Applied Soil Ecology 82 (2014) 38–43

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

# *Azospirillum brasilense* mitigates water stress imposed by a vascular disease by increasing xylem vessel area and stem hydraulic conductivity in tomato

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ARTICLE INFO

*Article history:*
Received 7 January 2014
Received in revised form 29 April 2014
Accepted 26 May 2014
Available online 6 June 2014

*Keywords:*
*Solanum lycopersicum*
*Clavibacter michiganensis* subsp. *michiganensis*
Bacterial canker
PGPB
Water stress tolerance

ABSTRACT

Water stress, with its negative consequences on plant growth and survival, can be mitigated by *Azospirillum brasilense* inoculation. In tomato, *A. brasilense* delays wilting caused by a vascular pathogen, *Clavibacter michiganensis* subsp. *michiganensis*, by yet unknown mechanisms. We studied morphological, anatomical and physiological changes induced by *A. brasilense* in tomato that relate to water stress tolerance, which could explain the deferral in symptom expression. For this purpose, tomato seeds were treated or not with *A. brasilense* BNM65, and 5 weeks later plants were challenged with *C. michiganensis* subsp. *michiganensis* or mock inoculated with water. There was a large growth promotion associated to *Azospirillum*: treated plants had higher total biomass and leaf area. In relation to water stress tolerance, *Azospirillum* treated plants had larger xylem vessel area, higher stem specific hydraulic conductivity, thicker stems, and lower shoot/root dry matter and specific leaf area. These changes were opposite to those induced by *C. michiganensis* subsp. *michiganensis*. We conclude that *A. brasilense* favoured a better adjustment of plant-water relations by several mechanisms, and thus, transitorily alleviated symptoms expression of a vascular disease.

## 1. Introduction

Because of the problems and concerns about the use of traditional pesticides and resource limitations, there is a great interest in the use of beneficial microorganisms in agriculture to improve crop nutrition and tolerance to biotic and abiotic stresses (Andrews et al., 2012). *Azospirillum* spp. is a soil free-living plant growth-promoting bacteria (PGPB) commercially available in many countries, including Argentina, Mexico, India, Italy, France (Bashan and de-Bashan, 2010) and Brazil (Hungria et al., 2010). Plant growth promotion is assumed to be a consequence of a combination of several mechanisms, possibly acting in an additive manner or as a cascade. Most studies refer to the production of phytohormones, such as indole-3-acetic acid, gibberellins and cytokinins; nitrogen fixation; general improvement of root growth and enhanced uptake of minerals and water; mitigation of environmental stressors and direct and indirect biological control of pathogens (recently reviewed in Bashan and de-Bashan, 2010; Creus et al., 2010). In tomato (*Solanum lycopersicum* L.), *A. brasilense* applied to seeds colonize roots, produce taller plants, with more leaves, higher leaf and root dry weight (Bashan et al., 1989, 1991; Molina-Favero et al., 2008; Romero et al., 2003), earlier flowering and higher yields (Bashan et al., 1989), and when treated plants are inoculated with the bacterial canker causal agent, there is a delay in plant wilting and death (Romero et al., 2003). *A. brasilense* also has shown activity as a biocontrol agent of bacterial speck (*Pseudomonas syringae* pv. tomato) in tomato (Bashan and de-Bashan, 2002) and fungal diseases in other crops (Russo et al., 2008; Tortora et al., 2011).

Bacterial canker is a vascular disease of tomato caused by the Gram positive bacteria *Clavibacter michiganensis* subsp. *michiganensis* (Davis et al., 1984). It is one of the most important diseases of this crop worldwide (de León et al., 2011). Bacteria enter the plant by natural openings, or most commonly by wounds, and invade the xylem vessels becoming systemic; bacterial cells can move 30 cm from the point of inoculation and reach the apical region in 15 days (Chalupowicz et al., 2012); subsequently, infected plants wilt and die. Depending on inoculum concentration, temperature, age of the plant at the time of inoculation and cultivar, infections can remain latent (Chang et al., 1992). It has been proposed that wilting is the result of the interruption of water movement from the roots to the leaves caused by bacterial cells and their extracellular

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http://dx.doi.org/10.1016/j.apsoil.2014.05.010
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polysaccharides that physically plug the xylem vessels, plus the action of extracellular enzymes which can attack the xylem vessels and adjacent parenchymatic cells (Jahr et al., 1999; Savidor et al., 2012). In earlier stages of infection, water flow could be reduced by embolism, which could be the result of a reduction on the surface tension of the sap or the degradation of the pit membranes between adjacent vessels, as was suggested for the Dutch elm disease and the diseases caused by the Gram negative bacteria *Xylella fastidiosa* (Tyree and Sperry, 1989; McElrone et al., 2008). In any case, the capability of the stems to conduct water is reduced.

Water stress in plants has been associated with a reduction of water uptake by roots, changes in stomatal behavior and occlusion of the xylem (Tyree and Sperry, 1989). *C. michiganensis* subsp. *michiganensis* primarily affects the xylem vessels, and thus, the main beneficial effect of *A. brasilense* could be an increase in the water conduction capability of the stems. Changes in hydraulic conductivity can be related to anatomical adjustments in xylem vessel diameter and/or frequency (Bauerle et al., 2011). In hydroponically grown wheat seedlings, *A. brasilense* reduces osmotic stress by an increase in the diameter of xylem vessels of the coleoptile (Pereyra et al., 2012) and in *Sorghum bicolor*, it improves hydraulic conductivity of the roots (Sarig et al., 1992). *Azospirillum* might help plants cope with water stress by other mechanisms: a more developed root system, changes in the elasticity of cell walls (Creus et al., 1998, 2004) or osmotic adjustments involving certain solutes (Creus et al., 2010; Groppa et al., 2012).

Information on the effects of *Azospirillum* spp. on water stress tolerance refers mostly to grasses, and there is no information about its effect on the water economy of plants affected by a vascular pathogen. Our hypothesis is that *A. brasilense* improves water stress tolerance in tomato by increasing the capacity of the stems to conduct water, delaying plant wilting. Our objectives were to determine the physiological, morphological and anatomical changes induced by *A. brasilense* that relate to water stress tolerance in tomato, and to establish how they relate to the development of a vascular disease, as the bacterial canker.

## 2. Materials and methods

Two independent experiments were conducted: one to determine the effects of *A. brasilense* and/or *C. michiganensis* subsp. *michiganensis* on the morphology and physiology of tomato plants, and the other to establish the anatomical changes induced on the xylem vessels of the stems by those treatments.

### 2.1. Microorganisms culture conditions

*A. brasilense* and *C. michiganensis* subsp. *michiganensis* strains were maintained as glycerol stocks at –80 °C. *A. brasilense* strain BNM65 (=Az65) is a native strain isolated from Argentinean soil which has proven to stimulate growth in tomato (Romero et al., 2003). BNM65 was kindly provided by the Instituto de Microbiología y Zoología Agrícola del Instituto de Tecnología Agropecuaria-EEA Castelar (IMYZA-INTA) and is preserved in the Banco Nacional de Microorganismos (Cátedra de Microbiología Agrícola, Facultad de Agronomía, Universidad de Buenos Aires, Argentina). BNM65 was cultured on nitrogen-free NFb semi-solid medium (Döbereiner, 1995) supplemented with 1 $g\,l^{-1}$ ammonium chloride for 44 h at 30 ± 2 °C, at 150 rpm. Cells were harvested by centrifugation (10,000 × g for 10 min), and washed twice in sterile tap water (STW). Washed cells were suspended in STW, adjusted with a spectrophotometer at OD 0.5 at 600 nm ($\sim 10^7$ CFU $ml^{-1}$), and used as inoculum. Bacterial concentration was verified by dilution plating on Congo red plates (Rodríguez Cáceres, 1982).

Cm9 is a strain of *C. michiganensis* subsp. *michiganensis*, originally isolated from a bacterial canker affected tomato plant in a commercial greenhouse in Florencio Varela, Buenos Aires province, Argentina (Romero et al., 2003). The strain was cultured on modified YDC medium (Ritchie and Dittapongpitch, 1991) at 28 °C for 48 h. Bacterial cells were suspended in sterile distilled water (SDW), and concentration was adjusted with a spectrophotometer at OD 0.3 at 600 nm, which corresponds to $\sim 10^8$ CFU $ml^{-1}$. The suspension was diluted 1:10 in SDW ($\sim 10^7$ CFU $ml^{-1}$) to be used as inoculum and plated on modified YDC to verify concentration.

### 2.2. Inoculation and plant growth conditions

Seeds of fresh-market tomato cultivar ACE55 (Asgrow Seed Co. Mendon, MI, USA) were washed with 0.2% commercial detergent for 10 min, exhaustively rinsed with tap water to eliminate detergent, disinfected with 2% sodium hypochlorite for 10 min and rinsed 6–7 times in STW. All disinfestations' steps were done under agitation. Seeds were aseptically transferred to a sterile filter paper to remove excess water, and then inoculated by immersion in the *A. brasilense* sp. suspension for 30 min with agitation. Seeds suspended in STW were used as controls. After pre-germination in a humid chamber in the dark for 48–72 h at 28 °C, one germinated seed was planted in each 300 ml plastic pot containing a mixture of commercial potting substrate (Gem™ 3, Agroquímicos Larrocca SRL, Buenos Aires, Argentina), perlite and soil (2:1:1).

Pots were placed in the greenhouse under natural temperature (18–28 °C) and light (approx. 14 h) conditions. Plants were watered daily with tap water. Commercial fertilizer, containing nitrogen, phosphorus and potassium (15:6.5:12.5), was applied weekly in order to prevent nutrient deficiencies; insecticides were used when necessary.

Five weeks after sowing, plants were inoculated with *C. michiganensis* subsp. *michiganensis* by removing the first true leaf of each plant with a flamed-sterilized scalpel immersed in the bacterial suspension ($\sim 10^7$ UFC $ml^{-1}$). Pathogen-free control plants were treated with a scalpel immersed in SDW. There were seven plants per treatment.

### 2.3. Evaluation of plant growth parameters

For the first experiment, plants were harvested 70 days after sowing. The experiment was repeated twice. The height of plants was measured, and the number of fully developed true leaves was counted. The roots of each plant were gently separated from bulk soil and washed under tap water. Turgid leaf area was measured with a leaf-area-meter (LI-COR, LI-3100). Dry (65 °C for 72 h) weight of turgid and flaccid leaves, stems and roots was determined. The specific leaf area (SLA) was determined dividing the leaf area by the dry weight of the leaves.

### 2.4. Hydraulic conductivity measurements

The stem hydraulic conductivity was determined by inducing water flow through excised sections under small pressure gradients (Sperry et al., 1988). Immediately before harvest, plants were kept well watered in darkness for 24 h to allow them to recover from any short-term embolism, so we could evaluate developmental responses. The first internode, between the first and the second true leaf, was used. Stems were always cut under water, and all measurements were done using deionized filtered (0.2 μm) water. Measuring pressures were created by a water column equivalent to 9 kPa. After reaching steady-state conditions, water flux was collected during 1 min in pre-weighed vials containing dry cotton. Stem conductivity ($K_h$; $mg\,s^{-1}\,MPa^{-1}\,m$) was calculated dividing the flow rate (known by the increase in vial weight) by the pressure gradient (water column pressure divided the length of the stem) (Trillo and Fernandez, 2005).

In order to compare treatments, hydraulic conductivity was expressed on three different ways: as stem-specific ($K_s$), leaf-specific ($K_L$) and partial-leaf-specific conductivity ($K_{Lp}$). $K_s$ was calculated as $K_h$ divided by the stem cross section at the first internode; it allows a comparison of the efficiency of stems to conduct water, and it depends on the number of vessels per unit cross section and the fourth power of their diameter (Tyree and Zimmerman, 2002). $K_L$ was determined as $K_h$ divided by the leaf area above the first internode. It is a measure of the hydraulic sufficiency of the stem segment to supply water to leaves distal to that segment (Tyree and Zimmerman, 2002). As in diseased plants, a part of their leaf area is wilted, we also expressed conductivity as a division of $K_h$ by the turgid leaf area, which we called partial-leaf-specific conductivity ($K_{Lp}$).

### 2.5. Number and area of xylem vessels

For the second experiment, sections of the fourth internode of plants were cut and fixed in FAA (absolute ethyl alcohol, glacial acetic acid, formaldehyde and distilled water, in a 50:5:10:35 ratio v/v) until ready to use. The stem sections were immersed in paraffin and cut into thin slices (8–10 μm) with a microtome, coloured with safranin-fast green and mounted in Canada balsam. Digital images were captured with an optical microscope (Leica 25 ED) and analyzed using the software UTHSCSA Image Tool Version 3.0 (http://ddsdx.uthscsa.edu.itdesc.html). All vessels with a diameter of 6 μm or larger were counted and the diameter (mean of two perpendicular measurements) registered. The contribution to the stem conductivity of smaller vessels was considered negligible. Six plants per treatment were evaluated.

### 2.6. Experimental design and statistical analysis

Treatments were a factorial combination of two levels of *A. brasilense* (pre-treated and not) and of *C. michiganensis* subsp. *michiganensis* inoculation (inoculated and not). Plants were arranged in a completely randomized design. Analysis of variance (ANOVA) was performed using the InfoStat/Professional system version 1.1 (Estadística y diseño, Facultad de Ciencias Agrarias, Universidad Nacional de Córdoba, Argentina). Data were transformed when needed to meet ANOVA assumptions of normality and homogeneity of variances.

## 3. Results

### 3.1. Plant growth

Plants treated with *A. brasilense* BNM65 were taller than non treated plants ($p = 0.0143$; Table 1). They also had larger root, leaf and stem dry weight ($p < 0.0001$; $p < 0.0001$ and $p = 0.0003$, respectively), and thus, had larger total dry weight ($p < 0.0001$) than non treated plants (Table 1). On the contrary, inoculation with *C. michiganensis* subsp. *michiganensis* caused a reduction on plant height ($p = 0.004$), stem diameter ($p = 0.0004$) and root, leaf, stem and total dry weight ($p = 0.0015$, $p = 0.0282$, $p = 0.0064$ and

**Table 1**
Effect of *Azospirillum brasilense* BNM65 and/or *Clavibacter michiganensis* subsp. *michiganensis* Cm9 inoculation on plant biomass, height and stem transversal surface. Values are mean ± SE (number of observations = 7; ANOVA *F*-tests were significant for *A. brasilense* and *C. michiganensis* subsp. *michiganensis* with no interaction between treatments for all variables; *p*-values are in the text).

| Variable evaluated | Untreated | BNM65 | Cm9 | BNM65 + Cm9 |
|---|---|---|---|---|
| Leaves dry weight (g) | 0.63 ± 0.08 | 1.56 ± 0.11 | 0.39 ± 0.08 | 1.18 ± 0.13 |
| Stems dry weight (g) | 0.10 ± 0.02 | 0.35 ± 0.08 | 0.07 ± 0.01 | 0.11 ± 0.02 |
| Root dry weight (g) | 0.25 ± 0.11 | 0.72 ± 0.11 | 0.07 ± 0.01 | 0.37 ± 0.06 |
| Total biomass (g) | 0.97 ± 0.19 | 2.63 ± 0.28 | 0.52 ± 0.10 | 1.66 ± 0.19 |
| Plant height (cm) | 18.9 ± 1.25 | 25.4 ± 2.29 | 12.9 ± 2.41 | 17.6 ± 1.69 |
| Stem transversal surface ($cm^2$) | 30.6 ± 5.09 | 31.0 ± 1.29 | 12.6 ± 1.94 | 24.0 ± 2.31 |

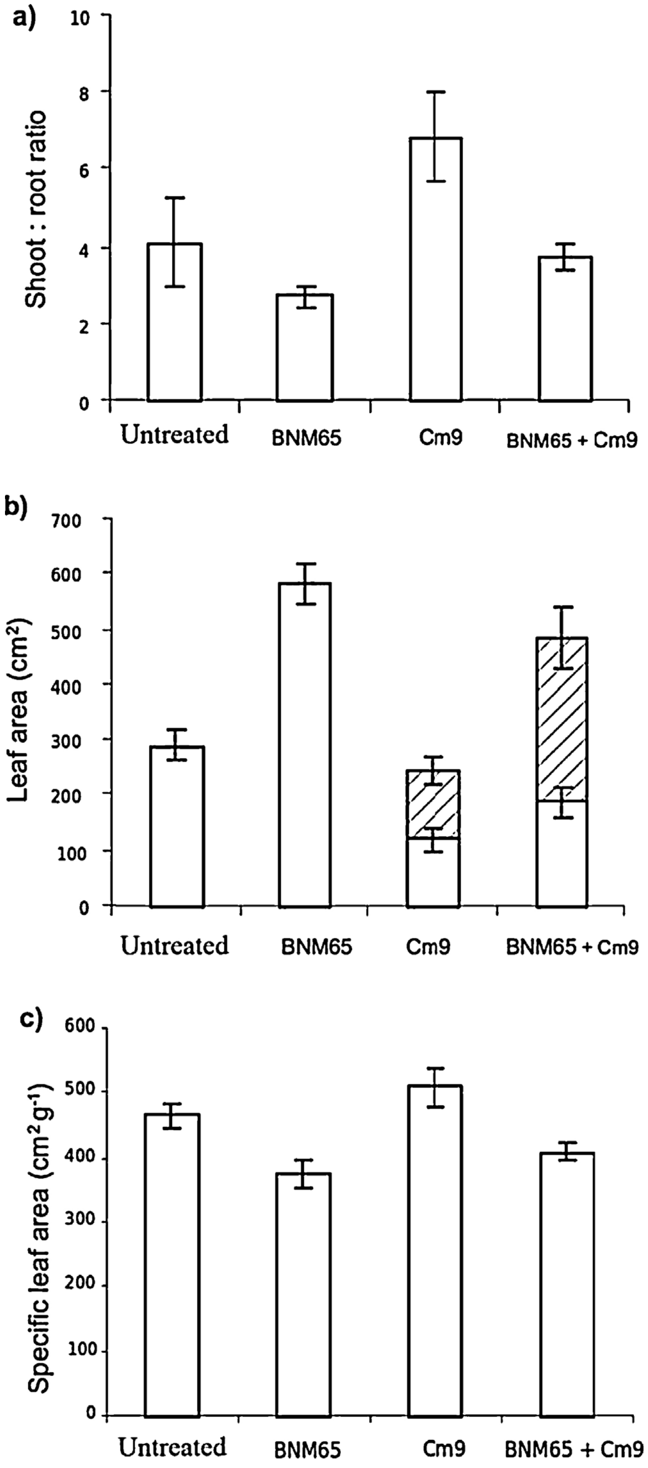

**Fig. 1.** Effect of *Azospirillum brasilense* BNM65 and/or *Clavibacter michiganensis* subsp. *michiganensis* Cm9 inoculation on (a) shoot: root ratio, (b) turgid and total leaf area ($cm^2$; stripped columns represent the wilted leaf area.) and (c) specific leaf area ($cm^2\,g^{-1}$; ratio of leaf surface to weight). Standard error of difference for treatment means is reported as a vertical bar (number of observations = 7; ANOVA *F*-test were significant for *A. brasilense* for the shoot: root ratio, total leaf area and specific leaf area and for *C. michiganensis* subsp. *michiganensis* for the shoot:root ratio, with no interaction between treatments; *p*-values are in the text).

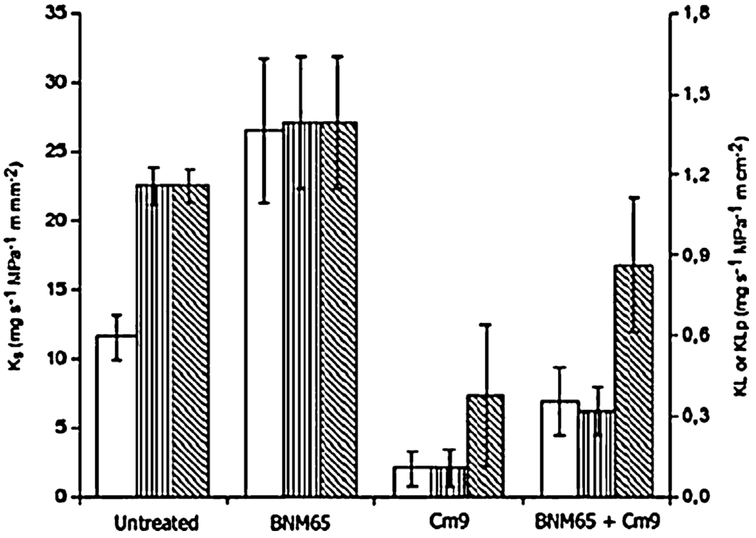

**Fig. 2.** Effect of *Azospirillum brasilense* BNM65 and/or *Clavibacter michiganensis* subsp. *michiganensis* Cm9 inoculation on $K_s$ (stem specific conductivity; (□), $K_L$ (leaf specific conductivity; ▥), and $K_{Lp}$ (partial leaf specific conductivity; ▨). The bars represent the standard error of means (number of observations = 7; ANOVA *F*-test were significant for *A. brasilense* for $K_s$ and for *C. michiganensis* subsp. *michiganensis* for $K_s$, $K_L$ and $K_{Lp}$, with no interaction between treatments; *p*-values are in the text).

$p = 0.003$, respectively). As a result of these opposite effects, the reduction of the root and shoot weight induced by the pathogen was less on *Azospirillum* pre-treated than on non-treated plants (48% and 32%, vs. 72% and 36%, respectively). The shoot/root ratio was also affected in opposite ways by the treatments: while *Azospirillum* caused a reduction ($p = 0.0181$), pathogen inoculation caused an increase ($p = 0.0282$) on this ratio (Fig. 1a), compared to untreated controls.

*Azospirillum* treatment was associated with an increase in the total leaf area of plants ($p < 0.0001$; Fig. 1b); *C. michiganensis* subsp. *michiganensis* inoculation did not modify this variable. On healthy plants, as expected, all the leaf area was turgid. On diseased plants, wilting was delayed on *Azospirillum* treated plants (~ 6–7 days); however, by the end of the experiment the area of turgid leaves was diminished in a similar level for plants treated or not (Fig. 1b). At that time, disease severity was of 67% and 71% for *Azospirillum* treated and control plants, respectively.

Treatment with *Azospirillum* significantly ($p = 0.0002$) reduced the SLA, so treated plants had thicker or denser leaves than non-treated plants (Fig. 1c).

### 3.2. Hydraulic conductivity, number and area of xylem vessels

The stem specific hydraulic conductivity ($K_s$) was increased by *Azospirillum* ($p = 0.0133$) and reduced by *C. michiganensis* subsp. *michiganensis* ($p < 0.0001$; Fig. 2).

The inoculation with the pathogen also reduced the leaf specific conductivity of plants ($K_L$; $p < 0.0001$), while there was no effect of *Azospirillum* on this variable ($p = 0.0999$) (Fig. 2). When only the turgid leaf area was considered to calculate the leaf partial hydraulic conductivity ($K_{Lp}$), the same trend was observed: diseased plants had lower $K_{Lp}$ than healthy plants ($p = 0.0227$), and there was no effect of *Azospirillum* ($p = 0.1772$; Fig. 2).

Plants pre-treated with *Azospirillum* had a larger xylem vessel area ($\mu m^2$) than non-treated plants ($p = 0.0254$). The effect was more evident for diseased (64%) than for healthy (16%) plants (Table 2 and Fig. 3). The number of vessels was not affected by treatments (Table 2).

**Table 2**
Number and area of xylematic vessels on stems of tomato plants treated as seeds with *Azospirillum brasilense* or not, and inoculated with *Clavibacter michiganensis* subsp. *michiganensis*. Values are mean ± SE (number of observations = 6; ANOVA *F*-test were significant for *A. brasilense* and *C. michiganensis* subsp. *michiganensis* with no interaction between treatments for both variables; *p*-values are in the text).

| Variable evaluated | Untreated | BNM65 | Cm9 | BNM65 + Cm9 |
|---|---|---|---|---|
| Xylematic vessel area ($\mu m^2$) | 1154 ± 109 | 1344 ± 242 | 1058 ± 148 | 1732 ± 111 |
| Number of xylem vessels | 195 ± 20 | 169 ± 12 | 155 ± 16 | 177 ± 11 |

## 4. Discussion

We have previously demonstrated that *A. brasilense* BNM65 can delay plant wilting induced by a vascular pathogen on tomato (Romero et al., 2003). Now, we demonstrate that *A. brasilense* BNM65 favours better plant-water relations of both healthy and diseased plants.

As reported previously (Bashan et al., 1989; Dobbelaere et al., 2001; Romero et al., 2003), *A. brasilense* treated tomato plants were taller and had larger total dry matter weight than non-treated plants. Because growth promotion of the roots was larger than that of shoots, the shoot/root ratio was smaller for treated plants. This is not surprising since in most cases the inoculation with *A. brasilense* improves the root system by promoting root elongation (Levanony and Bashan, 1989; Dobbelaere and Okon, 2007) and development of lateral roots and root hairs (Bashan and de-Bashan, 2010). However, this is not always the case with crop grasses inoculated with *Azospirillum* spp.: in a study of 79 reports from the literature, Bashan and Dubrovsky (1996) found that the shoot/root ratio decreased in half of them and increased in the other half. Also, Pereyra et al. (2009) did not detect a change in the partitioning of dry matter in *A. brasilense* inoculated wheat seedlings exposed to osmotic stress, while there was an increase in the ratio for un-stressed seedlings. The total leaf area was also increased in *Azospirillum* treated plants, but the SLA was reduced, which means that leaves were thicker or denser, i.e. better adjusted to drought, than those of non-treated plants. Also, the stems of *Azospirillum* treated plants were more efficient in conducting water (higher $K_s$) than those of non-treated ones, which can be explained by a larger xylem vessel area. The leaf specific conductivity ($K_L$) also tended to increase in relation to *Azospirillum* inoculation, although it was not statistically significant possibly because of a high variance of the data. $K_L$ is inversely related to the leaf area, which is more variable than the stem cross section

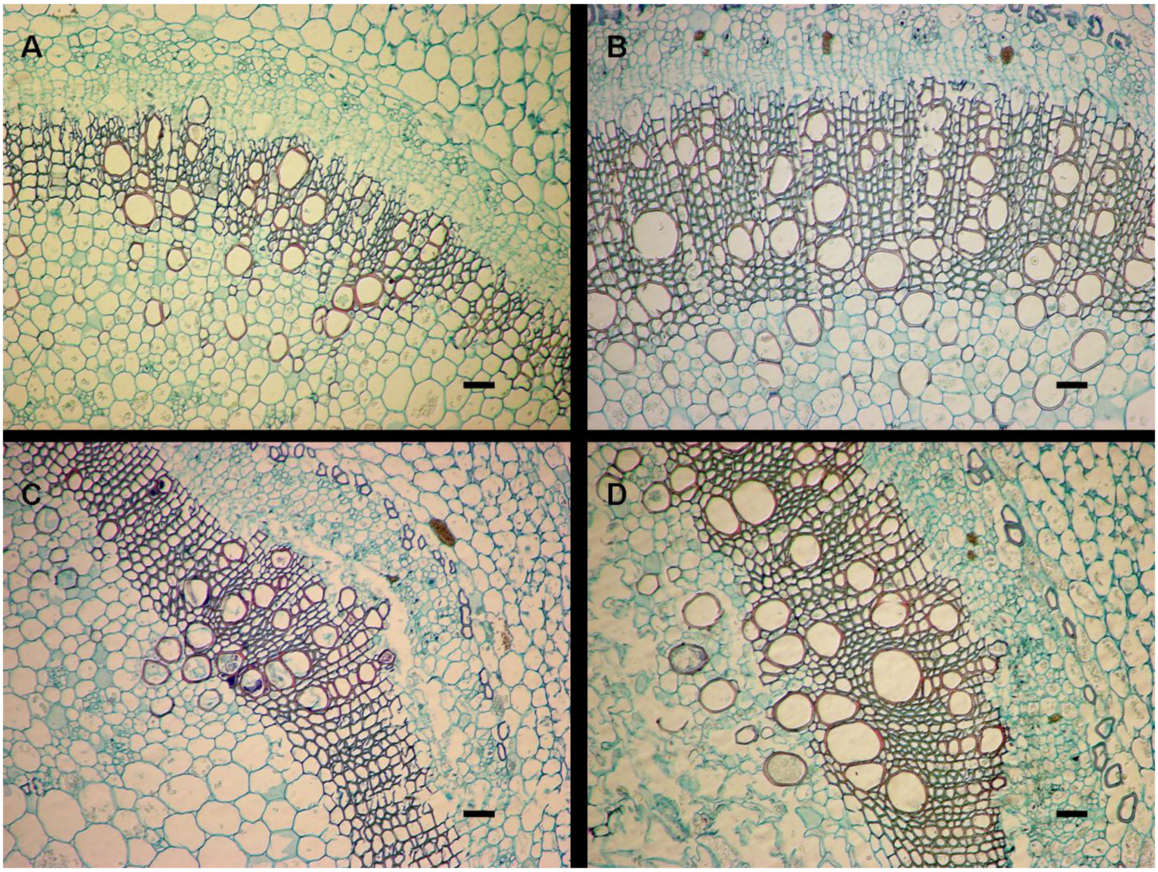

**Fig. 3.** Transversal sections of stems of tomato plants stained with safranin-fast green. (A) Healthy plants from untreated seeds. (B) Healthy plants from *Azospirillum brasilense* treated seeds. (C) Plants inoculated with *Clavibacter michiganensis* subsp. *michiganensis* and untreated as seeds. (D) Plants inoculated with *Clavibacter michiganensis* subsp. *michiganensis* and treated with *Azospirillum brasilense* as seeds. All pictures 100×. Black bars on the right bottom represent 50 $\mu$m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

used to calculate $K_s$. Nevertheless, a better provision of water to the leaves on *Azospirillum* treated, compared to non-treated, plants might have allowed higher leaf water potentials, and thus, an increase in cell expansion, which would have resulted in the larger leaf area observed on treated plants.

*C. michiganensis* subsp. *michiganensis* caused a reduction on plant height, stem diameter and dry weight of all plant parts, irrespective of *Azospirillum* treatment. The roots were more negatively affected than the aerial parts, so the shoot/root ratio increased. Also, the stems of diseased plants were impaired in their ability to conduct water. Their $K_s$ and $K_L$ were lower than those of healthy plants, even when only the turgid leaves were considered to calculate the $K_{Lp}$. So, it seems that the stems were at times unable to provide sufficient water to the leaves, which in turn wilted.

*A. brasilense* BNM65 inoculation induced developmental changes on the plants that might have played a role on the mitigation of the damage caused by *C. michiganensis* subsp. *michiganensis*. *Azospirillum* sp. has been related with a speeding of tomato plant phenology: treated plants have earlier fruit setting than non-treated plants (Bashan et al., 1989). Therefore, *Azospirillum* treated plants of the same chronological age as those non-treated might be physiologically older, and thus, less susceptible to bacterial canker (Chang et al., 1992). This theory is supported by the findings of Chalupowicz et al. (2012) who observed that *C. michiganensis* subsp. *michiganensis* preferentially attaches to protoxylem, the first xylem to develop during primary growth, as opposed to metaxylem vessels, which complete its development after cell elongation ceases. Phytohormones produced by *Azospirillum* spp., such as auxins, might be responsible for an increase in the rate of xylem formation (Bashan and de Bashan, 2010), and might be involved in the increase on vessel area on coleoptiles of *Azospirillum* treated wheat seedlings (Pereyra et al., 2012). Interestingly, vessel size was increased only when *Azospirillum* treated wheat plants were exposed to osmotic stress, but not when grown in water (Pereyra et al., 2012). In our experiments, the increase on vessel area related to *A. brasilense* BNM65 inoculation was four times larger on plants affected by a vascular disease (64%) than on healthy plants (16%). As a consequence, diseased *Azospirillum*-treated plants had higher (164%) leaf specific conductivity ($K_L$) than diseased control plants. As disease progressed, leaves began to wilt, but the specific conductivity of turgid leaves ($K_{Lp}$) was less impaired on *Azospirillum* treated than in non-treated plants, which meant that the stems were able to provide water to those leaves, possibly due to their larger xylem vessel area.

We conclude that *A. brasilense* BNM65 caused developmental changes on tomato plants that favoured a better plant-water relationship adjustment. The main effects were a larger xylem vessel area of the stems, a lower specific leaf area and a larger root system; wider vessels allowed a higher stem specific conductivity. These changes were opposed to those induced by a vascular pathogen such as *C. michiganensis* subsp. *michiganensis*, and thus, transitorily alleviated disease symptoms. The effect of *A. brasilense* on xylem vessel size could have an important role in the adaptation to unfavourable water conditions.

## Acknowledgements

We would like to thank Alejandro Perticari for kindly providing the strain BNM65 of *Azospirillum brasilense* and Romina Pizzorno and Esteban Fernandez for their assistance in the laboratory.

Support for this project came from UBACyT 20020100100656 and PICT-ANPCyT 1776/2010.

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