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Hydraulic and leaf reflectance alterations induced by *Clavibacter michiganensis* subsp. *michiganensis* on tomato plants

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Abstract *Clavibacter michiganensis* subsp. *michiganensis* causes bacterial wilt and canker in tomato. A detailed account of pathogen effects on growth and water parameters of affected plants is lacking. Besides, the long latent period of the disease represents a limitation for its management. Our objectives were to determine the physiological and morphological changes induced by this pathogen in tomato, in relation to water stress, and to evaluate two well-known leaf spectral reflectance indices for the diagnosis of this vascular disease before symptoms expression. Experiments were conducted in a greenhouse with pot-grown plants inoculated with a bacterial suspension (10^7 CFU/ml); controls were treated with sterile distilled water. Five weeks later plants were harvested and several growth parameters, the number and size of xylem vessels and the hydraulic conductivity of the stems were determined. Inoculated plants were shorter, with smaller leaf area and lower biomass than those of mock-inoculated plants. While the number and hydraulic radius of xylem vessels were not affected, the stem-specific (K_s) and the leaf-specific (K_L) conductivity were significantly reduced by inoculation. Wilting of leaves of diseased plants was associated to the reduction of K_L. Inoculated plants showed leaf spectral reflectance changes 1 week after inoculation, and 2 weeks before symptom expression, as shown by the index NDWI, an indicator of the water content of the leaves. We conclude that *C. michiganensis* subsp. *michiganensis* reduced the growth and altered plant-water relations of tomato plants by reducing the stem hydraulic conductivity. The NDWI index could be used for the early, non- destructive, diagnosis of bacterial canker.

Keywords Hydraulic conductivity · Xylem vessels · Vascular disease · Bacterial canker and wilt

Bacterial canker (*Clavibacter michiganensis* subsp. *michiganensis*) is one of the most important bacterial diseases of tomato (*Solanum lycopersicum* L.) worldwide (Gartemann et al. 2003; de León et al. 2011). It is a reemerging disease, with recent epidemics arising around the world (Milijašević-Marčić et al. 2012; Frenkel et al. 2016; Ialacci et al. 2016; Wassermann et al. 2017; Basım and Basım 2017). For many of the recent outbreaks it was suggested that the pathogen was introduced by the international trade of seeds, perpetuating locally on plant debris (Kawaguchi et al. 2010; Vega and Romero 2016; Wassermann et al. 2017).

C. michiganensis subsp. *michiganensis* usually enters the plant by wounds, especially those caused during

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common agronomical practices such as pruning lateral shoots or leaves (Carlton et al. 1994). Once the pathogen reaches the vascular tissue, it becomes systemic and produces typical wilting symptoms. Initially, there is a loss of turgidity of the leaflets on one side of the leaves, or the leaves on one side of the plant. Symptoms later progress on one or both sides of the plant until the whole plant succumbs and dies (Sharabani et al. 2013). Cankers may appear on stems or petioles. For vascular diseases, water stress is the result of a reduced hydraulic conductivity of the xylem (Dimond 1970).

C. michiganensis subsp. *michiganensis* is an effective endophyte (Gartemann et al. 2003) with a long latent period of up to 50 days, or even more, depending on the age of the plant at the time of infection, temperature, inoculum concentration and plant genotype (Chang et al. 1992; Sharabani et al. 2013). Symptomless plants might mislead farmers to reduce sanitation practices, favoring secondary spread of the pathogen. Early detection of those plants by a non-destructive technique could facilitate disease management. Leaf reflectance (the proportion of the incident radiation that is reflected by a leaf surface) can be measured in a non-invasive and non-destructive way (Sun et al. 2014). Leaf spectral reflectance indices, as the normalized difference water index (NDWI; Gao 1996) and the normalized difference vegetation index (NDVI; Gamon et al. 1995; Sims and Gamon 2002) have been widely used as plant stress indicators, in particular to evaluate water stress in plants (Sims and Gamon 2003; Katsoulas et al. 2016), and were also suggested as a means of monitoring diseases in a rapid, cost-effective, and reliable way (Sankaran et al. 2010; Yuan et al. 2014).

Many studies have been carried out in recent years on *C. michiganensis* subsp. *michiganensis* and have improved our knowledge on molecular interactions, epidemiology and infection. However, a detailed account of the effects of this pathogen on the growth and water condition of affected plants, and an early detection method of infected symptomless plants are still lacking. Our objectives were to describe the alterations induced by the vascular pathogen *C. michiganensis* subsp. *michiganensis* on the growth and the physiological parameters related to water translocation on tomato plants, and to evaluate two well-studied leaf spectral reflectance indices for the diagnosis of bacterial canker, prior to symptom expression.

All experiments were conducted in a greenhouse under natural light (approx. 14 h) and temperature (23 ± 5 °C) conditions. Plants were grown in pots to allow a more controlled manipulation of the plants, e.g. keeping them in the dark for 24 h before measurements to avoid any short-term embolism (Trillo and Fernández 2005). For the evaluation of plant growth and stem hydraulic conductivity, tomato plants were inoculated 4 weeks after emergence by removing the first true leaf with a flamed-sterilized scalpel immersed in the inoculum suspension (10^7 CFU/ml). Control plants were treated with a scalpel immersed in sterile distilled water. Growth conditions of tomato plants, bacterial strain and the inoculation procedure were as described in Romero et al. (2014). The experiment was repeated three times, with five or six replicates in each.

Six weeks after inoculation, plants were harvested, their height was registered and the leaf area of turgid leaves measured with a leaf-area-meter (LI-COR, LI-3100). The area of non-turgid leaves was estimated from their dry weight after determining the relationship between the leaf area and dry weight of turgid leaves. Roots, stems and leaves were dried at 65 °C for 72 h, to obtain their dry weight. Disease severity was estimated as the percentage of leaves with wilt symptoms at harvest.

Stem hydraulic conductivity (K_h; mg s^{-1} MPa^{-1} m) was determined by inducing water flow through excised sections under a small pressure gradient (Sperry et al. 1988), following the procedures described in Romero et al. (2014). The second internode, between the second and the third true leaf, was used. Stem-specific conductivity (K_s) was calculated as K_h divided by the stem cross section at the second internode; it allows a comparison of the efficiency of stems to conduct water. Leaf-specific conductivity (K_L) was determined as K_h divided by the total leaf area above the point of measure (the second 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).

Since the value of K_s depends on the number of vessels per unit cross section and the fourth power of their diameter (Tyree and Zimmerman 2002), both parameters were determined on thin sliced sections (8–10 μm) of the fourth internode of the stems. These measurements were taken on a different internode than K_h because both studies are destructive and under the assumption that responses along the stem are correlated. Also, the mean hydraulic radius of xylem vessels was calculated by dividing $\sum r^5$ by $\sum r^4$, where r is the radius of xylem vessels; this parameter is correlated to K_s (Pockman and Sperry 2000). Only vessels with a diameter (mean of two perpendicular measurements) of 6 µm or larger were considered, since the contribution to the stem conductivity of smaller vessels is negligible. Stem portions were fixed in FAA (absolute ethyl alcohol, glacial acetic acid, formaldehyde, and distilled water, in a 50:5:10:35 ratio *v*/v) immediately after harvest until anatomical and hydraulic measurements took place. Then, stem section slices were cut 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).

Another experiment was carried out to evaluate the possibility of detection of infected symptomless plants using two leaf spectral reflectance indices: NDWI and NDVI. Plant and bacteria growth conditions were similar to those described above. Plants were inoculated on the third leaf and the spectral measurements were registered on the fifth (fully developed at inoculation time) and seventh leaf (still enlarging at inoculation time), 1 week after inoculation. Spectral measurements were carried out with a portable spectroradiometer (FieldSpec Pro FR, ASD Inc., Boulder, Colorado, USA) with plant probe and leaf-clip accessories. The experiment was repeated twice with five replicates per treatment each time.

NDWI (Gao 1996) was defined as $(R_{860} - R_{1240}) / (R_{860} + R_{1240})$, where R_{860} (reflectance value at 860 nm) is used as a reference and R_{1240} (reflectance at 1240 nm) is related to the water content of a leaf because an absorption band of liquid water is centered there. NDVI (Gamon et al. 1995) is based on chlorophyll absorption features, and is thus directly related to the photosynthetic capacity. NDVI was formulated by Sims and Gamon (2002) as $NDVI = (R_{800} - R_{680}) / (R_{800} + R_{680})$, where R_{800} (reflectance at 800 nm) represents the high reflectance values related with leaf structure and R_{680} (reflectance at 680 nm) is situated at the strong chlorophyll absorption band.

Analysis of variance (ANOVA) and regression analysis were performed using the InfoStat/Professional system version 1.1 (Estadística y diseño, Facultad de Ciencias Agrarias, Universidad Nacional de Córdoba, Argentina).

All plants inoculated with *C. michiganensis* subsp. *michiganensis* expressed symptoms of bacterial canker, while mock-inoculated plants remained healthy. Microscopic observation of xylem vessels revealed the occlusion of the lumen of bacterial canker affected plants. Similar results were obtained in all independent experiments; the results of one of them are presented herein.

C. michiganensis subsp. *michiganensis* inoculation had a negative impact on all growth variables evaluated on tomato plants. Total dry weight was consistently lower (55%) on inoculated than on mock-inoculated plants (Table 1). The height of the plants and their leaf area were greatly reduced (21 and 52% respectively) by inoculation with *C. michiganensis* subsp. *michiganensis* (Table 1), especially the area of the leaves that remained turgid at the time of evaluation. Some leaves of inoculated plants had already wilted and died by the end of the experiment, which may have contributed to the observed decrease on leaf area. The pathogenic bacterium locates into xylem vessels (Chalupowicz et al. 2012), which provide water and nutrients to the shoots, so it was expected that the aboveground growth would be more affected than the belowground growth. Also, bacterial canker symptoms are reminiscent of those caused by droughts, where the growth of the aerial part of the plant is restrained on behalf of the roots (Lambers et al. 2008), and thus the shoot/root ratio is expected to be reduced. However, the shoot/root ratio was similar for inoculated and mock-inoculated plants. In a previous study this ratio was even increased in inoculated plants (Romero et al. 2014). Those results suggest that there are differences on the effects on the shoot/root ratio between this vascular disease and a drought. Xu et al.

Table 1 Biomass, height and leaf area of tomato plants inoculated with *Clavibacter michiganensis* subsp. *michiganensis* and their non-inoculated controls

Growth variables	Non-inoculated[a]	Inoculated[a]
Dry weight (mg) of:		
Leaves (all)	1.83 ± 0.33 a	0.90 ± 0.36 b
Stems	1.38 ± 0.20 a	0.52 ± 0.22 b
Roots	0.97 ± 0.22 a	0.47 ± 0.25 a
Total	4.21 ± 0.65 a	1.90 ± 0.73 b
Shoot/root ratio (*w*/w)	3.80 ± 1.85 a	4.81 ± 2.07 a
Plant height (cm)	45.97 ± 3.10 a	36.36 ± 3.46 b
Leaf area (cm^2): Total	387.1 ± 91.3 a	187.1 ± 58.0 a
Turgid	387.1 ± 91.3 a	53,4 ± 24.0 b

[a] Values are means per plant ± standard errors of six replicates; values in a row followed by the same letter are not significantly different according to the analysis of variance ($P < 0.05$; $n = 6$)

(2012) also observed a reduction on the fresh weight of roots of inoculated tomato seedlings, although they did not present aboveground data. *C. michiganensis* subsp. *michiganensis* typically colonizes xylem vessels, yet, on later stages of infection it can also colonize the phloem of upper plant parts (Jahr et al. 2000). The disintegration of phloem elements could impair their capacity to transport assimilates to the roots, which may cause an indirect reduction on the growth or functions of the root system. The pathogen can also cause a direct negative effect when it colonizes the root tissues. In our case, bacteria were isolated from all plant parts, including the roots, of all tested plants 2 weeks after inoculation (data not shown). In fact, *C. michiganensis* subsp. *michiganensis* moves more rapidly toward roots than shoots (Xu et al. 2012).

Neither the number of xylem vessels per stem surface area nor the mean hydraulic radius of the vessels was affected by the inoculation with the pathogen (Table 2). The conductivity of the stems, K_h, was greatly reduced (96%) on diseased plants, and since the diameter of the stems where the measurements were taken was not affected by inoculation, K_s was also lower on diseased than on mock-inoculated plants (95%; Table 2). K_s was positively and significantly correlated to the mean hydraulic radius of xylem vessels on mock-inoculated, but not on inoculated plants (Fig. 1). This indicates that the impairment of xylem water flow is related to the loss of functionality of xylem vessels, while their potential capability to transport water (mean hydraulic radius) remained unaffected.

Table 2 Effect of *Clavibacter michiganensis* subsp. *michiganensis* on stem (K_h), stem-specific (K_s) and leaf-specific (K_L) conductivity, stem diameter, and number and mean hydraulic radius of xylem vessels of tomato plants

	Non-inoculated[a]	Inoculated[a]
K_h	469.12 ± 154.97 a	20.51 ± 11.47 b
K_s	16.45 ± 3.28 a	0.77 ± 0.28 b
K_L	2.71 ± 0.30 a	0.50 ± 0.33 b
Stem diameter (mm)	5.40 ± 0.39 a	4.62 ± 0.44 a
Mean hydraulic radius (μm)	23.67 ± 1,47 a	24.2 ± 1.50 a
N° of xylem vessels / stem section	195 ± 18 a	155 ± 20 a
N° of xylem vessels mm^{-2}	8.13 ± 1.11 a	8.70 ± 1.24 a

[a] Values are means ± standard errors of six replicates; values in a row followed by the same letter are not significantly different according to the analysis of variance ($P < 0.05$; $n = 6$)

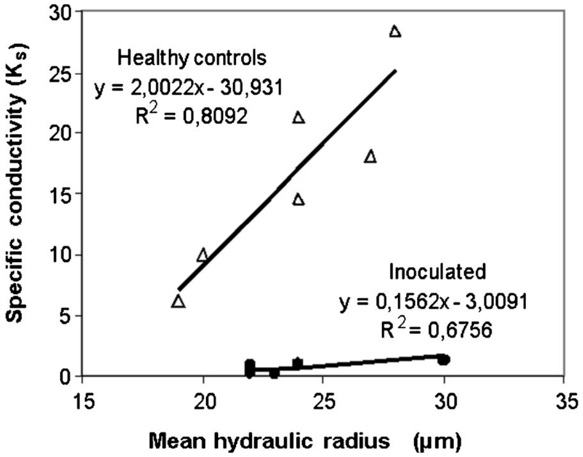

Fig. 1 Relation of stem-specific hydraulic conductivity (K_s) and mean hydraulic radius of xylem vessels for tomato plants inoculated with *Clavibacter michiganensis* subsp.*michiganensis* (black circles) and their controls (empty triangles), 6 weeks after inoculation. Equations and regression coefficients for both treatments are shown

The capacity of the stems to supply water to the leaves, K_L, was also diminished on diseased plants (81%; Table 2). Since cell expansion is very sensitive to the lack of water (Lambers et al. 2008), the observed reduction in the leaf area could be an early response to an impediment to water flow in the stems. At the same time, water supply might not have been enough to sustain the leaves already developed, as was evidenced by the reduced K_L and the number of wilted leaves per plant. Eventually the whole plant succumbed: mean disease severity was 71%.

Wilting of a particular leaf might be more influenced by the spatial distribution of the bacteria on the vascular tissue than the conductivity of the whole stem. Plugging or degradation of individual xylem elements might affect more the leaf connected with them than stem conductivity, which could explain the lack of pairwise correlations between K_h, K_s and K_L and disease severity (data not shown).

On the experiments carried out to determine the leaf spectral reflectance indices, the value of NDWI was significantly lower on inoculated than on mock-inoculated plants 1 week after inoculation for both leaf positions evaluated (Table 3). The reduction of NDWI observed for inoculated plants, with even negative values, resulted from an increase of R_{1240} (see formula above). This was a consequence of a reduced radiation absorption at 1240 nm (band of liquid water absorption), and is indicative of low foliar water contents (Gao

Table 3 Leaf spectral reflectance indices, normalized difference water index (NDWI) and normalized difference vegetation index (NDVI), for leaves 5 and 7 of tomato plants inoculated with *Clavibacter michiganensis* subsp. *michiganensis* 7 days before measurements, and their not inoculated controls

		Non-inoculated[a]	Inoculated[a]
NDWI	Leaf N° 5	0,026 ± 0.006 a	−0,161 ± 0.004 b
	Leaf N° 7	0,025 ± 0.016 a	-0,162 ± 0.004 b
NDVI	Leaf N° 5	0.755 ± 0.028 a	0.774 ± 0.006 a
	Leaf N° 7	0.788 ± 0.010 a	0.765 ± 0.021 a

[a] Values are means ± standard errors of five replicates; for each leaf and index, values in a row followed by the same letter are not significantly different according to the analysis of variance ($P < 0.05$)

1996). These results highlight the early effects of the pathogen on plant water stress. Symptoms were evident 3 weeks after inoculation and, thus, NDWI has the potential to be valuable for the detection of symptomless infected plants 2 weeks before symptoms expression. There was no difference between treatments for the NDVI index, which is consistent with other studies that conclude that it is not always a good indicator of water stress (Jones et al. 2004; Vicca et al. 2016). In our case, this would mean that the infection had no early effects on the chlorophyll absorption features or the photosynthetic capacity of the leaves.

We conclude that the leaf spectral reflectance index NDWI can be used as a rapid, non-destructive, and cost-effective method for the early diagnosis of bacterial canker. Also, we confirm that *C. michiganensis* subsp. *michiganensis* altered plant-water relations in a way that favoured plant wilting. The stem-specific conductivity (K_S) was reduced, reflecting a lower efficiency of the xylem vessels to conduct water, even though the hydraulic mean radius and the number of xylem vessels were not affected. The leaf-specific conductivity (K_L) was also reduced indicating a lower ability of the stem to supply water to the leaves which, as a consequence, wilted. In addition, aboveground growth was greatly reduced while the shoot/root ratio remained unaffected, reflecting a negative effect also on root growth, which could aggravate the water imbalance of the plants, especially under low soil water availability.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights This article does not contain any studies with human or animal subjects.

Informed consent All authors consent to this submission.

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