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Original article

Linking physiological parameters with visible/near-infrared leaf reflectance in the incubation period of vascular wilt disease

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ABSTRACT

The photosynthetic pigments are mainly responsible for absorbing the light intended to promote photosynthesis on the chloroplast of the leaves. Different studies have related the spectral response in the leaves of plants with the biotic stress generated by pathogens. In general, maximum differences in reflectance have been found in the range of 380–750 nm between plants subjected to biotic stress and healthy plants. In this study, it was possible to characterize and relate the spectral variance in leaves of *S. lycopersicum* infected with *F. oxysporum* with this physiological variation and pathogen concentration in tomato plants during the asymptomatic period of vascular wilt. Photosynthetic parameters derived from gaseous exchange analysis in the tomato leaves correlated related with four bands in the visible range (Vis). Additionally, five specific bands also present a high correlation with the increase in the concentration of *F. oxysporum* conidia measured at the root: 448–523 nm, 624–696 nm, 740–960 nm, 973–976 nm, and 992–995 nm. These wavelengths allowed a 100% correct classification of the plants inoculated with *F. oxysporum* from the plants subjected to hydric stress and the control plants in the asymptomatic period of the disease. The spectral response to biotic and abiotic stress in the measured Vis/NIR range can be explained by the general tendency to change the concentration of chlorophyll and carotene in tomato leaves. These studies also highlight the importance of the implementation of robust multivariate analysis over the multiple univariate analysis used in the applied biological sciences and specifically in the agricultural sciences. These results demonstrate that specific wavelength responses are due to physiological changes in plants subjected to stress, and can be used in indexes and algorithms applied to the early detection of diseases in plants on different pathosystems.

1. Introduction

Fusarium oxysporum (Schltdl. 1824) is a widely accepted model organism in studies for plants pathogenicity, currently considered as a complex of plant pathogenic fungal species (Baayen et al., 2000). This species has many pathogenic strains that parasitize a large number of plants, causing diseases in specific hosts of economic importance in Colombia, such as bananas, tomatoes, and potatoes (Bosland, 1988).

This fungus is a hemibiotrophous pathogen (Chen et al., 2014). In the biotrophic phase, the fungus grows on its host tissues, with the recognition, deposition or contact, adaptation, and inoculation or penetration of the infective unit or inoculum as a preliminary stage. Subsequently, colonization occurs in a tissue recognized as a susceptible host. The time elapsed between inoculation and the moment of symptomatic expression in the host is called the incubation period, this phase causes difficulties on detection of diseases caused by pathogens, since they are asymptomatic in its hosts for a period of time without causing visual changes, being foci of infection in natural ecosystems or crops. Once typical symptoms appear, observation is the traditional way to detect them. During the asymptomatic stage (incubation period) there are several methods that are currently used for the detection of diseases in plants, such as the Enzyme-Linked Immunoassay (ELISA) and the

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Polymerase Chain Reaction (PCR) (Sankaran et al., 2010). These generally take time, resources and destroy the plant. Despite the availability of these techniques, a system of early detection of the disease can help reduce losses caused in crops and prevent a greater spread of the disease. A fast and reliable method is needed, with the sensitivity, selectivity and that does not require the destruction of samples.

Indicators of biotic and abiotic stress commonly use the spectral quality of the light absorbed, reflected and transmitted by the plant leaves. More specifically, in the past 20 years, important advances focused on the application of spectroscopy to the early detection of diseases in plants (Khaled et al., 2017). However, as a result of increased interest in remote sensing, the study of reflectance deepens more than the absorbance and transmittance in the last decades as responses to stress in plants (Gregory et al., 2001). Additionally, the spectral characteristics of the radiation reflected by the leaves can provide a frame to physiological responses on plants with different types of pathogens (Carter and Knapp, 2001).

In the latest decades, the development of new equipment and techniques in the application of reflectance spectroscopy in early detection of plant diseases also intensified in terms of searching for spectral characteristics related to physiological responses (Khaled et al., 2017). These techniques are based on measuring the amount of radiation reflected by a surface as a function of the wavelengths to produce a unique reflectance spectrum for each material, which can be used as a "fingerprint" (spectral signature) that allows to detect infected plants (Zhang et al 2003, Huang et al., 2004, Larsolle and Muhammed, 2007, Mahlein et al., 2010). Previous research has related some regions of the visible spectrum (400–750 nm) and the physiological parameters obtained from the classical measurements, similar to those obtained from gaseous exchange analysis, and that these are determined for specific absorbance-reflectances patterns for the photoactive pigments, mainly chlorophylls, and carotenoids (Sims and Gamon, 2002).

These two types of pigments are mainly responsible for absorbing the light intended to promote photosynthesis in the chloroplast: chlorophylls ("a" and "b") and carotenoids (alpha-carotene, beta-carotene and xanthophyll), with chlorophyll being the chromophore biomolecule that intervenes most directly in the absorption and conversion of light energy (Amthor, 2010). Consequently, changes in total chlorophyll foliar concentration and chlorophyll proportions "a" and "b" are indicators of physiological variation due to stress, leaf development, senescence and factors directly related to the primary production rate (Blackburn and Ferwerda, 2008). Reflectance in the leaf changes significantly due to the stress generated by pathogens at specific wavelengths in the visible range (Vis, 380–750 nm), and a general variation in the far red range (690–720 nm) provides an indication of earlier or more consistent infection than the reflectance in other regions of the electromagnetic spectrum (Carter, 1994; Carter and Knapp, 2001). However, the level at which different pathogens can produce different spectral signatures in plants and the degree to which the spectral response to a particular stress factor can vary between species, are questions yet still unresolved on many pathosystems due to specificity.

Based on this, a large number of indexes and algorithms have been developed for the non-destructive estimation of chlorophylls and carotenes, which stand on the spectral reflectance in the Vis range in a wide variety of species, plants and organs (Gitelson et al., 2002a, Gitelson et al., 2003; Merzlyak et al., 2003; Gitelson et al., 2007; Solovchenko et al., 2005). The detection and discrimination of diseases in plants use these indices and algorithms based on specific wavelengths, related to physiological changes in plants (Naidu et al., 2009; Song et al., 2011; Zhang et al., 2012; Mahlein et al., 2013). However, the mechanisms responsible for close relationships between reflectance and plant physiology at early stages of infection, even when symptoms are not visible, need more detailed research.

The objective of this study was to relate the changes in specific spectral traces present due to the invasion of *F. oxysporum* in tomato, with the invasion of the pathogen in the tissue and associated physiological changes, which allow verifying that process of pathogenesis cause specific spectral variations. Additionally, since water stress in the plant is one of the consequences of *F. oxysporum* invasion in the tissue, hydric stress and its spectral fingerprint and other parameters were compared with infected plants, in order to verify the specificity of the spectral response found with the pathogen.

2. Methodology

2.1. Biological material

The plants used in this study were maintained under semi-controlled greenhouse conditions, located at the Universidad Nacional de Colombia in Medellín (Antioquia, Colombia). The environmental conditions presented average temperatures between 18 and 24 °C, relative humidity between 60 and 70% and a photoperiod of 12 h during the time the experiments lasted. In this study, the tomato variety Ponderosa was used. This variety is susceptible to all races of *F. oxysporum* (Reis and Boiteux, 2007). *F. oxysporum* Fo5 strain isolated from Passiflora edulis (passionfruit) was used because it is highly pathogenic on tomato plants. This strain showed an incubation period of 24 dpi on tomato plants. The maintenance protocol of the plants, the process of pathogen inoculation (Ortiz and Hoyos-Carvajal, 2016), and the infectivity tests, were described extensively in the previous chapter and a publication made with preliminary results (Marín-Ortiz et al., 2018).

2.2. Physiological parameters on foliar tissue

Some important photosynthetic parameters were measured on all plants (infected plants before the appearance of disease symptoms, those subjected to hydric stress and controls): the net assimilation rate of CO_2 (A), intercellular CO_2 concentration (Ci), stomatal conductance (gs) and transpiration rate (E), using an Infrared Gas Analyzer (ADC Scientific Ltd., model LCi, UK). It was also calculated the intrinsic water use efficiency (A/gs), transpiration efficiency (A/E) and the ratio of internal (Ci) and atmospheric (Ca) CO_2 concentration (Ci/Ca). The quantitative yield of PSII (ΦPSII) and continuous fluorescence (Ft) performance were measured with a modulated fluorometer (FluorPen 100 WP) to evaluate the efficiency of photosystem II in tomato leaves subjected to different treatments as indicators of biotic stress in plants. The measurements were made in 30 plants for each treatment, with five repetitions per plant, in the second developed leaf. Samples were taken on day 0 and 12 (the asymptomatic period for plants inoculated with *F. oxysporum*) and at the end of the experiment, at 24 dpi (the symptomatic period in plants inoculated with *F. oxysporum*).

2.3. Data analysis

Five reflectance spectra of the adaxial face of the second leaf of each tomato plant were measured in the spectral range between 380 and 1000 nm with a spectral resolution of ~0.5 nm, using an Ocean Optics HR2000 spectroscope with a tungsten halogen light source HL-2000-HP (wavelength range of 360–2400 nm). A completely randomized design was carried out to compare two treat-

ments: susceptible plants inoculated with *F. oxysporum*, plants subjected to water stress at 60% field capacity; additionally, no infected plants were measured with 100% field capacity. Reflectance measurements were performed every three days after infection. Physiological parameters derived from the gas exchange and chlorophyll fluorescence analysis were performed at day 0 dpi, at day 12 dpi (when symptoms are not visible) and at day 24 dpi (with visible symptoms). Due to this design, we collected three groups of data with 150 spectra each (30 leaves per treatment), for each sampling day (450 spectra/day of sampling).

We performed a selection of the spectra and those with noise were removed, either because the spectra was deformed and/or because of a reading error. These spectra showed very different patterns. The differences were confirmed with an outlier analysis identified in a principal component analysis (PCA) without prior data treatment. The standard normal variate (SNV) transformation was chosen as one of the best pre-treatments that allow a good grouping of the plants in the treatments carried out, according to the results of the analyses carried out in previous works (data not shown).

After performing the pretreatment, we built a matrix graph of the correlation coefficients initially calculated as a measure of the relationship between the growth of *F. oxysporum* in roots and leaves (CFU/mg) and the reflectance in the spectral range measured. The evaluated treatments were then compared using only the physiological parameters derived from the gas exchange analysis and the chlorophyll fluorescence, testing the difference between the means with a one-way ANOVA with multiple samples and represented with bar graphs. Then, the determination coefficients for linear regressions plotted with the objective of relating the spectral variance in leaves of *S. lycopersicum* submitted to the three treatments evaluated, with the variation in physiological parameters measured with the Infrared Gas Analyzer. Finally, we proceeded to make the adjustment of the regression lines with the largest R^2. Linear discriminant analysis (LDA) were made to carry out supervised classifications with the photosynthetic variables, and the specific wavelengths selected above. All analyses were done with the Software R.

3. Results

Plants inoculated with *F. oxysporum* developed visual symptoms between 21 and 24 dpi under moderate environmental conditions (temperature between 18 and 24 °C and relative humidity between 60 and 70%). The leaves suffered gradual chlorosis, starting from the lower layers upwards, but without loss of turgor evident in most of the plants (Fig. 1). Plants subjected to water stress presented visual symptoms after 18 dpi (data not shown).

3.1. Colonization of F. oxysporum in tomato plants and relationship with the spectral response in leaves during the incubation period of the disease

The growth of an isolate of *F. oxysporum* (Fo5) on root and stem, in a variety of susceptible tomato plants, was evaluated during the incubation period of vascular wilt. The increase in the concentration of *F. oxysporum* conidia in the course of the experiment describes the classical "J" type growth curves for both organs measured. During the first 12 dpi, a similar tendency, coincidently, was observed in the spectral response of the control plants, even presenting a slight decrease in the reflectance in the infected plants in the range Vis/NIR measured (Fig. 2A and 2B). After this period, there is a higher growth of fungi, up to 1.5×10^3 CFU/mg on inoculated roots (Fig. 2), and in terms of reflectance, after 12 dpi there is an increase of reflectancia in the Vis/NIR region in infected plants with respect to the non-infected plants (Fig. 2C and 2D).

Disease symptoms were clearly observed in most infected plants after 24 dpi. These symptoms coincide with a marked increase in reflectance in the VIS range and a decrease in the NIR range evaluated in the infected plants (Fig. 1E).

On Fig. 3A the five spectral bands most highly correlated with the growth of *F. oxysporum* in root and leaf ($r \geq 0.8$, $p \leq 0.05$) on infected tomato plants are shown. Two of them in the visible range, 448–523 nm and 624–696 nm, and three in the near infrared (NIR) range measured, 740–960 nm, 973–976 nm, and 992–995 nm. In general, higher values were observed in the correlation coefficients and lower values for p-value on the *F. oxysporum* measurements

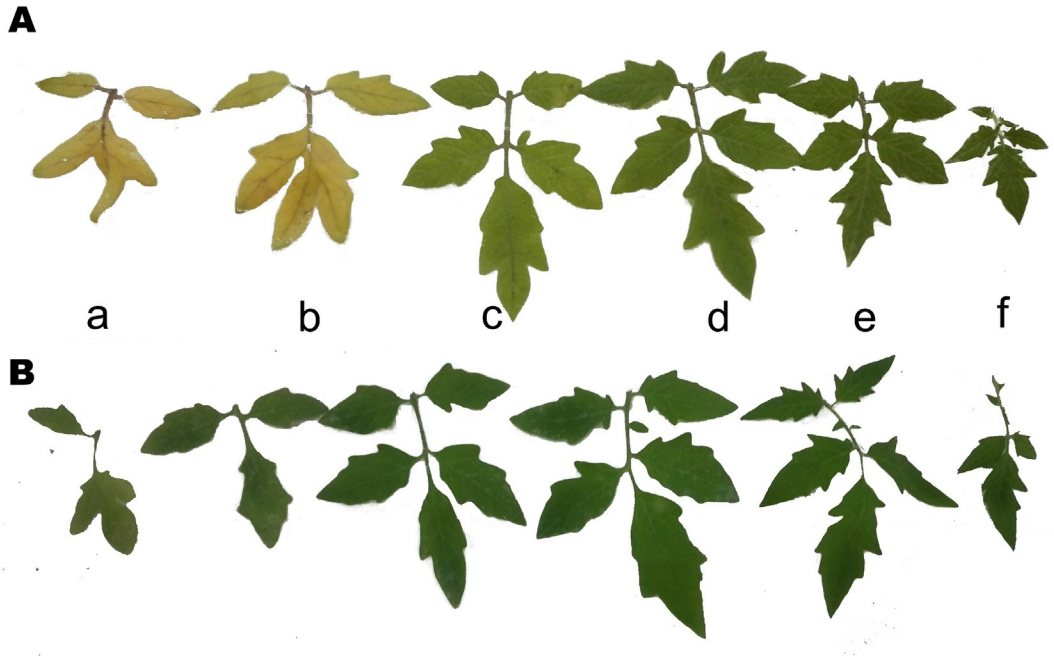

Fig. 1. Tomato plant leaves inoculated with *F. oxysporum* of 4 weeks-28 dpi (A) and healthy control (B). (a) leaf 1, (b) leaf 2, (c) leaf 3, (d) leaf 4, (e) leaf 5, (f) leaf 6.

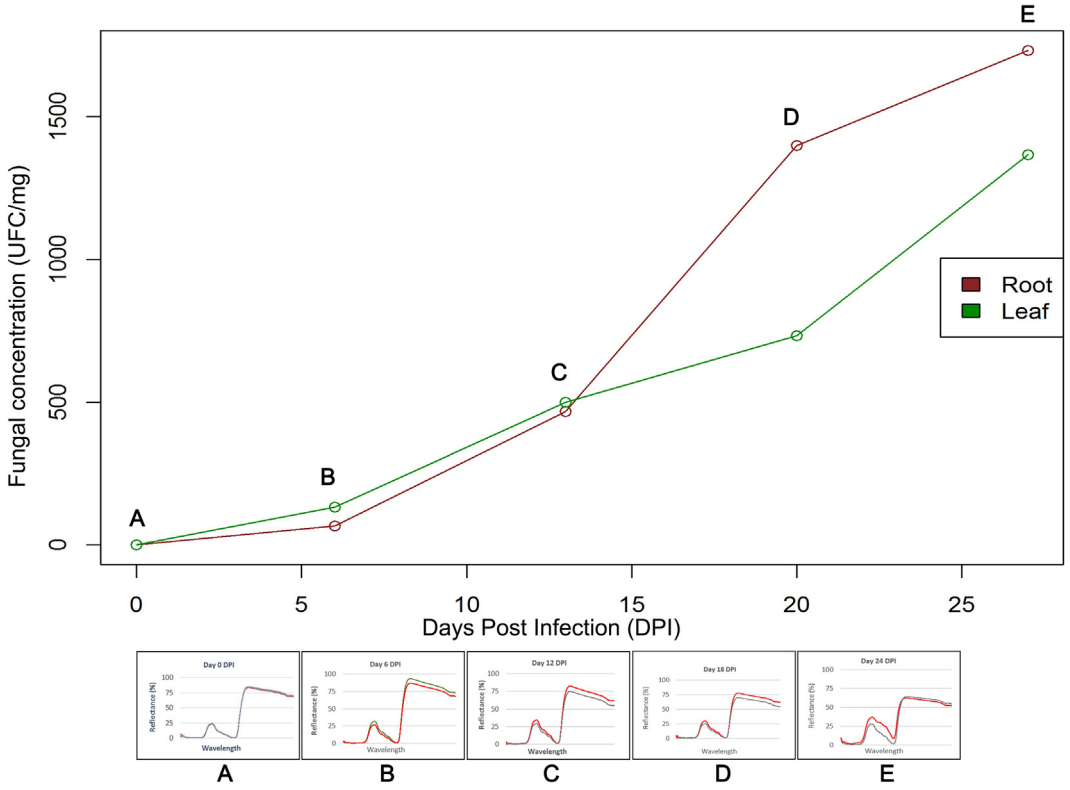

Fig. 2. Inoculum density of *F. oxysporum* (UFC) by mg in fresh plant tissue on the incubation period. A: day 0 dpi; B: day 6 dpi; C: day 12 dpi; D: day 18 dpi; E: day 24 dpi. Red series in boxes: Plants infected with *F. oxysporum*; Green series in boxes: control plants (without infection).

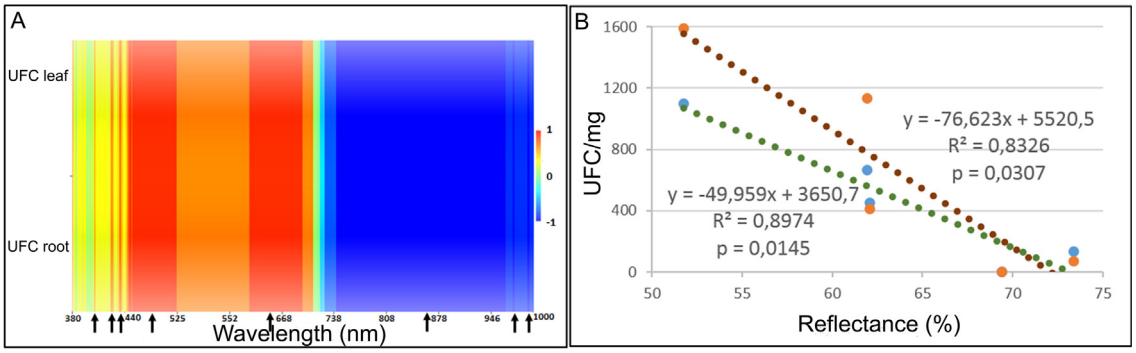

Fig. 3. Quantitative relationship between the growth of *F. oxysporum* on roots and leaves (CFU/mg) and the reflectance in tomato leaves. A) Matrix plot showing eight bands with high R2 values ; B) Linear regression for the dependent variable "CFU" measured in roots (brown) and leaves (green) as a function of reflectance at 950 nm.

made on the leaves of the plants, in Fig. 3B a specific example can be seen in the 992–995 nm band.

3.2. Physiological response of tomato plants inoculated with F. oxysporum during the incubation period and subjected to water stress.

Regarding the physiological parameters evaluated by means of classical instruments, no significant differences were detected in the plants infected with *F. oxysporum* and subjected to water stress with respect to the control plants at 12 ppi (asymptomatic period, Fig. 4C, 4E), suggesting a minor differentiation between inoculation, water stress, and healthy plants. At 24 dpi, when the symptoms of the disease were already visible, the significant variation was observed in most of the physiological parameters evaluated in the plants subjected to the two types of stress with respect to the control plants. Specifically, in the plants infected with *F. oxysporum* there was a significant reduction with respect to the control plants for A, E, A/gs, A/E and Ft (Fig. 4A, 4C, 4E, 4F and 4H), and an increase in gs, Ci and Ci/Ca (Fig. 4B, 4D and 4I). Plants subjected to water stress presented the same pattern as infected plants, but there was no significant difference in gs (Fig. 4B).

3.3. Spectral variance in S. lycopersicum infected with F. oxysporum vs physiological responses

In all groups of plants, the determination of the coefficient resulting from each linear regression between reflectance vs physiological parameters (A, E, Ci and gs) result in high values on the

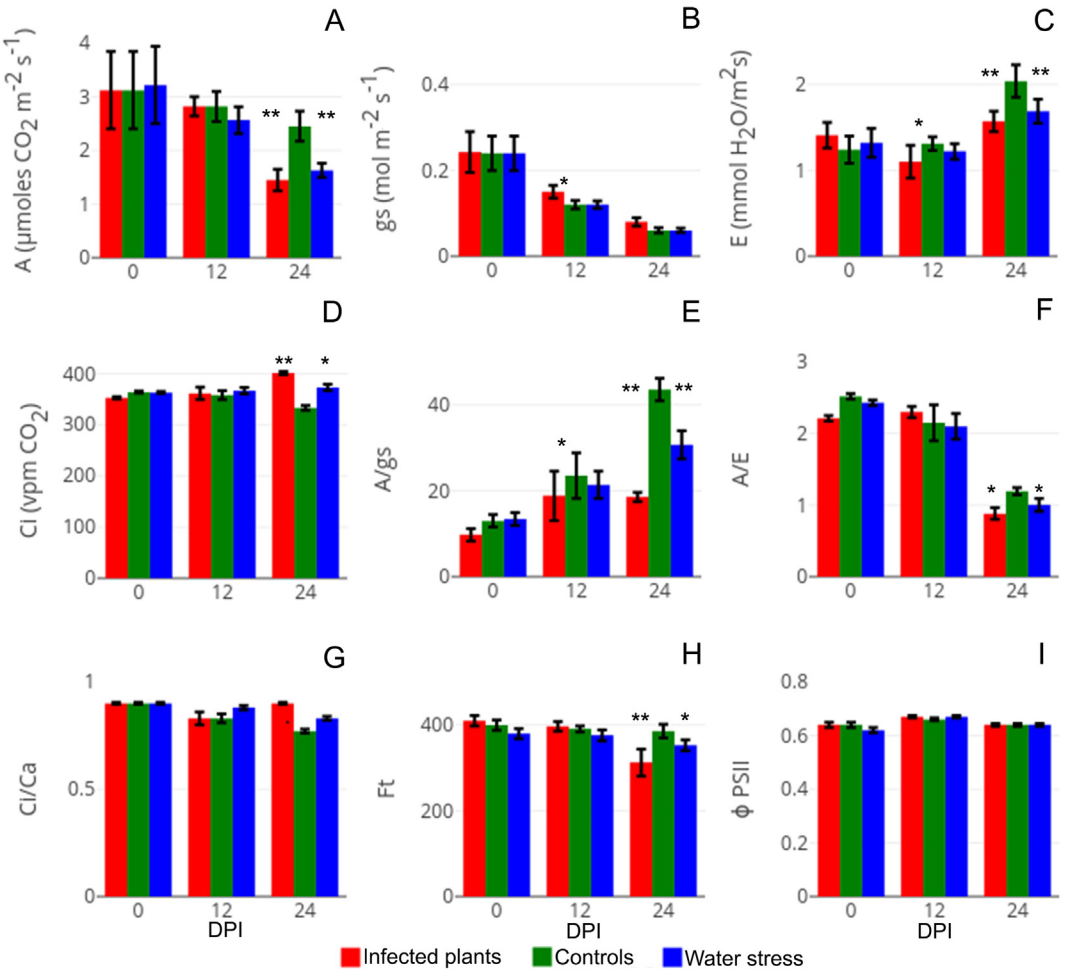

Fig. 4. Bar plots showing the mean values (±standard error) of A, gs, E, Ci (measured with an Infrared Gas Analyzer), A/gs, A/E, Ci/Ca, Ft' and Qy' (Φ PSII) (measured with FluorPen 100 WP modulated fluorometer) under constant actinic light, in tomato leaves infected with *F. oxysporum* (red), subjected to water stress (blue) and control plants (green). Estimates indicated by * and ** were significant at the 5% and 1% levels, respectively.

range of 420–490 nm (blue light), and had secondary peaks close to 560 nm (green), 680 nm and 710 nm (red) (Fig. 5).

Fig. 5 shows the correlation coefficients (R^2) calculated for each wavelength in the measured spectral range, with respect to each monitored physiological parameter. This R^2 are maximum on ranges 420–490 nm, 560 nm, and 680 nm, mainly in plants inoculated with *F. oxysporum* (Fig. 5A). On plants subjected to water stress, r^2 was also slightly greater than 0.7 in the wavelengths of blue (Fig. 5C). In gs and E, the majority of wavelengths mentioned above had high correlation values (Fig. 5G–5L), except for the peak at 560 nm that had low correlations with these physiological parameters in plants subjected to water stress (Fig. 5I, 5L), and in the particular case of E in the control plants (Fig. 5K). The graph referring to r^2 for linear regressions between reflectance and intercellular CO_2 exhibits an inverse pattern to that observed in the other parameters (A, gs and E) (Fig. 5D–5F), in which they presented high peaks ($R^2 > 0.7$) at 510 nm, 658 nm, 694 nm, and 750 nm only in plants subjected to water stress (Fig. 4F). The reasons why this inverse pattern occurs and other particularities will be discussed later in this manuscript.

Once the wavelengths with a higher coefficient of determination are defined, adjusted regression lines with the highest R^2 (and r and p-value) were drawn and shown in Fig. 6. The blue range is represented by a wavelength of 440 nm where some of the physiological parameters showed R^2 maxima. It is important to highlight the similarity between the linear regressions of the wavelengths in the blue (440 nm) and the first peak in the red (680 nm), with positive relationships between predictor (X) and response variables with low values of p ($p \ll 0.05$). This suggests that fluctuations in the predictor variables (A, Ci, gs and E) are highly associated with the response variable (reflectance at 440 nm and 680 nm). This pattern is not equivalent on particular cases of intercellular CO_2 in plants inoculated with *F. oxysporum* and controls on which there is a negative relationship betwen reflectance (440 nm and 680 nm) and "Ci" (Fig. 6D and 6E); but since p values are high ($p \gg 0.05$), predictor variable (Ci) and response are not associated. In the green wavelength (~560 nm) the reflectance decreases with increasing A (Fig. 6A, 6B and 6C), gs (Fig. 6G, 6H and 6I) and E (Fig. 6J, 6K and 6L) with p values $\ll 0.05$, instead, a positive relationship between intercellular CO_2 was noticed with the explanatory variables. A similar pattern occurs in the red light wavelength (710 nm) even with lower p values, except Ci on plants subjected to water stress ($p = 0.340$) (Fig. 6F), revealing that changes in Ci are not linked with reflectance at this point.

As a result of the Principal Components Analysis (PCA), the first two Principal Components (PC) explain 83.3% of the variance of the data (Fig. 7A), the quality of the variables is represented in

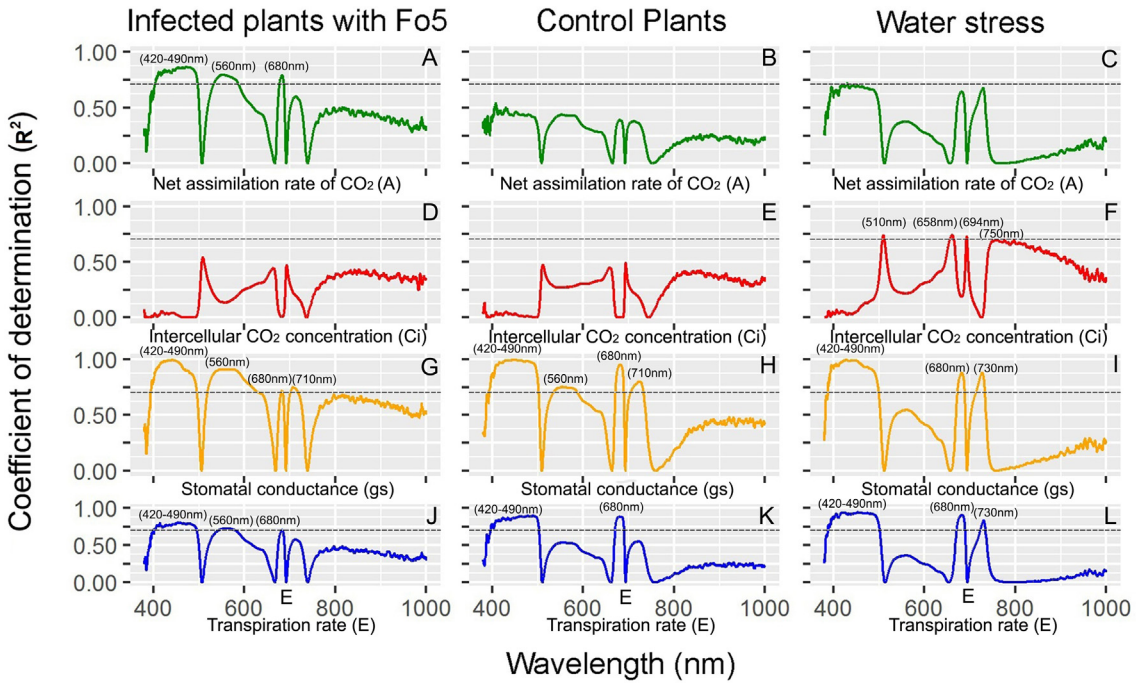

Fig. 5. Coefficient of determination (R^2) vs wavelength for simple linear reflectance relationships in tomato leaves with photosynthetic parameters (measured with an Infrared Gas Analyzer) in plants infected with *F. oxysporum* (Fo5), subjected to water stress and controls. The wavelengths in which the best fit relationships were identified are indicated in parentheses (the black line indicates the $R^2 = 0.7$).

the factor map by means of the square cosine or square coordinates (cos^2). Since high values of cos^2 indicate a satisfactory contribution of the variable in the PCs. Most variables are placed near the correlation circle on this study, indicating a good representation of the variables in the first PCs (red); others like Ci, Ft, and A/E present moderate correlation values (yellow), and A/gs, close to the center, have a reduced representation in the first PCs (blue).

To perform a dimensional reduction for the use of relevant variables in future prediction models, percentages of the contributions of the variables in each principal component were represented (Fig. 7A, 7B). Variables correlated with PC1 (Dim-1) are the physiological parameters gs, Qy, E, A, and the wavelengths at 718 nm, 564 nm, 484, 683, and 700 nm (Fig. 7B); and with PC2 (Dim-2) are Ci, Ft and the wavelengths at 510 nm, 800 nm, 650 nm, 750 nm, 700 nm, and 950 nm. These are critical variables to explain the variability in the data set. Others that do not correlate with any PC or with last dimensions correspond to variables with poor contribution and could be eliminated to simplify the general analysis.

A statistical Linear Discriminant Analysis (LDA) with transformed data (SNV) was done to achieve a supervised classification with the photosynthetic variables and the specific wavelengths selected above. Three groups were defined a priori: (i) plants inoculated *with F. oxysporum* (12 dpi, asymptomatic period), (ii) plants subjected to water stress and (iii) no-infected plants (Fig. 8). The LDA with photosynthetic parameters obtained from the analysis of gaseous exchange and fluorescence of the chlorophyll achieved an acceptable classification percentage of 82%, plants inoculated with *F. oxysporum* are well separated from the other treatments, nevertheless the model does not differentiate plants subjected to water stress from the controls at 12 dpi (asymptomatic period) (Fig. 8A). Finally, the LDA done with the spectral variables achieved a correct classification of 100% (Fig. 8B), perfectly discriminating the three treatments at day 12 after the inoculation, when the symptoms are not visible yet.

4. Discussion

4.1. Relationship of F. oxysporum concentration with the spectral response during the incubation period of the disease

The measurement of conidial concentration of *F. oxysporum* in tomato and its relation with spectral response in leaves during the incubation period of the disease is a basic requirement to allow comparisons, repetitiveness, and genetic analysis of disease tolerance and standardization of the results in many experiments on controlled and semi-controlled environments (Caligiore-Gei and Valdez, 2015). The inoculum concentration of *F. oxysporum* influenced the severity of the disease. Specifically, it has been shown that the increase of inoculum of *F. oxysporum* showed an increase on the incidence, mortality (20%), the severity of the disease and number of lesions on gladiolus roots (Riaz et al., 2008). Additionally, it was found a high coefficient of determination ($R^2 = 0.94$) on the linear relationship between the severity of the disease generated by *F. solani* in bean plants and the concentration of chlamydospores in the substrate (Nicoli et al., 2013).

Results on this investigation suggest a high correlation between inoculum concentration and spectral response of tomato plants inoculated with *F. oxysporum* before the symptoms are visible, particularly in the blue (448–523 nm) and red (624–696 nm) regions of the visible spectrum and in the infrared plateau between 750 nm and 1100 nm. The mechanism underlying this correlation is different for each region of the electromagnetic spectrum. Primarily the main photosynthetic pigments (chlorophylls and carotenoids) determine reflectance in the visible region, while in the infrared plateau the high reflectance is due to multiple dispersion within the leaf, in relation to air spaces within the tissue (internal structure) and its water content (Jacquemoud and Ustin, 2001). Maximum absorption peaks for chlorophyll and carotenoids in the blue range (chlorophyll *a* = 428 nm, chlorophyll *b* = 453 nm, carotenoids = 450 nm) and red range (chlorophyll *a* = 661 nm, chlorophyll *b* = 642 nm) (Guidi et al., 2017) are coincident with

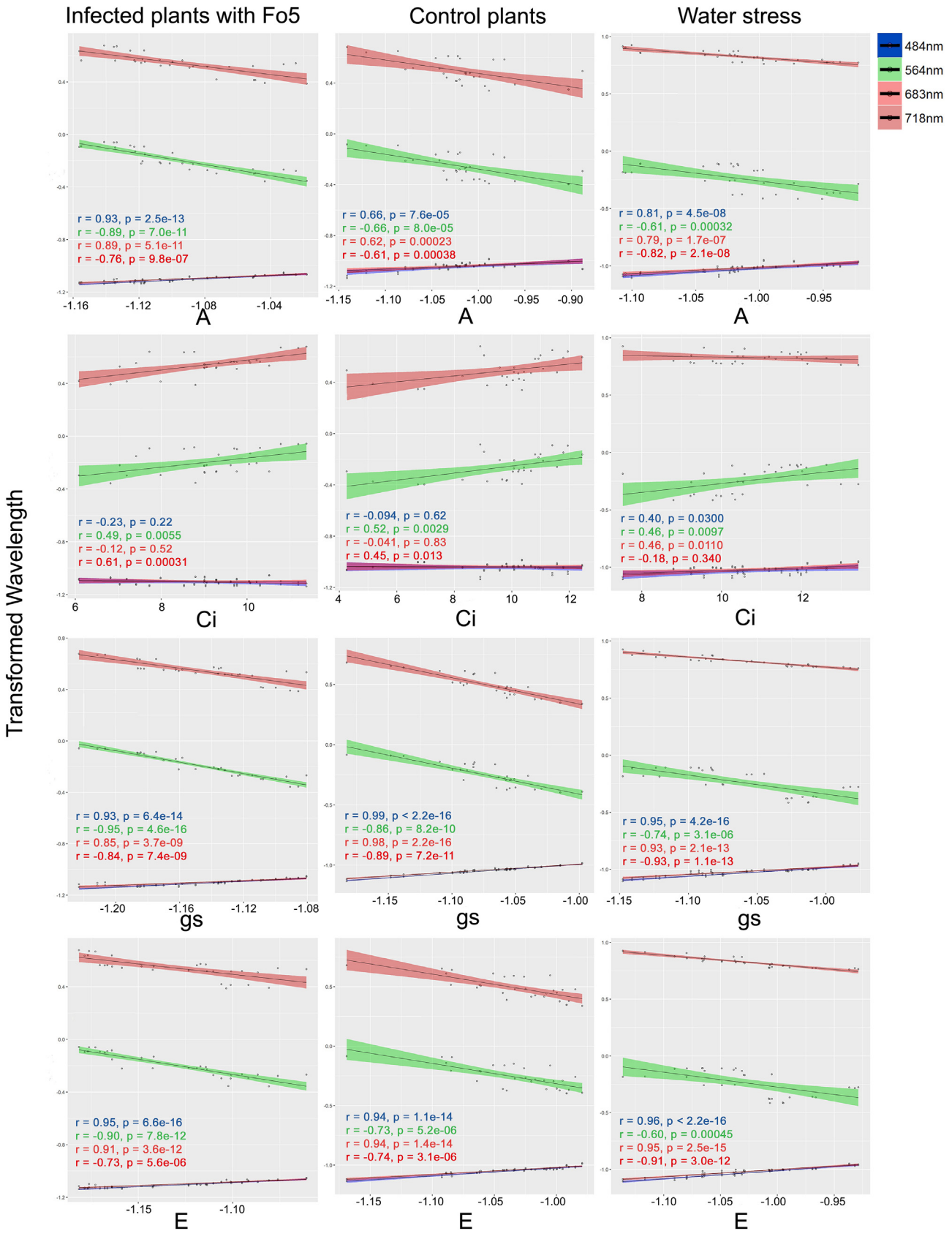

Fig. 6. Best adjusted linear reflectance near 440 nm (blue), 564 nm (green), 683 nm (light red) and 718 nm (dark red) with A, E, gs and Ci on tomato leaves inoculated with *F. oxysporum*, subjected to water stress and control plants on day 12 after inoculation (incubation period of the disease).

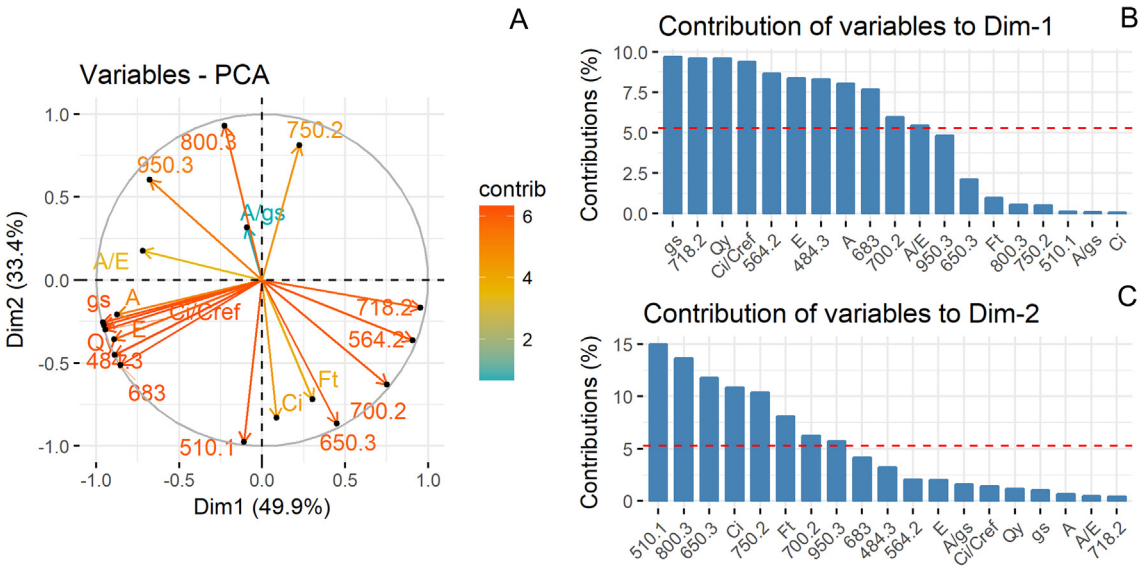

Fig. 7. A) Principal Components Analysis in which the contribution of representative physiological variables and wavelengths with high coefficients of determination to the first PCs by means of the square cosine is represented. B) Contribution of variables to PC1 (%). C) Contribution of variables to PC2 (%).

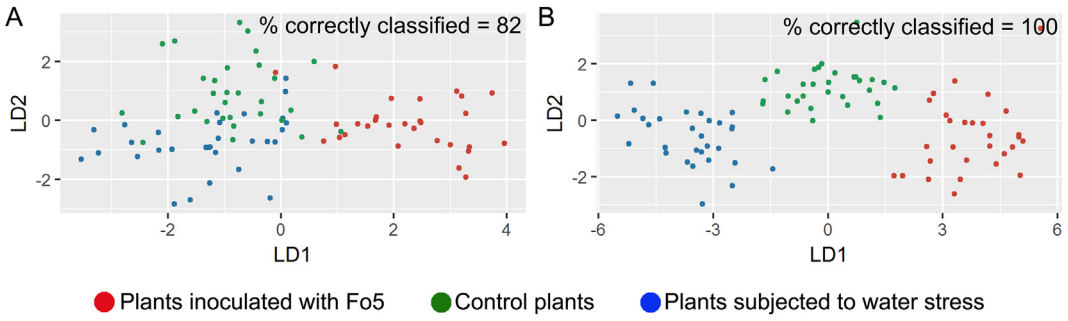

Fig. 8. Linear Discriminant Analysis (LDA) with transformed data (SNV) in plants inoculated with *F. oxysporum* (12 dpi), plants with water stress and controls. A) Physiological variables: A, gs, Qy, E, A, Ci, Ft, and Qy B) Reflectance data of the selected wavelengths: 484 nm, 510 nm, 564 nm, 650 nm, 683 nm, 700 nm, 750 nm, 800 and 950 nm.

wavelengths on the Vis spectrum correlated with the concentration of the pathogen (448–523 nm and 624–696 nm) under our experimental conditions. These results suggest a direct relationship between the inoculum concentration and the photosynthetic response in the plant that can be used for the design of models to quantify pathogen densities in asymptomatic periods of different vascular diseases. Spectral responses assessed on tomato plants for this research were complete at leaf scale, in order to observe specific changes on spectral characteristics in the course of the infection process. The inoculum concentration is indirectly measured by intermediate responses in tissue. However, to measure the direct concentration of spores or structures of the fungus in a particular tissue, the use of hyperspectral microscopes is necessary (Thomas et al., 2017).

4.2. Analysis of physiological changes in tomato plants

In this section, the effects generated in the exchange of gases from photosynthesis and the fluorescence of chlorophyll by the inoculation of *F. oxysporum* and the submission to water stress, on leaves, of a susceptible variety of tomato, in the incubation period of the disease are reported. Meanwhile during the asymptomatic period of the disease (12 dpi), a significant difference found on the gs, E, A/gs and A/E, which suggests a water imbalance on plants inoculated with *F. oxysporum* respect to the control plants, thus corroborating water stress as an important factor for vascular wilt diseases (Nogués et al., 2002; Ghaemi et al., 2009; Ochola et al., 2015). Vessel obstruction is one of the relevant effects of *F. oxysporum* in the photosynthesis of leaves, but little is known about the effect of water stress induced by pathogens. However, the reduction of the diameter of the vascular bundles by species of the *Fusarium* spp., their metabolites, and enzymes or by inducing the accumulation of gummy and hairy substances, generating resistance to the movement of water, has been widely reported (Aguirreolea et al., 1995, Jensen, 2002).

Under the experimental conditions here, non-significant differences were found on net photosynthesis at 12 dpi using univariate analysis with the Infrared Gas Analyzer (IRGA), neither with parameters of chlorophyll fluorescence (discussion of multivariate analysis will be addressed later), although it has been reported to be one of the first symptoms of vascular wilt. The aforementioned is often reported indicating that the water imbalance generates a decrease in the rate of CO_2 fixation, on electron transport chain in chloroplasts, and lack of activity of ATP synthase (Yordanov et al., 1997, Flexas et al., 1999a, Flexas et al., 1999b). However, different authors have also reported the absence of significant differences up to 17 dpi in gas exchange parameters measured with an infrared gas analyzer and changes in quantum yield of photosystem II only until 27 dpi in tomato plants infected with *F. oxysporum* (Lorenzini et al., 1997; Nogués et al., 2002).

The pattern development of the wilt by *Fusarium* reduces the photosynthetic activity depending on different mechanisms. Photosynthesis variation is difficult to detect on the asymptomatic period of the disease with these techniques. This can also be explained according to the type of vascular wilt (type I or II) described by Pshibytko et al. (2006), which depends on environmental conditions such as temperature, relative humidity and moisture content of the floor. In this research, the pattern of disease development on inoculated tomato plants was Type I, according to the classification of Pshibytko et al (2006). In this study, plants inoculated with Fo5 exhibited yellowing, and slow-wilting from lower to upper leaves (Fig. 1), on moderate environmental conditions (average relative humidity between 60–70%, temperature of 18–24 °C), and gradual symptom display, only after 21 dpi. The authors suggest that on infection type I wilt, the mycelium of *F. oxysporum* partially obstructs xylem and grows extensively within the parenchyma, being able to absorb the nutrients and regulate the metabolic processes of the plant. Finally, in this type of wilting, plants die due to nutrient deficiency and poisoning by fungal toxins, and not due to water deficit. This hypothesis plausibly explains the chronology of the symptoms described above and why in this research the leaves of plants infected with *F. oxysporum* only showed loss of turgor shortly before wilting.

The difficulty in detecting responses associated with the disease in the asymptomatic period could be due to the lack of sensitivity in the equipment used for measuring gas exchange and fluorescence parameters of chlorophyll. Since in both cases few parameters are assessed on the plant, such as the absorbance in the NIR to 1640 cm^{-1} for the case of the IRGA, and a relation between the pulse of saturating light generated by the fluorometer (usually ultraviolet light) and its consequent response of lower energy generated by the sample (in the Vis or NIR). The conclusions obtained from the limited univariate analysis can also lead to erroneous conclusions. Therefore, it is necessary to implement more robust multivariate analyses (as in Figs. 7 and 8), which will be discussed later.

Finally, when the plants inoculated with *F. oxysporum* have finished the incubation period (24 dpi) and the symptoms of the disease on the leaf and on plants subjected to water stress can be observed, net photosynthesis decreases, as well transpiration rates, intrinsic efficiency in water use, transpiration efficiency (A/E), and the proportion between intercellular CO_2. Nevertheless, there is no evidence that this variation on parameters can lead to permanent damage on energy transduction phases, nor by stomatal or hydric limitations, coincident with results obtained in *P. edulis* with the same isolate of *F. oxysporum* (Cruz, 2012). Chlorophyll fluorescence parameters, especially the transient fluorescence (Ft), which significantly decreased on this study between evaluated treatments, remarks the importance to obtain information about the pigment complexes, the organization and the transfer of excitation energy between them, and different specific electron transfer reactions in photosystem II (Stirbet, 2012). Additionally, plant samples have a transient fluorescence that level off in a new stable state before any change in the quality or quantity of the light to which they are exposed, regardless of whether the sample was adapted to darkness or light. Although Ft is an important tool to assess plant stress (Strasser et al., 2000), nevertheless in this study it is not an indicator of early stress on plant diseases.

4.3. Linking the physiological parameters with the leaf reflectance in the Vis/NIR during the incubation period of the disease

Chlorophyll (mainly "a" and "b") and carotenoids are essential pigments for the conversion of light energy into stored chemical energy. The amount of solar radiation absorbed by a leaf is a function of the content of photosynthetic pigments; therefore, the chlorophyll content can determine directly photosynthetic potential and primary production (Filella et al., 1995, Gitelson et al., 2003). Higher coefficients of determination (R^2) between some regions of the Vis spectrum and the parameters obtained from the gas exchange measurements are determined by the specific absorbance-reflectance patterns of the photoactive pigments, mainly chlorophylls and carotenoids (Gitelson et al., 2002, Gitelson et al., 2003; Merzlyak et al. 2003; Solovchenko et al., 2005). In this research, plants inoculated with *F. oxysporum* showed high correlations with A, gs and E on 4 bands of the Vis spectrum. The first high correlation zone is located between 420 nm and 490 nm and coincides with the spectral region where the maximum absorbance of chlorophyll *a* (430 nm), chlorophyll *b* (453 nm) and carotenoids (450 nm and 485 nm) occurs, while secondary peaks of the chlorophylls in the far-red (642 nm and 662 nm) match with regions of low correlation (Fig. 9). Chlorophylls and carotenes absorb light on mesophyll mainly at wavelengths between 400 and 500 nm (blue), its measurement is a signal of the physiological variation due to biotic and abiotic stress, leaf development, senescence and factors directly related to the photosynthetic primary production rate (Blackburn and Ferwerda, 2008). The results of this study support the idea that infection by *F. oxysporum* has correlation with photosynthetic pigments concentration on early stages of the disease. Additionally, the wavelengths with the highest coefficients of determination with the physiological parameters in the gas exchange analysis could be used in mathematical models for early detection of plant diseases based on spectral data in the Vis.

Plants under water stress, show high correlations in the band of 420 nm-490 nm and the peak of 680 nm, coincident with the absorbance of main photosynthetic pigments, due to the decrease in water potential on the leaf that suppresses the photosynthetic activity of the plant (discussed previously). However, on tomato, water stress has a high relation between reflectances at 730 nm and 750 nm, which are not affected by the absorption of chlorophyll. These are linked with water stress or changes in tissues (Carter and Knapp, 2001). The control plants did not show high coefficients of determination (>0.7) between the A and Ci on none of the wavelengths in the measured spectral range, confirming the lack of significant variation of these parameters within the individuals for this treatment. The correlation found in these plants between gs and E, and the spectral bands 420–490 nm, 560 nm, 680 nm, may suggest differences in the concentration patterns of photosynthetic pigments due to gaseous exchange in the leaves (mainly water vapor) without significantly affecting net photosynthesis. This hypothesis is reinforced by the high correlation between stomatal conductance and reflectance at 710 nm in this treatment.

Reflectance spectroscopy is widely applied for the non-destructive estimation of leaf chlorophyll as an indirect measurement of the photosynthetic potential and primary production of the plant (Richardson et al., 2002). The bands of 420–490 nm, 560 nm and about 700 nm (far red), which presented high values in the correlation coefficients with gas exchange parameters in this research, have been widely used in the form of indexes and simple functions to relate the concentration of chlorophyll in a variety of plant species and in a wide range of photosynthetic pigment composition (Gitelson and Merzlyak, 1996; Gitelson and Merzlyak, 1997; Sims and Gamon, 2002; Gitelson et al., 2003; Merzlyak et al., 2003). Additionally, some bands in the Vis/NiR regions can be used to develop biotic stress indices during the incubation period and the moment that symptoms are already visible in the plant with vascular wilt disease (Sankaran et al., 2010; Khaled et al., 2017).

Some of the bands identified in this study have been used independently and/or to develop indices, sensitive to plant disease pro-

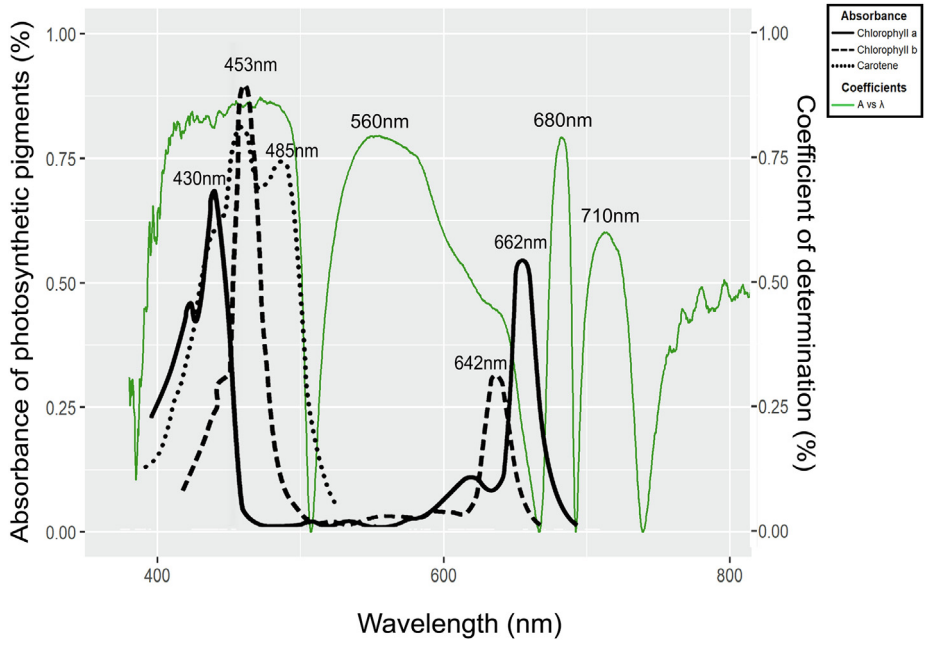

Fig. 9. Line plot illustrating the superposition of the relationship between the r^2 net photosynthesis vs wavelength in plants inoculated with *F. oxysporum* (green line) and the optimal absorption of light from the main photosynthetic pigments (lines black, absorption curves extracted from "The light-dependent reactions of photosynthesis: Figure 4," by OpenStax College, Biology [CC BY 3.0]).

cesses, such as: the Photochemical Reflectance Index [PRI = (R570 – R531]/[R570 + R531)], Physiological Reflectance Indices [PhRI = (R550 – R531)/(R550 + R531)], Standard chlorophyll pigment rate index [NPCI = R680 – R430)/(R680 + R430)] and the Index of Anthocyanin Reflectance [ARI – (R550) –1 – (R700) –1] (Song et al., 2011; Zhang et al., 2012; Krishna et al., 2014). On plants exposed to water stress, main water absorption information in the spectral range measured in this research is in the range of short wave NIR (750–1000 nm) (Zhang et et al., 2012; Genc et al., 2013). Due to the intention to relate reflectance to the physiological parameters measured from a gaseous exchange analysis, it is expected that no specific bands are related to the water content in this spectral range.

The results obtained in the biplot of the PCA (Fig. 7) and the LDA (Fig. 8) reinforced the previous idea. On these analysis, some wavelengths that belong to the range of short wave NIR were added, which were highly correlated with the concentration of conidia in the plants inoculated with *F. oxysporum* (Fig. 3). In general, the wavelengths selected from the proposed methodology provided a high contribution to the first two main components, while only the parameters gs, Qy and E (derived from the gas exchange analysis) offered a contribution classified as high on main components.

The advantages of multivariate analysis over the multiple univariate analysis shown in Fig. 4 should be highlighted, as a possible way of identifying system constructs of response variables, selecting subsets of variables and determining the relative value for each stand out variable (Huberty and Morris, 1989). Precisely, the addressing of this analysis with the multivariate approach, allowed to obtain high percentages of classification (82%) in the LDA, during the incubation period (12 dpi) with the variables derived from the analysis of gas exchange, while in the same period with multiple univariate analysis did not find significant difference for most parameters. Even with the multivariate analysis, the use of parameters derived from gaseous exchange analysis have limitations for the realization of applicable models in the early detection of plant disease. For example, the model applied in this study using these parameters did not allow to efficiently separate the plants subjected to water stress from the control plants (healthy and with 100% field capacity), while the model completed from the spectral reflectance with relevant specific wavelengths allows correct classification of 100% of the three treatments. The relevant specific wavelengths related to the physiological response of the plant to *F. oxysporum* can be used in the development of portable instruments for the early detection of vascular wilt, easily, quickly and non-destructively.

5. Conclusions

It is possible to relate, by means of an indirect methodology such as reflectance spectroscopy in the Vis/NIR, the concentration of conidia with the spectral response on the leaves of inoculated plants of *F. oxysporum* during the incubation period of the disease. The increase in the concentration of pathogen on the vascular system of the plant coincides with the increase of reflectance in the Vis region (380–750 nm), but has an inverse relationship in the range of the infrared plateau (750–1000 nm), at least in the last week of the incubation period. Five specific bands were found highly correlated with the increase in the concentration of *F. oxysporum* conidia measured at root and leaves, two on the Vis range (448–523 nm, 624–696 nm), and three near the infrared measured (740–960 nm, 973–976 nm, and 992–995 nm).

Tomato plants inoculated with *F. oxysporum* did not present a significant difference in net photosynthesis with respect to control plants in asymptomatic period of disease, using only using only univariate analyzes. Multiple univariate analyzes only allowed to detect a significant decrease in this period in the transpiration, the transpiration efficiency and the proportion between the intercellular CO_2 and the environmental CO_2. However, there is no evidence of permanent damage in the energy transduction phase, nor by stomatal or water limitations. When the disease symptoms were observed, there was a marked decrease of the net

photosynthesis, but the parameters derived from the chlorophyll fluorescence analysis showed no decrease in the electron transport efficiency of the photosystems in the plants infected with *F. oxysporum*.

Four bands in the Vis range correlated to the photosynthetic parameters derived from the gaseous exchange analysis in the tomato leaves subjected to biotic and abiotic stress. The sensitivity of the relationship between reflectance with net photosynthesis, stomatal conductivity and transpiration was higher on spectral ranges such as 420–490 nm, 560 nm, 680 nm, and 710 nm. Particularly, the far-red bands correlated more with those on net photosynthesis in the plants inoculated with *F. oxysporum*, allowing the estimation and detection of the infected plants during the early stages of infection.

Based on the relationships between the photosynthetic parameters and the spectral response of the plants subjected to two different types of stress, several wavelengths were selected in the blue (480 nm and 510 nm), the green (560 nm), the red (650 nm, 680 nm, 700 nm, and 718 nm) and NIR (750, 800, and 950) ranges to perform the Linear Discriminant Analysis. These wavelengths allowed classifying correctly 100% of the plants inoculated with *F. oxysporum*, the plants subjected to water stress and the control plants in the asymptomatic period of the disease. These results allow a significant increase in the knowledge in the area of early detection of diseases in the specific pathosystem *S. lycopersicum*-*F. oxysporum*.

Based on the findings of this research, the use of robust multivariate analysis is recommended for the applied biological sciences and, specifically in the agricultural sciences. The efficacious use of these multivariate application tools can avoid falling on wrong conclusions on specific areas of interest, such as the identification of variable response systems, subset selection of variables and determination of the relative value for each variable in highly complex matrices.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

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