (Table 3).

Table 2. The effect of seedling age and abiotic stress on *Azospirillum brasilense* adhesion

Genotype	Adhesion of *Azospirillum brasilense* ($\log_{10}$ CFU.mL^{-1})						
	Inoculated 7-day-old seedlings						
	Seedling age (days)			Salt treatment ($dS.m^{-1}$)		Nitrogen treatment	
	4	7	8	0	12	with	without
Opata M85	3.36[a]	3.34[a]	3.52[a]	3.35[a]	3.34[a]	3.91[a]	3.73[a]
WSHCPI208	nil	nil	nil	nil	nil	nil	nil
WSHYAV498	nil	nil	nil	nil	nil	nil	nil
WSHDOY458	nil	nil	nil	nil	nil	nil	nil
WSHD67.2-257	nil	nil	nil	nil	nil	nil	nil

Mean values followed by a different superscript letter differ from one another at $P < 0.05$.

Discussion

Several *Azospirillum* strains have been trialled in an attempt to improve the grain yield of wheat, but with rather inconsistent results (Okon and Labandera-Gonzalez 1994; Rodriguez-Sala et al. 2007; Díaz-Zorita and Fernández-Canigia 2009). The expected benefits of adhesion from the host plant's point of view include the provision of organic nitrogen, a source of indole-3-acetic acid, the production of nitric oxide, and the de-amination of the

* Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

Table 3. Variation for adhesion in the germplasm panel

Azospirillum brasilense (log_{10} CFU.mL^{-1})												
	Inoculated	Adhered		Inoculated	Adhered		Inoculated	Adhered		Inoculated	Adhered
BW			SH			SLs			SLs		
Bacanora	7.07	4.12[a]	WSHCHEN205	6.70	0.00	1A	8.00	0.00	5B	8.00	0.00
Chinese Spring	7.13	0.00	WSHCPI208	6.70	0.00	1B	8.00	0.00	5D	8.00	3.86
Ciano	7.07	4.10[b]	WSHDOY458	6.70	0.00	1D	8.00	0.00	6A	8.00	0.00
Flycatcher	7.07	3.71[a]	WSHD67.2-257	6.70	0.00	2B	8.00	0.00	6D	8.00	3.35
Oasis	6.70	0.00	WSHMAYOR222	7.07	3.79[a]	2D	8.00	0.00	7B	8.00	0.00
Opata	6.70	4.34[a]	WSHSABUF895	7.07	3.95[d]	3A	8.00	0.00	7D	8.00	0.00
Pilón	6.70	0.00	WSHSCOTT314	6.70	0.00	3B	8.00	0.00			
			WSHTUR/BUC205	6.70	3.65[ac]	3D	8.00	0.00			
			WSHS6x	7.97	3.43[abcd]	4B	8.00	0.00			
			WSHYAV498	6.70	0.00	4D	8.00	0.00			
						5A	8.00	0.00			

The different superscript letters mean significant difference at $P < 0.05$ by one-way ANOVA. BW stands for bread wheat; SH for synthetic hexaploids; SLs for substitution lines.

ethylene precursor 1-aminocyclopropane-1-carboxylate (Lambrecht et al. 2000; Creus et al. 2005; Blaha et al. 2006). These factors should all serve to enhance root proliferation and elongation, with consequent improvements to both water and mineral nutrient uptake (Okon and Kapulnik 1986; Glick et al. 1998; Jacoud et al. 1999; Steenhoudt and Vanderleyden 2000). It has been proposed that a key trigger for this set of root responses is provided by exudates from the host, which acts as an attractant of *Azospirillum* (van de Broek et al. 1998). The presence of root exudates in the present *in vitro* system was able to maintain the viability of *A. brasilense* cells for up to 48 h. Although the outcomes of the experiment were largely consistent with Vancura and Hanzlikova (1972) suggestion that wheat seedling exudate is benefical for *Azospirillum* survival, many wheat accessions are unable to establish the adhesion of *A. brasilense* cells to their roots.

The variation in adhesion ability appears to be under genetic control, partly because of the association of the trait with pedigree, and partly because of the behaviour of the SLs. The cvs. Opata M85, Flycatcher and Bacanora T88 share cv. Jupateco as a common ancestor, while cvs. Opata M85 and Bacanora T88 are both descended from cv. Bluejay, as are cvs. Jupateco and Bluejay from cv. Lerma Rojo 64. In the other hand, cvs. Bluebird and Penjamo are ancestors of cv. Ciano T79, and in turn, along with cv. Lerma Rojo 64, are descendants of cvs. Norin 10 and Brevor (Fig. S1). The group of bread wheat/synthetic hexaploid derivatives showing adhesion to *A. brasilense* can also be traced back to cvs. Norin 10 and Brevor (Fig. S1). Since WSHCHEN205 was unable to support *A. brasilense* colonization, the implication is that the *Triticum tauschii* D genome donors 895, 222 and 205 contributed no alleles promoting adhesion. In contrast, as suggested by the performance of the SLs, the genetic basis of the adhesion trait in WSH6x lies on chromosomes 5D and 6D inherited from its *T. tauschii* progenitor.

Inoculation with *A. brasilense* Sp245 has been shown to only inconsistently promote seedling and agronomic performance of *T. durum* (Pereyra et al. 2007), while the NH strain seemed able to readily colonized the roots of salinity stressed *T. durum* cv. Waha, and was associated with the restoration of vegetative growth and grain productivity (Nabti et al. 2010). Whether this benefit is a direct effect of NH adhesion or an indirect one operating via the stimulation of other microbial species is unclear. Of all the *Triticeae* species, Stancheva and Dinev (1992) concluded that the two which most readily interacted with *A. brasilense* were bread wheat and the diploid grass *Dasypyrum villosum*. The present results are consistent with the notion that the D genome is the source of genetic variation for the capacity of *A. brasilense* to adhere to the seedling roots.

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at http://www.akademiai.com/content/120427/

Electronic Supplementary *Table S1*. The effect of wheat root exudates on the viability of *Azospirillum brasilense* cells

Electronic Supplementary *Table S2*. Effect of salt stress and nitrogen stress on the growth of seven days root seedlings of wheat

Electronic Supplementary *Figure S1*. The pedigree of the bread wheat and derivatives of various bread wheat × synthetic hexaploid crosses