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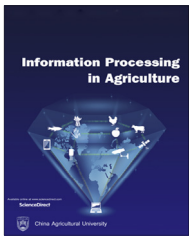

Early detection of tomato bacterial canker by reflectance indices

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ABSTRACT

Early detection of infected asymptomatic plants could prevent the dissemination of tomato bacterial canker throughout the crop and, thus, reduce associated economic losses. Reflectance spectroscopy has been proposed for the detection of diseases in plants, mainly caused by foliar fungal pathogens. In contrast, bacterial canker is a vascular disease that does not produce visible foliar symptoms until it is too late to avoid contagion to other plants. The effects of the disease on the conductivity of the xylem have an impact on the water content of the leaves; initially, a subtle effect that cannot be assessed with the naked eye but can be evaluated by reflectance spectroscopy. The objective of this work was to identify indices, based on the reflectance spectral signature of the plants, for the detection of tomato plants affected by bacterial canker prior to the appearance of symptoms. To verify that the proposed methodology is applicable to a broad range of tomato genotypes, two experiments were carried out with different tomato cultivars grown in pots and another on the ground in a greenhouse. Plants were inoculated with the pathogenic bacteria and the reflectance spectrum of the apical leaflet of two leaves per plant were obtained before symptoms expression; several spectral indices were calculated from the reflectance spectra. Three indices, of the shortwave-infrared zone, allowed the detection of bacterial canker inoculated plants in a fast and non-destructive way, up to one week before symptoms were visible: Normalized Difference Water Index (NDWI), Simple Ratio of Water Index (SRWI) and Water Index 1 180 (WI_{1180}). Our research demonstrates, for the first time, the usefulness of spectral indices sensitive to water content for the identification of diseased plants. These indices arise as a promising tool for the early detection of vascular plant diseases, that could be integrated into sustainable management plans.

1. Introduction

Bacterial canker is one of the most damaging diseases of tomato (*Solanum lycopersicum* L.) worldwide; this vascular disease is caused by *Clavibacter michiganensis* subsp. *michiganensis* [1]. Symptoms begin with the loss of turgidity of the leaves on

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one side of the plant, and end with the death of the plant [2]. Other symptoms include cankers on stems and petioles and necrotic spots on fruits. The global distribution of the disease has been favored by the commercialization of infected/infested seeds, which are the main source of inoculum of the pathogen. This bacterium can also survive locally on plant debris [3]. Bacteria enter plant tissues through natural openings, such as stomata or hydathodes, or more commonly through wounds, especially those created during pruning and other cultural practices [1]. Once the pathogen enters the plant it colonizes and destroys the xylem vessels, reducing the hydraulic conductivity of the stem [4]. Until now, there are no tomato cultivars resistant to this pathogen [5].

Because bacterial canker causes the disintegration of vascular tissues, there is no curative treatment. On affected crops, the only possibility is to modify cultural practices to reduce secondary dispersal of the pathogen and associated economic losses [6]. For this reason, the early detection of diseased plants becomes crucial.

There are innovative techniques for the diagnosis of plant diseases, that include visible and infrared spectroscopy of reflectance, fluorescence spectroscopy, and fluorescence and hyperspectral imaging (see the reviews [7;8] for more details). Image processing is an innovative technique that has also allowed the identification of foliar diseases in recent years [9,10]. All these analytical techniques based on image analysis and spectroscopies present many advantages, mainly: rapidness, effectiveness, accuracy, and objectiveness. Moreover, they are non-destructive, do not need sample treatment, and are able to assess the whole area of the product despite the presence of uneven features [11].

Plant reflectance spectroscopy can be measured with portable equipment like radiometers, spectroradiometers or even imaging sensors. The spectral signature of vegetation is characterized by low reflectance values in the visible zone of the electromagnetic (EM) spectrum due to strong light absorption by the photosynthetic pigments [12]. Chlorophyll *a* and b have maxima of absorption in the blue (400 ~ 500 nm) and the red (around 670 nm) zones; carotenoids (i.e. xanthophylls and carotenes together) also absorb in the blue region. Any disease that causes destruction of pigments, chlorosis or even tissue necrosis could produce reflectance changes in the visible region. In the near-infrared (near-IR) zone, between 700 ~ 1 000 nm, the reflectance values are high. This zone is controlled by the anatomical structure of the leaves [13]. Usually, there is a direct relationship between the intercellular air content and the values of reflectance [14,15]. So, if a pathogen produces any change in the cellular structure of the leaves, i.e. cell lysis, or plasmolysis due to water stress, it would be reflected in this zone of the EM spectrum. Finally, in the shortwave-infrared (SWIR), between 1 000 ~ 2 500 nm, reflectance values are closely related to the water content of the leaves. Water absorption maxima, due to OH bonds of water, are located approximately at 760, 970, 1 450, 1 950 and 2 250 nm, where minima of reflectance appear [12,16]. The major compounds of the cell wall, lignin and cellulose, and compounds of the cytoplasm, such as proteins and sugars, also absorb in the SWIR [17].

Spectroscopy of reflectance was successfully used in different plant species for the detection of biotic stresses that produce foliar symptoms, caused mostly by fungi, such as scab in apple trees [18] and Cercospora leaf spot, powdery mildew and rust in sugar beet [19]. It was also employed in tomatoes for the assessment of late blight [20,21], insect damage by a leaf miner [22] and a combination of foliar diseases (late blight, target spot and bacterial spot) [23]. In two recent works, reflectance spectroscopy even allowed to distinguish the wilt produced by the vascular fungus *Fusarium oxysporum* from that caused by drought in tomato [24,25]. Recently we communicated a preliminary study on bacterial canker [26].

The information contained in reflectance spectra can be synthesized by means of indices, which combine the reflectance signal at specific wavelengths depending on the information that is required. For example, to evaluate pigment contents, the reflectance values of the visible region are combined algebraically, while if the intention is to evaluate the water content of the plants, combinations of the near-IR or SWIR zone are used. This strategy allows the amplification of the spectral differences, providing additional details for stress detection [27], while creating synthetic variables that make it simple to compare tissues components between plants or crops [28–31]. Two recent studies showed the sensitivity of indices related to water stress and those related with chlorophyll content and photosynthesis, for the assessment of abiotic stresses in tomato plants [32,33]. The advantage of using indices is reflected by the fact that more than 150 vegetation indices have been developed during the last three decades and many of them are used to make management decisions such as when to fertilize or irrigate crops [31]. However, most of the studies on disease diagnosis do not use indices, for example those referred to the vascular disease induced by *F. oxysporum* on tomato [24,25].

Although reflectance spectroscopy has been evaluated as a tool for the diagnosis of several fungal foliar diseases, information on vascular diseases is scarce or even not available for those of bacterial origin. Also, the potential of the use of spectral indices for disease detection during the incubation period, before symptoms expression, has not been fully exploited yet [8]. Trying to fill these information gaps, the main objective of this work was to assess the use of spectral indices for the early detection of a vascular disease, bacterial canker, in asymptomatic tomato plants. We hypothesized that the reduction in the water content of the leaves, imposed by *C. michiganensis* subsp. *michiganensis* negative effect on the xylem conductivity, could be detected by spectral indices developed specifically to detect water stress situations, even before wilting symptoms become apparent. Since water stress also produces restrictions that strongly impact the photosynthetic process (like stomata closure), indices related to chlorophyll content and photosynthesis were also evaluated. The use of spectral indices for the detection of tomato bacterial canker during the incubation period in a fast, non-destructive way, would allow growers to apply control measures to prevent secondary dispersal of the pathogen, reducing economic losses.

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2. Materials and methods

2.1. Growth conditions of plants, treatments, and experimental design

The trials were conducted in two different greenhouses situated at Facultad de Agronomía, Buenos Aires University, Argentina (34°35′41″S, 58°28′41″W). Three independent assays were done to test the robustness of the proposed methodology, using three tomato cultivars widely planted by local growers, to verify that the results were independent of the tomato genotype. On two of the trials plants were grown in pots and in the third on the ground. The two pot trials were done in a greenhouse of 17 m^2 under natural light (approx. 14 h) with heating and cooling systems; the temperature was kept around 22 °C (±2 °C). In these cases, 5 weeks old seedlings of tomato cultivar ACE 55 (Asgrow Seed Co.; trial A) and hybrid El Coya (Seminis; trial B) were transplanted in 5 000 ml pots containing a mixture of soil and compost (1:1). Plants were watered daily and fertilized weekly with nitrogen, phosphorus and potassium (15:15:15). The ground trial (trial C) was carried out in a greenhouse of 200 m^2, to test if the results obtained in pots were replicated in a situation that resembles a real situation-commercial crop. For this assay, seedlings of tomato hybrid Houdini (Florensa) were planted on two furrows of 0.90 m by 12 m in length, with two rows per furrow and a density of 1.6 plants m^{-1} linear. Plants were drip irrigated and maintained under natural light (approx. 14 h). The temperature was kept around 25 °C (±5 °C).

Treatments consisted on the inoculation of half of the plants of each trial with *C. michiganensis* subsp. *michiganensis*; the rest of the plants were mock inoculated with water, to be used as controls. All trials had a completely randomized block design. In trials A and C there were five plants for each treatment (control, inoculated) while in trial B there were eight plants per treatment.

2.2. Bacterial strain and inoculation procedure

Plants were inoculated with *C. michiganensis* subsp. *michiganensis* strain Cm9. This strain was isolated from a diseased tomato plant from Florencio Varela, Buenos Aires province, Argentina, and stored at −80 °C [34]. Cm9 was cultured on modified YDC medium [35] at 28 °C for 48 h. Bacterial cells were suspended in sterile distilled water and its concentration was adjusted to an OD_{600} of 0.3 with a spectrophotometer (*c.* 10^8 CFU mL^{-1}). The suspension was diluted (10^7 CFU mL^{-1}) to be used as inoculum; a sample was plated to verify its concentration. Tomato plants were inoculated when they had 5 ~ 6 leaves, by placing a drop of 20 μl of the inoculum in the axil of the third leaf, counting from the soil, and pricking the stem with a needle through the suspension. Control plants were treated in the same way, using water instead of the bacterial suspension.

2.3. Spectral reflectance signatures

Reflectance measurements were made 3 days after inoculation (DAI) for trial A, 7 DAI for trial B, and 12 DAI for trial C. To determine if the distance from the inoculation point was important for the detection of infected plants, measurements were done on the fifth and seventh leaf (closer and farther to the inoculation site, respectively) of each plant. Reflectance spectra of the apical leaflet of the leaves were registered with a portable hyperspectral spectroradiometer FieldSpec Pro FR ASD (Analytical Spectral Devices Inc., Boulder, USA) using the plant probe and leaf-clip accessories. The fiberoptic contact probe has an external 50 W halogen lamp which provides lighting for measurements, it has a space at the top to insert the optical fiber at 45° which reduces the field of view to 23 mm. The leaf-clip accessory has a double-sided rotatable plate, a white reference (WR) plate for calibration and a black reference for leaf reflectance measurements. The FieldSpec® RS2 software of the spectroradiometer ASD allowed to record the reflectance values at each wavelength (λ) according to Equation (1).

$$R_\lambda = \frac{\text{leaflet Rad}_\lambda}{\text{WR Rad}_\lambda} \quad (1)$$

where R_λ is the reflectance value and Rad_λ is the radiance value of the leaflet or the white reference, at the wavelength λ.

To make each measurement, the white reference was recorded using the WR plate of the leaf-clip. Then, the background of the leaf-clip was changed to its black reference, where the leaflet was rested, the adaxial face of the leaflet was clamped with the plant probe to prevent the entry of ambient light and finally, the reflectance spectrum of the adaxial face of the leaflet was recorded. The spectral range of this equipment varied between 350 nm and 2 500 nm and its spectral resolution was 3 nm in the range 350 ~ 1 000 nm with a sampling interval of 1.4 nm. In the zone between 1 000 to 2 500 nm of the electromagnetic spectrum the spectral resolution was 10 nm with 2 nm of sampling interval. Fig. 1 shows a diagram of the temporal sequence of the trials and a photograph of how the measurements were made. Fig. 2 clarifies the sequence of data collection and analysis; this figure shows the flowchart of data processing. The indices calculated in this work from the reflectance spectra of the leaflets are presented in Table 1. These indices can be separated in two categories, those that analyze the pigment contents of the leaves: NDVI, PRI, NRI and SIPI, and those that assess the water status and structure of leaves: MSI, SRWI, NDWI, WI_{970}, WI_{1180} and WI_{1450}. We have included WABI in the second group (Table 1) although it considers reflectance values of both groups (531 nm and 1 500 nm). Thus, WABI integrates two complementary zones of the electromagnetic spectrum, the visible zone related with photosynthetic pigments and the shortwave-infrared related with the water status of plants.

2.4. Reflectance spectra simulations with the Prospect model

To confirm the effects of the disease on the reflectance spectra, simulations were carried out with the Prospect model. The software used, Visual Prospect, was downloaded from an education site of the Universidad of Alcalá [47]. Prospect

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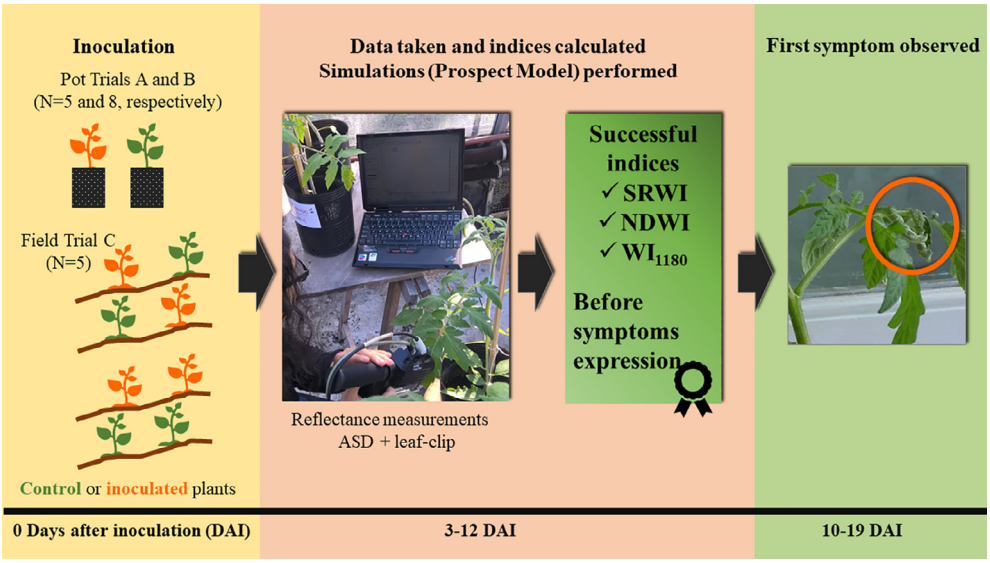

Fig. 1 – Temporal sequence of the experiments. In the three trials, half of the plants were inoculated, and the other half were used as controls (yellow box). Then, the reflectance signatures were measured, and the spectral indices calculated (pink box). Three indices detected the presence of the bacteria prior to the appearance of the first visual symptoms (green boxes).

is a radiative transfer model [48] which allows to simulate leaf reflectance and transmittance spectra between 400 and 2 500 nm based on four biochemical parameters. The inputs of the model were obtained from LOPEX93 database [49]. This database compiles the information of 45 different species, one of which is tomato. It contains spectral and physiological information of five different tomato leaves. In the simulations, inputs for the control treatment were calculated as the average values of these five leaves: leaf structure parameter (N) = 1.6, chlorophyll content (Cab) = 52 μg/cm^2, water content (Cw) = 0.018 g/cm^2 and dry content (Cm) = 0.002 g/c m^2. The reflectance spectrum of a water stressed leaf was recreated by changing the value of the water content to 50% of the control, Cw = 0.009; the rest of the parameters remained unchanged. To simulate a water stressed leaf which also had its internal structure altered, the simulation was performed with Cw = 0.009 and with a value of N = 2, higher than the control; the other two parameters remained unchanged (Table 2), According to [48], the parameter N takes values between 1.5 ~ 2.5 in dicotyledonous leaves; it is greater than 2.5 in senescent leaves with a disorganized internal structure. In this case, N = 1.6 for control leaf (average value taken from LOPEX93 database) and its value was increased to 2 to simulate leaves where the internal structure is affected by dehydration.

2.5. *Data analysis*

The effect of the treatments on the spectral indices of the leaves was analyzed by mixed general linear models using the InfoStat system version 1.1 through the R interphase [50]. *C. michiganensis* subsp. *michiganensis* moves through the xylem vessels of the plant [51]. A priori, it was unknown if the effects of the bacteria would be seen before in the leaves closer to the inoculation point (third leaf from the ground) or not. For this reason, measurements were made on the fifth and seventh leaf. The models were then run with two fixed factors (inoculation and position of the leaf assessed in each plant) and each individual tomato plant was considered as a random factor; mean differences were determined by Fishers LSD test at a significance level of $p \leq 0.05$.

Prior to the analysis, the assumption of normality of the residues was evaluated through the Shapiro-Wilks test and the homogeneity of variances by plotting residuals versus the values predicted by the proposed model. When appropriate, the heteroscedasticity was modelled with varIdent variance function. The selected models were those that presented the lowest AIC value (Akaike Information Criterion).

3. Results and discussion

There was no interaction between the effects of the inoculation and the position of the evaluated leaf (fifth or seventh) on the indices calculated from the reflectance spectra of the leaves ($p \geq 0.05$), in any of the three assays. For this reason, the data of both leaves were combined in subsequent analyzes. This result suggests that the position of the leaf, in relation to the point of inoculation, does not need to be considered at the time of measurements, which is important because in natural infections the site of inoculation is unknown. Measurements should be done in leaves farther apart in future experiments, to verify the consistency of the observation.

The average leaf spectral signature differed between inoculated and not inoculated plants. Fig. 3 shows the spectra of the leaves of trial B; similar spectra were obtained in the other two assays (data not shown). Regions of the EM spectrum and the major factors influencing in each region are indicated.

C. michiganensis subsp. *michiganensis* colonizes the xylem vessels of tomato plants [2,6], causing a decrease in the hydraulic conductivity of the stems [4]. The symptoms that

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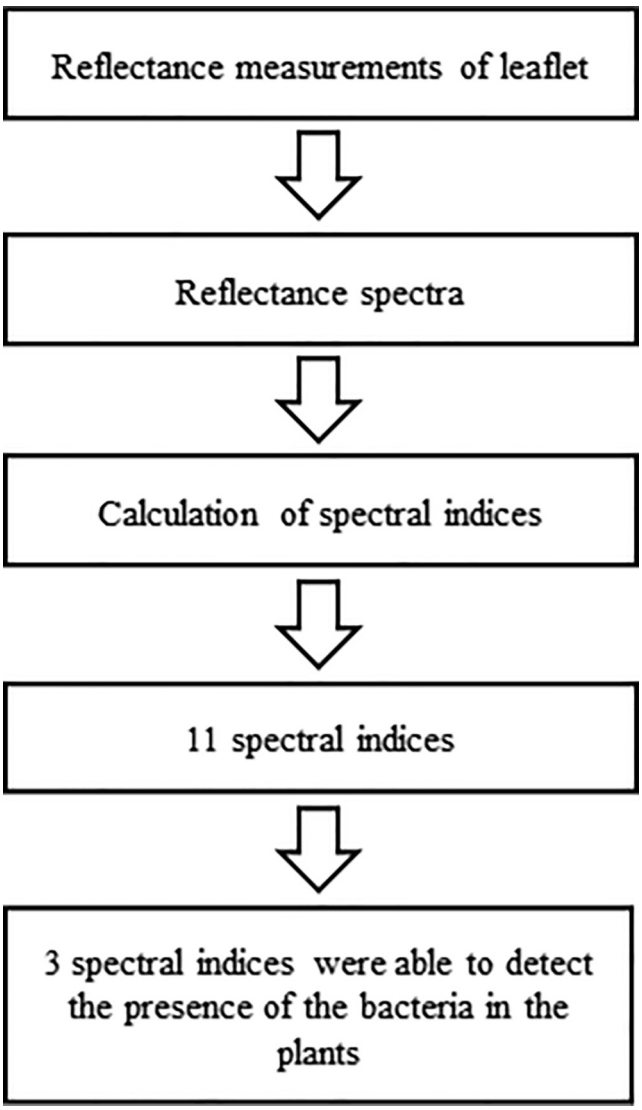

Fig. 2 – Flowchart of data processing.

follow resemble other forms of water stress: leaves first loss turgidity, then wilt and finally the whole plant succumbs [1]. As happens with plants under water stress [27,41,45,52], plants inoculated with *C. michiganensis* subsp. *michiganensis* had higher values of leaf reflectance than control plants. Simulations performed with the Prospect model showed that the changes in the reflectance signature in the infrared zone (near and middle) are associated with the water content and with the structure of the leaf. In Fig. 4, the simulated water stressed leaf (magenta line), in which the water content (Cw) was reduced by half, had an increase in the reflectance values beginning at 950 nm, with respect to the control (green line). In the leaf in which not only the water content (Cw) was reduced but also a degradation of its internal structure (N) was simulated (orange line), the reflectance values increased with lower wavelength values, beginning at 740 nm. The latter simulation produced a reflectance spectrum with a very similar shape to that obtained in the leaves of plants inoculated with the bacterium *C. michiganensis* subsp. *michiganensis* (compare Fig. 3 and Fig. 4). The results of the simulations would further suggest that the disease modified both the water content and the structure of the leaf.

On the contrary, in the work carried out on tomato plants infected with *F. oxysporum*, a vascular pathogenic fungus, a strong increase in the reflectance values in the visible region (510 ~ 520 and 650 ~ 670 nm) and a light decrease in the reflectance values in the near-IR (750 ~ 1 000 nm) were found during the incubation period of the disease [25]. According to the authors, these small differences in the near-IR zone, between the infected plants and their controls, could indicate a minor disturbance in the water status of the infected leaves. Although they also investigated a vascular disease in tomato, their results with a fungus were very different from those obtained in this work with a pathogenic bacterium.

The indices that assess foliar pigments content, NDVI, PRI, NRI and SIPI, did not show significant differences between control and inoculated plants in the performed assays ($p \geq 0.05$), except for PRI in trial B ($F_{1,14} = 8.12$, $p = 0.012\ 9$). Although NDVI and PRI were previously used in literature to detect early water stress in plants, they were not created for this purpose. Furthermore, several authors [28,32–33,53–54] reported that NDVI is less sensitive to the water content of plants than those indices that use water absorption bands in their calculation. Regarding PRI, although in occasions it was considered effective for the evaluation of the plant water status by other researchers [55,56] and in one case in this study, in [57] no correlation was found between PRI and the relative water content in the rooting medium where plants grew. Therefore, the usefulness of PRI to assess water stress has not yet been well established.

On the contrary, the indices related to the structure and water content of the leaves were effective for the early detection of the effects of the pathogenic bacteria, in varying degrees depending on the index and the experimental conditions. Three of the calculated indices, NDWI, SRWI and WI_{1180}, were able to detect the infected plants before symptoms expression in the three assays (Fig. 5; Table 3). In all the cases, the values of the indices of the infected plants were lower than those of control plants. This is because the reflectance values of the water-sensitive bands, 1 240 and 1 180 nm, are located in the denominator of the formula of SRWI and WI_{1180} (see Table 1). In the case of NDWI, the reflectance value of the band sensitive to water content (1 240 nm) corresponds to the subtrahend of the normalized difference (Table 1). If the values of subtrahend are higher than the values of reflectance in the reference band, the numerator will have a negative value (and therefore a negative value of the index), as occurred in assays B and C.

The wavelengths considered for the indices NDWI, SRWI (860 and 1 240 nm) and WI_{1180} (900 and 1 180 nm) are shadowed in Fig. 3. These three indices use wavelengths located in the same spectral range: they combine a reference band in the near-IR zone and a band sensitive to the water content of the SWIR. This combination of bands maximizes the differences between control and inoculated plants. Other authors have also found that the spectral region of the SWIR used by theses indices is slightly more advantageous than other regions in which leaf (or canopy) water absorption is usually evaluated, i.e. 950 ~ 970 nm and 1520 ~ 1540 nm [29,58]. The strongest absorption band of water is located at 2 950 nm

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Table 1 – Description of the indices used in this study separated into two groups: those related with the content of pigments in the leaves and those related to foliar structure and water content.

Vegetation index	Equation	Brief description	Reference
Indices related to pigment contents			
Normalized Difference Vegetation Index (NDVI)	$(R_{800} - R_{680})/(R_{800} + R_{680})$	It integrates two characteristics of photosynthetic tissues: low reflectance values in the red zone (680 nm) of the EM spectrum due to the strong absorption of light by chlorophyll and high reflectance values in the NIR (800 nm) due to light scattering caused by the mesophyll structure of the leaves.	[36]#1,[37]#2
Photochemical reflectance index (PRI)	$(R_{531} - R_{570})/(R_{531} + R_{570})$	It allows estimating fast changes (daily scale) in the relative levels of the pigments of the xanthophyll cycle (photo-protection mechanism) from changes in the spectral signal around 531 nm. When measurements are made at higher spatial or temporal scales, PRI is strongly influenced by the chlorophyll/carotenoid ratio. R_{570} is the reference band.	[38]
Nitrogen Reflectance Index (NRI)	$(R_{570} - R_{670})/(R_{570} + R_{670})$	It estimates nitrogen content of plants, due to its association with the chlorophyll *a* content. NRI compares R_{670} (chlorophyll absorption) with R_{570} (reference band).	[39]
Structure-Insensitive Pigment Index (SIPI)	$(R_{800} - R_{455})/(R_{800} + R_{680})$	This index estimates the proportion of carotenoids with respect to chlorophyll *a*. Values greater than 1 indicate higher absorption of carotenoids (445 nm) than of chlorophyll (680 nm). Values lesser than 1 indicate the opposite.	[40]
Indices related to foliar water status and foliar structure			
Normalized Difference Water Index (NDWI)	$(R_{860}–R_{1240})/(R_{860} + R_{1240})$	The index shows a linear relationship with the water content of the leaves. It establishes a relationship between a band affected by changes in leaf water content (R_{1240}) and a reference band (R_{860}). The value of the index drops with lower water contents.	[41]
Moisture Stress Index (MSI)	R_{1600}/R_{820}	MSI is sensitive to the status hydric of leaves. If the leaf water content increases, the absorption of light at 1 600 nm also does so (R_{1600} decrease). Absorption at 820 nm is little affected by this type of change, it acts like a reference. There is an inverse relationship between the values of the index and the leaf water content, when the water content decreases the index values increase.	[42]
Simple Ratio of Water Index (SRWI)	R_{860}/R_{1240}	It is sensitive to the status hydric of leaves. If the leaf water content increases, the absorption of light at 1 240 nm also does so (R_{1240} decrease). Absorption at 860 nm is little affected by this type of change, it acts like a reference. When the water content drops the index values too.	[43]
Water Index 970 (WI_{970}) Water Index 1180 (WI_{1180}) Water Index 1450 (WI_{1450})	R_{900}/R_{970} R_{900}/R_{1180} R_{900}/R_{1450}	These three indices have the same logic. In all of them, R_{900} is the reference band while R_{970}, R_{1180} and R_{1450} are bands sensitive to the status hydric of leaves. WI_{970} was created by Peñuelas and colleagues [44] whilst WI_{1180} and WI_{1450} were proposed by Sims and Gamon [29] considering the proposal of the first one. A decrease in the indices is expected while the water content decreases	[44,29]
Water Balance Index (WABI)	$(R_{1500}–R_{531})/(R_{1500} + R_{531})$	WABI uses a band related to thermal deactivation (R_{531}), the same used by the PRI, with a band related to the water content of the leaves (R_{1500}). Due to the way in which both bands are combined, when foliar water stress is greater, the index has higher values.	[45]

#1 First bibliographic citation of this index, #2first use of NDVI as a descriptor of vegetation [46].

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Table 2 – Biochemical parameters used in the simulations performed with the Prospect model.

Biochemical parameters of Prospect model	Control leaf	Water-stressed leaf	Water-stressed + degraded internal structure leaf
Leaf structure, N	1.6	1.6	**2**[a]
Chlorophyll content, C_{ab} (μg/cm^2)	52	52	52
Water content, C_w (g/cm^2)	0.018	**0.009**[a]	**0.009**[a]
Dry content, C_m (g/cm^2)	0.002	0.002	0.002

a Notice that this parameter value changed with respect to control leaf.

and becomes progressively weaker at shorter wavelengths [59]. Several authors argue that it is preferable to use radiation that is more weakly absorbed by water molecules, such as 970 nm [44,59] or 1 240 nm [29,58], than those bands of the shortwave-infrared. The former radiation penetrates deeply into the leaf (or canopy) and therefore it is better to produce a reflectance signal more closely related with the actual water content. Stronger absorption bands of water (i.e. 1 450, 1 900 or 2 250 nm) often produce worse correlations with water content of samples [29,44,59].

Other indices commonly used to assess the water content of the leaves produced inconsistent results. The indices MSI and WABI detected symptomless infected plants only in the assays carried out in pots, trials A and B, but not in the assay carried out on the soil, trial C. For both indices, the leaves of inoculated plants resulted in higher values than of control plants. In MSI the sensitive band is placed in the numerator (Table 1); as the water content of the leaves decreases, the reflectance values at 1 600 nm increase, resulting in a higher value of the index. Regarding WABI, the normalized difference between the wavelengths 1 500 and 531 nm produced a similar effect, higher values of WABI when reflectance values increase due to water stress. As already mentioned, it should be taken into account that stronger absorption bands of water (as 1 500 and 1 600 nm) often produce worse correlations with water content of leaves [29,44,59].

The indices WI_{970} and WI_{1450} were only able to detect infected plants in trial B ($F_{1,8}$ = 7.51, p = 0.025 4 and $F_{1,8}$ = 8.98, p = 0.017 2, respectively) (data not shown). As shown in Table 1, mathematically both indices are the ratio between a reference band (900 nm) and a band sensitive to the water content of the leaves (970 nm or 1 450 nm). According to Fig. 3, in the case of WI_{970} it seems that the proximity between the reference band and the sensitive band makes it an index with low sensitivity to variations in the water content of the leaves. However, in two recent works, the WI_{970} index was successfully used to detect water stress of abiotic origin in tomato plants [32,33], which emphasizes the differences in the sensitivity of the indices depending on the origin, biotic or abiotic, of the water stress. The differences in the reflectance values at 1 450 nm between leaflets of infected and control plants were not large enough compared to the differences observed at 900 nm (reference band), see Fig. 3.

The first visible symptoms of the disease were observed on inoculated plants at 10 and 19 DAI in pot trials A and B, respectively. That was seven and 12 days after spectral

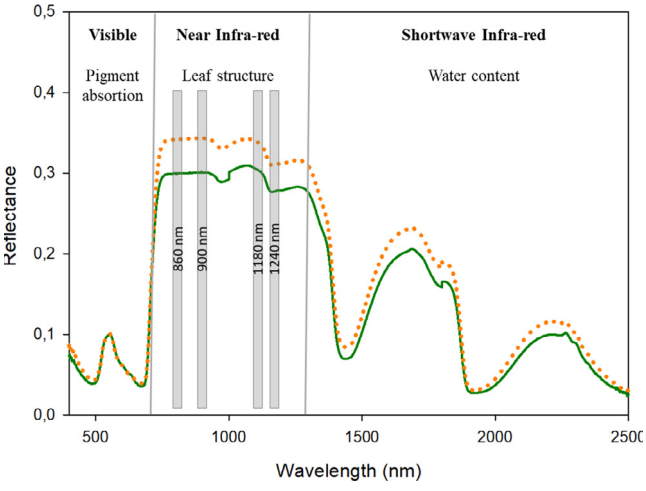

Fig. 3 – Reflectance spectra of leaves of tomato inoculated with *Clavibacter michiganensis* subsp. *michiganensis* (orange dotted line) and control plants (green line), one week after inoculation, trial B. Regions of the EM spectrum and the major factors influencing in each region are indicated. The wavelengths considered for the indices NDWI, SRWI (860 and 1 240 nm) and WI_{1180} (900 and 1 180 nm) are shadowed.

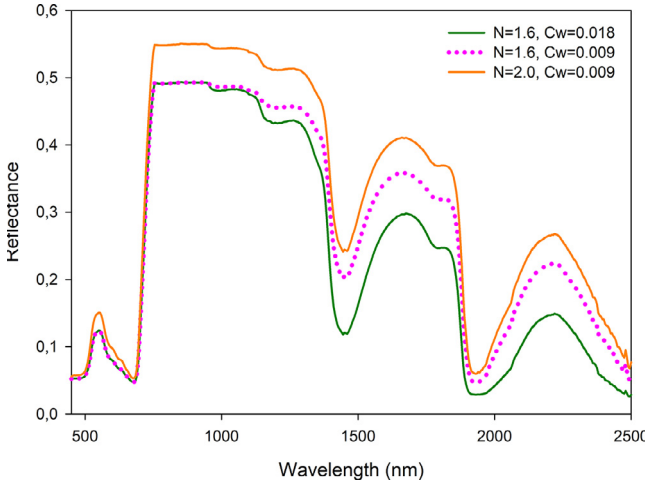

Fig. 4 – Simulated reflectance of tomato leaves with Prospect model. Control leaf (green line), water-stressed leaf (magenta dotted line, water content (Cw) corresponds to half of the control leaf) and water-stressed leaf with a degraded internal structure (orange line, Cw corresponds to half of the control leaf and the value of N increases respect to the control).

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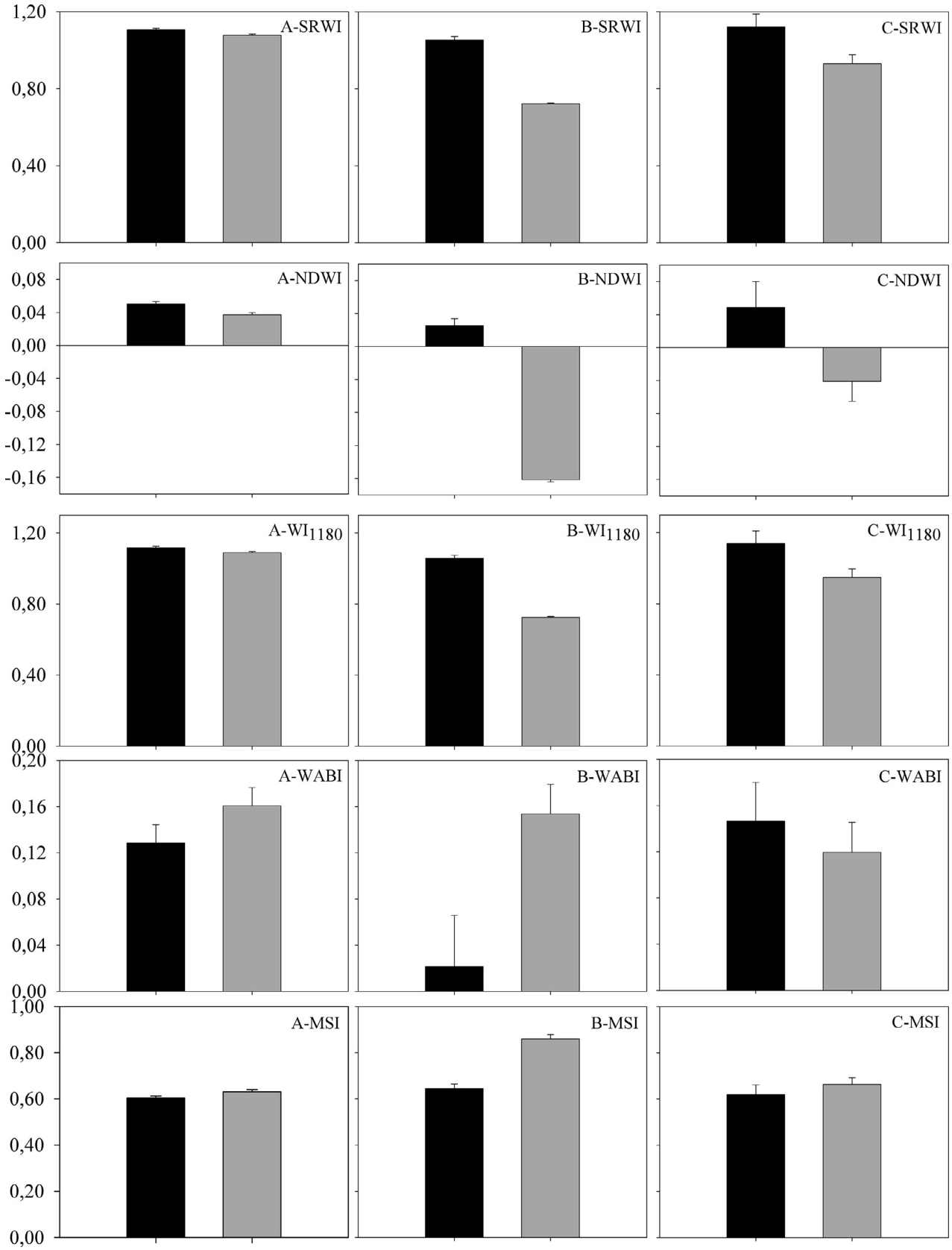

Fig. 5 – Values of the indices SRWI, NDWI, WI_{1180}, WABI and MSI of control (black bars) and inoculated (gray bars) plants. The error bars correspond to the standard error (trial A, n = 16, trial B, n = 10, trial C, n = 10). The letters A, B and C correspond to the trials A, B and C, respectively. The differences were significant for all the indices except for MSI and WABI of trial C (see Table 3).

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Table 3 – Variance ratio (F) and p-values of the indices shown in Fig. 4.

		SRWI	NDWI	WI_{1180}	WABI	MSI
Trial A	$F_{1,14}$ (p)	6.69 (0.021 5)	6.64 (0.022 0)	5.65 (0.032 3)	5.12 (0.040 2)	7.13 (0.018 3)
Trial B	$F_{1,8}$ (p)	411.71 (0.000 1)	409.29 (0.000 1)	386.78 (0.000 1)	23.75 (0.001 2)	99.80 (0.000 1)
Trial C	$F_{1,17}$ (p)	4.87 (0.042 2)	4.77 (0.043 3)	4.52 (0.049 4)	0.52 (0.480 6)	0.69 (0.419 9)

Mean differences were determined by Fisher´s LSD test at a significance level of $p \leq 0.05$.

Fig. 6 – Tomato plants at the beginning of trial B. On the right, an example of a leaf with first visible symptoms of the disease, a subtle loss of turgidity of a leaflet.

reflectance was measured and infected plants were successfully detected by changes in leaf reflectance in both assays, respectively. At that time, leaf wilting was very subtle and was only detected in one or two leaves per plant (see illustrative example in Fig. 6). In plants grown in soil (assay C) reflectance readings were done later (12 DAI) than on the other two assays (3 or 7 DAI), and at that time two plants presented two leaves with a flaccid leaflet. Control plants never expressed symptoms of the disease throughout the experiments.

In this work we explored the use of spectral indices, an approach that was not used in most of the investigations that evaluated the use of reflectance spectroscopy to detect diseases in tomato; instead, most researchers used the full reflectance spectrum or specific spectral regions [20–22,24–25]. One of the exceptions is the work by Lu and colleagues [23], who evaluated the feasibility to detect multiple foliar diseases of tomato in different stages of progress, by means of spectral indices. While our research was centered on the detection of a vascular disease using spectral indices, their focus was on foliar diseases detected by means of a set of spectral indices, using principal component analysis. Also, we took data from whole plants whereas they used excised leaves, three hours after removal. This procedure could explain, at least in part, the little changes detected by them between different stages of disease (from healthy leaves to those in a late stage of disease) beyond 700 nm, the zone where water content distortions are detected [23].

The spectral indices NDWI, SRWI and WI_{1180}, developed to reveal water stress situations, were able to detect the presence of infected plants in a non-destructive way as early as three days after inoculation. On the contrary, indices related to chlorophyll content and photosynthesis were not useful for this purpose; it is possible that during the first stages of this disease the physiological changes that those indices detect were not strongly affected.

The detection of infected plants several days before symptom expression makes reflectance indices NDWI, SRWI and WI_{1180} a potentially useful sensitive tool for early diagnosis of tomato bacterial canker, as part of sustainable management strategies. It would be interesting to evaluate all the indices analyzed in this work for other vascular diseases of different biotic origin on tomato, such as the wilt induced by *F. oxysporum* or bacterial wilt, caused by *Ralstonia solanacearum*. Based on the results obtained for Fusarium wilt [24] it is possible that the selected indices would be different from those useful for bacterial canker. It would be very convenient if different vascular diseases could be differentiated from each other with distinct indices. Development of fast, non-destructive techniques for the detection of asymptomatic tomato plants infected with bacterial canker and other vascular diseases would allow growers to identify and remove infected plants, preventing secondary dispersal of the pathogens, reducing economic losses.

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4. Conclusions

We have demonstrated, for the first time, that single spectral indices can be used to detect plant diseases in tomato. The analysis of reflectance indices was effective to detect the presence of *Clavibacter michiganensis* subsp. *michiganensis* infected plants as early as three days after inoculation, and a week before the first symptoms were visible. This gives time to isolate the diseased plants and avoid contagion to the rest of the crop.

The most effective indices for the detection of bacterial canker infected plants were NDWI, SRWI and WI_{1180}. These indices proved to be robust: they were able to detect the presence of the bacteria in asymptomatic plants both in pot and ground tests, in three different tomato cultivars.

MSI and WABI only detected diseased plants on the assays carried out in pots. More tests are needed to determine the usefulness of these indices for the detection of bacterial canker on plants grown in soil.

Growers are beginning to become familiar with the use of spectral indices to make management decisions. This has led to the development of simple commercial sensors that directly calculate indices like NDVI and PRI. Other sensors, designed to calculate spectral indices useful for the early detection of plant disease, could follow. In this sense, these results are a promising starting point in the search for useful tools for the sanitary management of tomato crops using reflectance indices. NDWI, SRWI and WI_{1180} allowed the detection of bacterial canker infected plants not only very early, before symptoms expression, but also in a fast, non-invasive, and non-destructive way. These characteristics favor the potential applicability of this technology in the daily work of the tomato growers.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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