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EUPHRESO III - Special Issue on Plant Health Research Priorities RESEARCH PAPERS

A hyperspectral phenotyping platform for studying plant responses to *Xylella fastidiosa* infection

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Summary. The plant pathogenic bacterium *Xylella fastidiosa* is able to infect a wide range of hosts. Its long incubation period and frequent asymptomatic infections hinder early visual detection, allowing infected plants to remain undetected facilitating disease spread. This paper describes a hyperspectral imaging-based phenotyping platform for early, non-destructive assessment of woody plant responses to *X. fastidiosa* infection under controlled conditions. The platform includes a vertical-scanning structure that integrates controlled illumination with a push-broom VNIR hyperspectral camera, and is supported by an automated hyperspectral image processing pipeline. This workflow includes calibration, spectral smoothing, supervised machine-learning-based image segmentation, and extraction of pure-vegetation spectral signatures, ensuring quantitative and reproducible spectral data. The platform performance was evaluated using asymptomatic olive cultivars inoculated with two *X. fastidiosa* strains. The resulting spectral signatures showed characteristic vegetation reflectance patterns, along with subtle but consistent differences from non-inoculated control plants, and for qPCR-positive and qPCR-negative *X. fastidiosa* inoculated plants. These differences were supported by univariate and multivariate statistical analyses, which confirmed a significant treatment-related spectral effect. The developed system can provide quantitative evidence that hyperspectral imaging can capture pre-symptomatic physiological alterations associated with *X. fastidiosa* inoculation status. This platform is a robust and versatile tool for high-throughput phenotyping, disease monitoring, and resistance screening in olive, and is readily transferable for use on other woody crop hosts. It has strong potential to support future research on vascular plant diseases and pathogen resistance plant breeding programmes.

Keywords. Asymptomatic infection, hyperspectral imaging, plant-pathogen interactions, plant phenotyping, spectral reflectance.

INTRODUCTION

Xylella fastidiosa is a xylem-limited plant pathogenic bacterium capable of infecting a wide range of hosts, including 728 plant species reported to date (EFSA, 2026). This pathogen causes severe diseases in economically important crops and forests, resulting in significant economic, social, and environmental impacts (Sánchez *et al.*, 2019). *Xylella fastidiosa* infections disrupt host xylem functionality and produce physiological and metabolic changes in host plants, including reduced transpiration and alterations in photosynthesis and pigment composition (Zarco-Tejada *et al.*, 2018; Camino *et al.*, 2021; Surano *et al.*, 2022). These alterations influence canopy and leaf temperature, chlorophyll fluorescence and spectral traits related to pigment degradation (Ribeiro *et al.*, 2004; Zarco-Tejada *et al.*, 2021).

The key epidemiological characteristics of *X. fastidiosa* arise from its pathogenesis, which is a complex process combining bacterial virulence factors and host plant physiological responses (Purcell, 1997; De la Fuente *et al.*, 2024). Infections are frequently asymptomatic and characterized by prolonged incubation periods, allowing infected plants to act as reservoirs for pathogen spread by insect vectors, even in absence of visible symptoms (Hopkins and Purcell, 2002). Asymptomatic infections, long incubation periods, and nonspecific symptom expression hinder timely and reliable bacterial detection, particularly during early stages of infection (Velasco-Amo *et al.*, 2022; De la Fuente *et al.*, 2024).

As a quarantine pathogen in the European Union, *X. fastidiosa* requires robust and effective monitoring systems to support effective disease management. Early detection is essential to enable timely eradication or containment measures before outbreaks become widespread, thereby minimizing yield losses (Mastin *et al.*, 2022). Although conventional diagnostic methods, including pathogen isolation or molecular techniques, remain essential for confirming pathogen presence, their application at large scales can be costly, time-consuming and constrained by detection thresholds (Loconsole *et al.*, 2014; Velasco-Amo *et al.*, 2026). Optical sensing technologies, including spectral sensors, chlorophyll fluorescence quantification and thermography, offer complementary approaches for rapid, non-destructive and high-throughput screening of plant health status. By capturing pathogen-induced physiological and biochemical alterations prior to the onset of visible symptoms, these approaches can enable continuous plant health monitoring across different spatial and temporal scales, from proximal to remote sensing platforms (Zarco-Tejada *et al.*, 2018; Tanner *et al.*, 2022; Cubero *et al.*, 2024; Román-Écija *et al.*, 2026).

Hyperspectral sensors capture high spectral resolution plant signatures across hundreds of narrow bands, covering the visible (VIS), near-infrared (NIR), short-wave infrared (SWIR) and thermal infrared (TIR) regions, enabling the precise detection of subtle plant physiological changes (Cubero *et al.*, 2024). These sensors can be deployed across diverse platforms, including microscopes, laboratory-based systems, field machinery, robot units and high-throughput phenotyping facilities, providing new insights into complex plant-pathogen interactions and supporting applications in precision agriculture, plant phenotyping and crop breeding programmes (Mahlein, 2016). Automated image acquisition and analysis enable detailed, quantitative spectral measurements of plants, facilitating estimation of disease-related traits with greater precision and accuracy than conventional visual disease assessments (Mutka and Bart, 2015). Collectively, these advances highlight the significant potential of hyperspectral phenotyping approaches for plant disease research.

This paper outlines development of a phenotyping platform designed to acquire high-spatial resolution hyperspectral images of woody plant species, with a specific application for olive plants (*Olea europaea*). The primary objective was to obtain high-resolution spectral signatures of *X. fastidiosa*-infected plants that would enable subsequent detailed physiological characterisation of infection-related responses in future studies. Results showed that the proposed platform presented here is a robust tool for plant phenotyping, and for studying physiological response patterns associated to infection by vascular pathogens, thereby enabling rapid evaluation of olive genotypes for resistance, and their integration into olive breeding programmes.

MATERIALS AND METHODS

The methodological framework developed in this study enabled the acquisition of high-resolution hyperspectral plant images and extraction of quantitative vegetation spectral signatures through a controlled and reproducible workflow (Figure 1). It integrates: (i) a dedicated phenotyping platform, (ii) standardized hyperspectral image acquisition under controlled optical conditions, and (iii) a multi-step data-processing pipeline that converts raw hyperspectral data into calibrated, noise-reduced, and segmented vegetation spectra. This end-to-end framework was implemented to support phenotyping studies of vascular plant diseases, with particular application to *X. fastidiosa*.

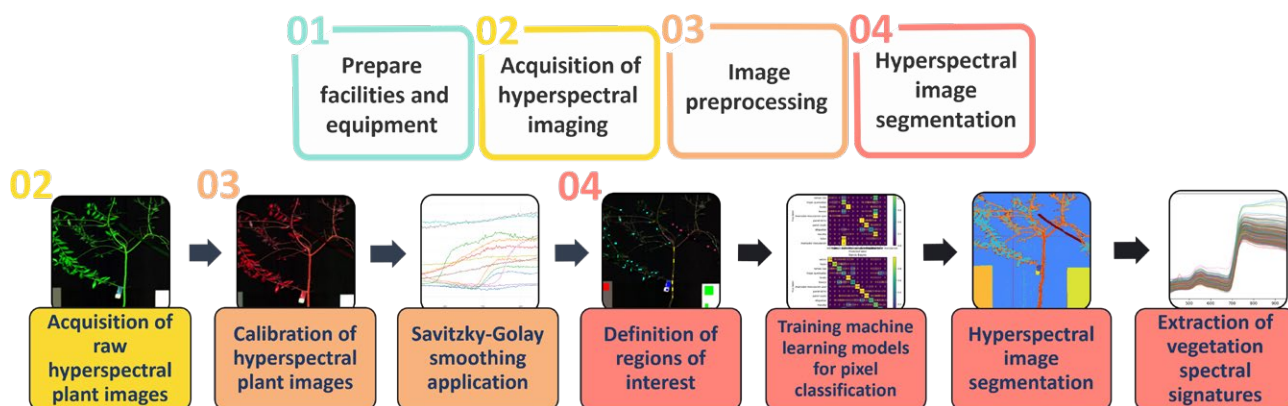


Figure 1. Overview of the hyperspectral phenotyping platform and data-processing workflow developed in this study. The workflow is structured into four main stages: (01) facility and equipment preparation, (02) hyperspectral image acquisition, (03) image pre-processing, and (04) image segmentation. Raw plant images are acquired and radiometrically calibrated, followed by Savitzky-Golay spectral smoothing. Regions of interest (ROIs) are manually defined to train supervised machine learning models for pixel classification, enabling automated segmentation. Reflectance spectra from pure-vegetation pixels are then extracted to derive representative vegetation spectral signatures.

Design of the phenotyping platform

The phenotyping platform was developed to acquire hyperspectral images of plants using a vertical-scanning approach. The platform comprises a mobile support structure, a motorised vertical linear-stage module, a dedicated lighting system based on a 400 W halogen lamp, and a hyperspectral imaging sensor (nano-hyperspectral imager, Headwall Photonics) mounted on a custom bracket with an integrated control/electronics box (Figure 2).

The support structure was designed specifically for this application and has overall dimensions of 100 × 75 × 190 cm. It was constructed from galvanised steel with a matte black finish to minimize reflection. The structure includes wheels to facilitate repositioning and adjustable levelling feet to ensure mechanical stability during operation.

The vertical movement of the hyperspectral sensor was driven by a motorised linear-axis module with a toothed-belt drive system (drylin® ZLW, Igus), providing smooth, low-maintenance movement with high positional repeatability. The linear module was controlled using a stepper motor with brake (drylin® E, Igus). Positioning was defined by proximity and limit sensors integrated into the belt-drive system. Motor operation and motion parameters, including limit definition, speed, real-time position and working cycles, were managed through a web-based motor control system housed in the control box (drylin® D1, Igus). The controller was connected to a computer via a local Ethernet link, enabling full remote control of vertical motion. The nominal scanning speed was set to 20 mm s⁻¹ and the maximum scanning height



Figure 2. Phenotyping platform for hyperspectral image acquisition using vertical scanning of plants. The platform consists of a mobile support structure, a motorized vertical linear-stage module, a dedicated lighting system, a hyperspectral imaging sensor mounted on a custom bracket, and a control and electronics unit. Videos of the platform in operation during hyperspectral data acquisition are available in the CSIC institutional repository at <https://doi.org/10.20350/digitalCSIC/17945>.

was 1.28 m, resulting in an effective acquisition time of approx. 1 min per plant.

For image acquisition, a 400W IP54-rated halogen projector (R7s J118 lamp) was mechanically attached to the motorised vertical linear-stage module. A custom L-bracket mounting plate supported the sensor. The bracket was custom 3D-printed in acrylonitrile styrene

acrylate (ASA) and measured 119×133×105 mm. This configuration provided uniform broadband illumination, mechanical stability, and precise alignment of the hyperspectral sensor with the vertical scan axis.

Spectral data were acquired using a push-broom hyperspectral sensor (Nano-Hyperspec® model, Headwall Photonics) mounted on the phenotyping platform. The sensor acquired complete image lines and continuously records data during vertical motion. In spectral mode, the sensor provided 272 bands with a full-width at half-maximum (FWHM) spectral resolution of 6 nm over the 400-1000 nm range, covering the visible and near-infrared (VNIR) regions. A 4.8 mm focal-length lens provided an angular field of view (FOV) of 50.7°. At an imaging distance of ~1.6 m from the potted plants, the sensor's 7.4 µm pixel pitch corresponded to a spatial resolution of ~2.5 mm per pixel. The integration time was set to 150 ms. Data were transmitted via Ethernet using Hyperspec® III software (Headwall Photonics), generating an average data volume of about 300 MB per hyperspectral image.

Design of the phenotyping room

The phenotyping platform was installed within a dedicated phenotyping room in a plant biosecurity level 2 (PBSL2) greenhouse at the Institute for Sustainable Agriculture-CSIC in Córdoba, Spain. The platform enabled scanning of woody species grown in pots, such as olive, inoculated with *X. fastidiosa* (Figure 1-01).

To ensure controlled, reproducible optical conditions during image acquisition, the walls of the phenotyping room were lined with black plush fabric and the floor was covered with a black nonslip rubber mat (Figure 1-01). This provided a uniform low-reflectance environment and safe working conditions.

Plant material

An experiment was carried out using ten olive cultivars, including 'Leccino', 'FS17', 'Ogliarola' (3 to 4 years old), 'Frantoio', 'Arbequina', 'Arbosana', 'Cornicabra', 'Gordal', 'Hojiblanca' and 'Picual' (1.5 to 2 years old), that were maintained in a plant biosecurity level 2 (PBSL2) greenhouse under controlled conditions (24 ± 5 °C, 60% RH) (Figure 1-01). To minimise potential confounding effects of plant age and vegetative size on spectral responses, all comparisons between non-inoculated and *X. fastidiosa* inoculated plants were made within each cultivar, where plants had uniform age and similar vegetative development. A total of 262 olive plants were

used in the study, of which 94 (from six to ten plants per cultivar) were inoculated with the Spanish strains *X. fastidiosa* subsp. *pauca* XYL1961/18 (ST80) and 88 (from seven to ten plants per cultivar) were inoculated with *X. fastidiosa* subsp. *multiplex* XYL1966/18 (ST81). At least three stem points (IPs) per plant were inoculated with a bacterial suspension (1.6×10^8 cells per mL). Additionally, 80 plants (eight plants per cultivar) were treated with PD3 medium only, as non-inoculated controls.

Hyperspectral images were acquired from potted woody olive plants at 12 months post-inoculation (mpi) with the different *X. fastidiosa* strains (Figure 1-02). Images were captured prior to the appearance of visible disease symptoms to monitor the physiological and biochemical responses associated to infection under the different experimental combinations (i.e. olive cultivar and *X. fastidiosa* strain).

Plants were sampled 1 mpi, by collecting the petioles of the two leaves of each plant located approx. 5 cm above the IP. At the time of image acquisition, one of the three inoculated branches (length approx. 5 to 10 cm) branches per plant was sampled at 15 cm above the inoculation point. Plant extracts and DNA extraction from these samples were carried out as described in Román-Écija *et al.* (2026). The presence or absence of *X. fastidiosa* in the olive plant tissues was determined using a duplex qPCR protocol (Bonants *et al.*, 2019).

Sensor calibration

An *in situ* camera calibration was carried out at the beginning of the experiment to produce a pixel-by-pixel calibration map that accounted for the spectral response of each spatial and spectral pixel in the camera, and for light non-uniformity across spatial pixels. Full scan images were acquired, where the x axes were each covered with three reference surfaces: i) a white EVA foam panel (70-80% reflectance); ii) a grey EVA foam panel (25 to 40% reflectance); or iii) the background and black plush fabric (2 to 4% reflectance). The spectral reflectances of these panels were measured using a Spectravista Corporation (SVC) XHR-1024i spectroradiometer (Spectra Vista Corporation), equipped with a contact probe for surface reflectance. Before image capture, a dark-current image was captured by covering the sensor with its lens cap.

The digital numbers, with dark current subtractions, extracted from the panel images were related to the known reflectance values using linear regression, yielding two arrays of gain and offset parameters. Therefore, raw hyperspectral plant images were converted to reflectance units according to the following linear model:

Calibrated plant image = [(raw plant image – dark current) × gain] + offset [1];

where dark current is thermal and electronic detector noise, gain accounts for variations in illumination and sensor sensitivity, and offset correction compensates for systematic errors in the acquisition system.

Preprocessing of hyperspectral plant images

For plant image acquisition, the grey and white panels were placed, respectively, on the left and right sides of the image, with the plant centred on the X-axis. These surfaces, along with the black background, provide three reference targets for verifying the calibration process. The images were converted to reflectance using Equation [1] using a custom Python script (Figure 1-03).

Savitzky-Golay smoothing application

To reduce high-frequency noise in the reflectance while preserving relevant spectral information, the calibrated hyperspectral data were smoothed using a Savitzky-Golay filter (Savitzky and Golay, 1964) (Figure 1-03). A second-order polynomial with a 21 nm window size was applied, as this configuration provides an effective balance between noise mitigation and preservation of vegetation spectral features.

Hyperspectral image segmentation

Once all plant hyperspectral images were calibrated, regions of interest (ROIs) were defined on the images to identify representative pixels for each scene category (vegetation, pot, background, calibration panels, trunk, labels) (Figure 1-04). ROIs for each cultivar were manually defined in calibrated olive images using ENVI software (L3Harris Geospatial Solutions Inc.). These ROIs were exported to shapefile format and imported into QGIS (v3.34 Prizren) using the EnMAP-Box plugin v3.15.3 (Jakimow *et al.*, 2023). EnMAP-Box was used to extract the pixels for each category from the ROIs in JSON format. These JSON files were then imported into a custom Python script and used as training data for supervised machine learning classification algorithms (Figure 1-04). Each model was trained to assign a category label (vegetation, pot, background, stem) to individual pixels based on spectral characteristics learned from the ROIs. To account for diverse classification behaviours and modelling assumptions, multiple super-

vised algorithms were implemented, including instance-based methods (K-Nearest Neighbors), support vector machines (Linear and RBF SVM), tree-based models (Decision Tree, Random Forest, and AdaBoost), neural networks implemented via a Multilayer Perceptron, and probabilistic classifiers (Gaussian Naive Bayes, Gaussian Process, and Quadratic Discriminant Analysis). The individual classifiers were then integrated into an ensemble model specifically for the leaf category. For this approach, a pixel was assigned to the leaf class only when it was consistently predicted as such by most models (at least six out of nine). This ensemble approach mitigates algorithm-specific biases, reduces sensitivity to individual modelling assumptions, and yields more robust and stable classifications than any single algorithm alone. Using the spectral information retrieved from the ensemble model, mean reflectance spectra were generated for each treatment group (qPCR-positive inoculated plants, qPCR-negative inoculated plants and mock-inoculated control plants) and cultivar (Figure 1-04). This standardized extraction procedure ensured that the spectral profiles represented only pure leaf pixels by implementing a fully automated, reproducible workflow applicable to successive image acquisitions of potted plants (Figure 1).

All image preprocessing, calibration, segmentation, and spectral extraction steps were implemented in an automated pipeline developed in Python (v3.10, Python Software Foundation). The libraries used throughout this workflow were: Spectral v0.23.1 (Boggs *et al.*, 2022), Matplotlib v3.8.0 (Hunter, 2007), NumPy v1.26.4 (Harris *et al.*, 2020), Pandas v2.2.2 (McKinney, 2010), SciPy v1.13.0 (Virtanen *et al.*, 2020) and Scikit-learn v1.7.2 (Pedregosa *et al.*, 2011).

Multivariate analysis of spectral signatures

To assess associations between hyperspectral reflectance data with *X. fastidiosa* inoculation status, univariate and multivariate analyses were conducted. Principal component analysis (PCA) was first applied to reduce dimensionality of the 272 spectral bands and address multicollinearity among adjacent wavelengths. Treatment effects were then evaluated by analysis of variance (ANOVA) for the scores of the first ten PCs, with olive cultivar as a blocking factor. Tukey's *post hoc* test was used for pairwise comparisons among treatments. In addition, a PERMANOVA was carried out on the spectral dissimilarity matrix computed from the PC scores that showed significant treatment effects ($P < 0.05$) in the ANOVA analysis (PC1, PC5 and PC7), using Euclidean distances and 999 permutations, with olive cultivar

included as a blocking factor. Homogeneity of multivariate dispersion among treatment groups was assessed to determine whether significant PERMANOVA results ($P < 0.05$) could be attributed to differences in group location rather than differences in within-group dispersion.

All statistical analyses were carried out in R (v4.2.3, R Foundation for Statistical Computing). ANOVA and PCA were conducted using base R functions, and PERMANOVA and *post hoc* tests using *vegan* v2.6.4 (Oksanen *et al.*, 2022) and *emmeans* v1.8.5 (Lenth *et al.*, 2023), respectively.

RESULTS AND DISCUSSION

Olive plants inoculated with *X. fastidiosa* and non-inoculated controls were used as a case study to evaluate performance of the phenotyping platform. Success of inoculations with the two *X. fastidiosa* strains was verified by qPCR assays at 1 mpi in the leaf petioles closest to the IP. At 12 mpi, at stem level the frequency of detection at 15 cm from IP varied depending on the strain and cultivar, ranging for strain XYL1961/18 from 40% for cultivar Gordal to 90% for Arbequina, and for strain XYL1966/18 from 14.3% for cultivar Frantoio to 88.9% for Arbosana (Table 1).

Although the frequencies of detection sometimes were low, these were consistent with values reported in previous studies based on artificial inoculations of olive with different *X. fastidiosa* strains (Saponari *et al.*, 2017; Burbank *et al.*, 2024; Román-Écija *et al.*, 2026). Detection by molecular methods can be affected by several

factors, including *X. fastidiosa* strain virulence, cultivar susceptibility, time after inoculation, and portion of the stem sampled. In the present study, samples were collected from approx. 10-15 cm from the IPs, which may have reduced probability of detecting the pathogen in all infected plants.

Previous studies have shown that *X. fastidiosa* detection in stem tissues can decrease with increasing distances from IPs, whereas greater detection rates are generally observed in highly susceptible cultivars from plant tissues close to IPs at 1 year post inoculation (Román-Écija *et al.*, 2026). Therefore, even in cases where molecular detection is negative, this does not necessarily exclude successful infection. The bacterium may remain restricted to specific tissues or be unevenly distributed within host xylem. In such cases, infections can still trigger systemic host plant defence responses and physiological alterations, even when molecular detection at sampled positions is negative. Based on these assumptions, plants in the present study were categorised into three groups for spectral analysis comparisons: mock-inoculated control plants, plants confirmed as infected by *X. fastidiosa* via qPCR, and inoculated plants in which infection was not detected by molecular analyses.

Figure 3 summarises the automated hyperspectral image segmentation obtained for an olive plant of 'Arbosana' as a representative example. Figure 3A shows the mean smoothed reflectance spectra from regions of interest (ROIs) corresponding to scene categories within the images, which were used to train supervised machine-learning models for image segmentation. ROIs exhibited distinct spectral signatures, supporting their suitability for supervised training. Given these spectral differences among scene categories, accurate segmentation of pure vegetation pixels is critical to minimize spectral contamination and avoid confounding effects on plant trait retrieval associated with pathogen infection (Lowe *et al.*, 2017). Figure 3B illustrates a representative segmentation result obtained using a Linear SVM, presented as an example of the different classifiers used in the present study.

The model successfully classified the main structural components of the olive plants (leaves, secondary branches, trunk), enabling automated extraction of pure vegetation leaf pixels. However, all subsequent analyses were based on the ensemble model described above, which was quantitatively evaluated and revealed high performance, with an overall accuracy of 0.989, sensitivity of 0.842, specificity of 0.999, and a Cohen's kappa coefficient of 0.904. The high specificity values indicate a low rates of false positives, which is critical for avoiding spectral contamination from non-vegetation elements,

Table 1. Frequencies of detection (and numbers of cultivar detections) for two *Xylella fastidiosa* strains detected by using qPCR from stem tissues of different olive cultivars, each sampled at 15 cm from inoculation points at 12 months post inoculation.

Cultivar	<i>Xylella fastidiosa</i> strain	
	XYL1961/18 (%), (Cultivar No./total)	XYL1966/18 (%), (Cultivar No./total)
Arbequina	90.0 (9/10)	85.7 (6/7)
Arbosana	88.9 (8/9)	88.9 (8/9)
Cornicabra	70.0 (7/10)	44.4 (4/9)
Frantoio	50.0 (3/6)	14.3 (1/7)
FS17	60.0 (6/10)	60.0 (6/10)
Gordal	40.0 (4/10)	50.0 (5/10)
Hojiblanca	70.0 (7/10)	70.0 (7/10)
Leccino	50.0 (5/10)	33.3 (3/9)
Ogliarola	66.7 (6/9)	66.7 (6/9)
Picual	60.0 (6/10)	50.0 (4/8)

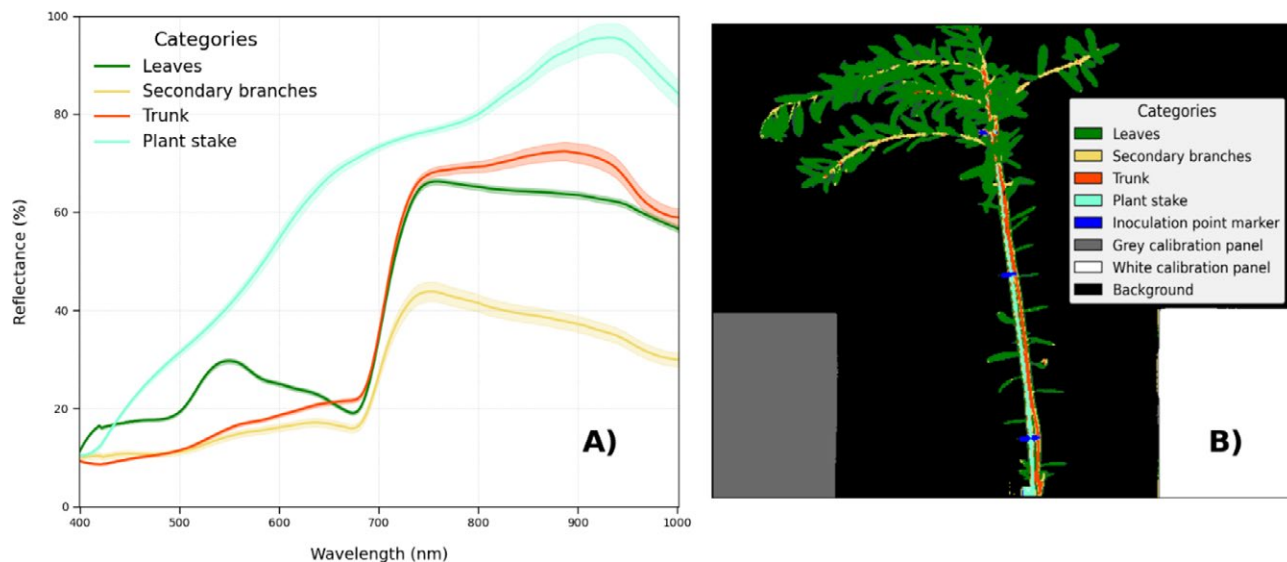


Figure 3. Spectral information generated from automated acquisition and processing of hyperspectral images using the phenotyping platform. A) Mean and smoothed leaf reflectance spectra (%) between 400 and 1000 nm, calculated from regions of interest (ROIs), and used to train machine learning algorithms for image segmentation. Shaded bands represent standard errors of the means. B) A classified hyperspectral image of the olive cultivar Arbosana, produced using a Linear SVM, as a representative example of an individual classifier.

while slightly lower sensitivity values reflect conservative classification that prioritizes extraction of pure leaf pixels. Accurate image segmentation using robust classification is therefore a critical step for reliable spectral assessments (i.e., automated extraction of pure leaf pixels), as the spectral mixing with branches, trunk tissue, or background elements is known to mask subtle spectral signals associated with plant physiological status or stress response (Berry *et al.*, 2025).

Figure 4 presents mean leaf reflectance spectra derived from hyperspectral images for the ten olive cultivars, that were assessed 12 months after inoculation with the two *X. fastidiosa* strains. Spectral signatures were calculated from pure leaf pixels automatically extracted from each image by segmentation and were grouped according to inoculation status. All the inoculated plants were asymptomatic at the time of image acquisition.

The spectral signatures exhibited expected shapes and relative reflectance levels for vegetation (Liu *et al.*, 2020), indicating adequate hyperspectral data acquisition and calibration by the phenotyping platform. The spectra displayed the characteristic reflectance pattern of vegetation, with low reflectance in the visible region (VIS, 400–700 nm) due to photosynthetic pigment absorption (approx. 5 to 10% reflectance), followed by sharp increases across the red-edge (680–750 nm), and high reflectance in the near-infrared region (NIR, 700–1000 nm), reaching values of ~40% (Oerke *et al.*, 2014; Ahmed *et al.*, 2023).

PCA of the hyperspectral reflectance data showed that PC1 (88.3% of the variance) and PC2 (10.2%) explained 98.5% of the variance. However, the score plots did not give clear separation among the treatments, as variation was largely driven by cultivar effects, masking treatment differences. Cultivar was therefore included as a blocking factor in subsequent analyses, to reduce background variability and improve detection of treatment-related responses. ANOVA carried out for the first ten PCs revealed statistically significant treatment effects on PC1 ($F = 4.08$, $P = 0.018$), PC5 ($F = 11.83$, $P < 0.001$) and PC7 ($F = 3.80$, $P = 0.024$). *Post hoc* Tukey tests indicated that for PC5, non-inoculated plants differed from the plants in both inoculated groups ($P < 0.05$), with no differences ($P > 0.05$) between inoculated qPCR-negative and qPCR-positive plants (Figure 5 B). In contrast, for PC1 and PC7, differences ($P < 0.05$) were only found between non-inoculated and inoculated qPCR-positive plants (Figure 5, A and C).

Analysis of the PCA loadings showed that each statistically significant component was associated with different spectral regions. PC5 was mainly associated with the blue (399–435 nm) and red-edge (718–740 nm) regions, both with negative loadings, with more negative scores in inoculated plants. These indicated greater reflectance in these bands (Figures 4 and 5 B). PC1 showed positive loadings across the full spectral range, with strongest contributions in near infrared (880–945 nm). Therefore, the greater scores observed in inoculated qPCR-positive plants indicated overall increases

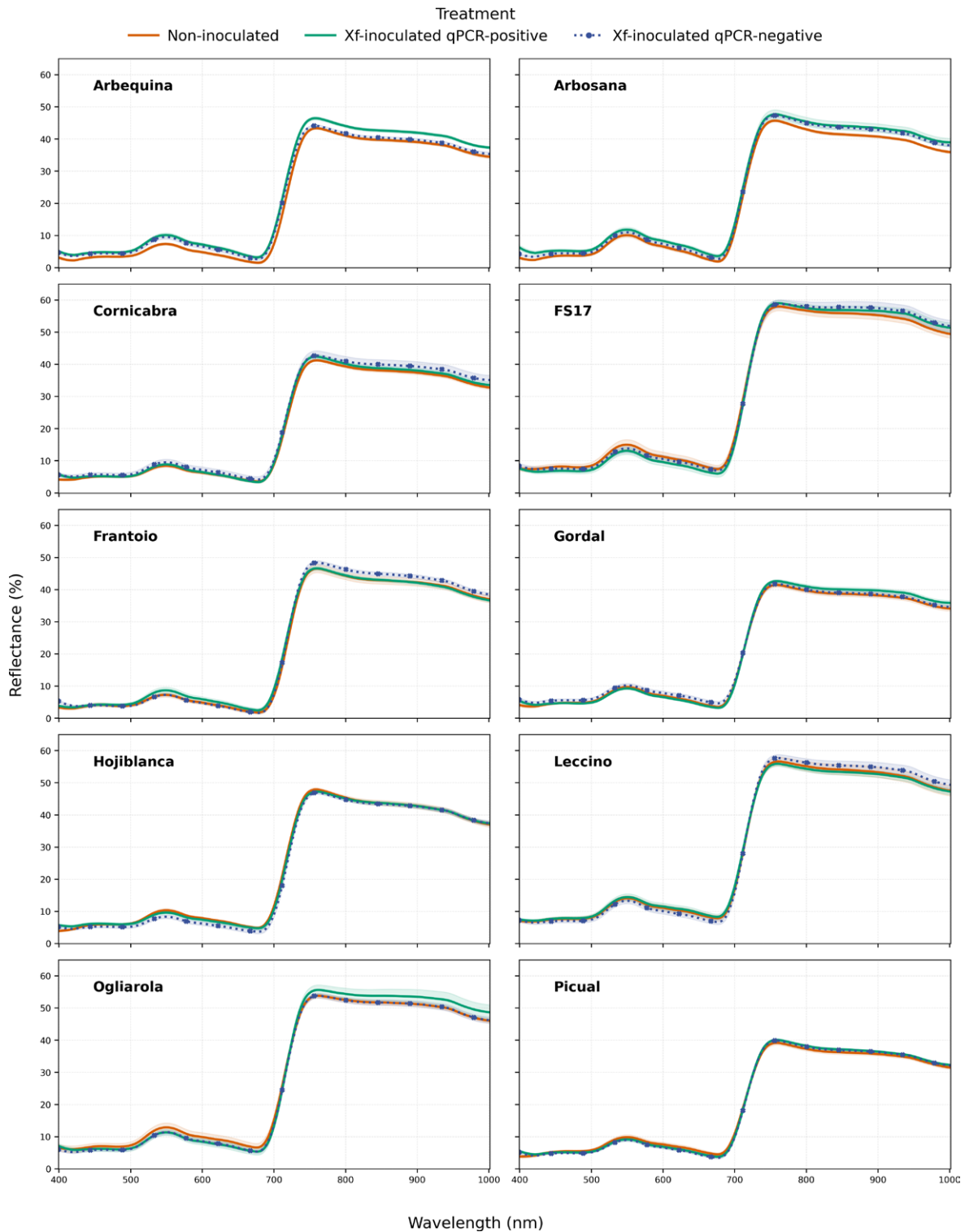


Figure 4. Mean leaf reflectance spectra (between 400 and 1000 nm) for ten olive cultivars 12 months after inoculations with two *Xylella fastidiosa* strains. All the *X. fastidiosa* (Xf)-inoculated olive plants were asymptomatic at the time of hyperspectral image acquisition. Spectral signatures were obtained through automated acquisition and processing, using a phenotyping platform. The three curves for each cultivar represent, respectively, non-inoculated plants, qPCR-positive inoculated plants, and qPCR-negative inoculated plants. Shaded bands represent standard errors of means. The number of qPCR-positive and negative inoculated plants varied among cultivars and *X. fastidiosa* strains, according to qPCR detection frequencies shown in Table 1. Non-inoculated controls included eight plants per cultivar.

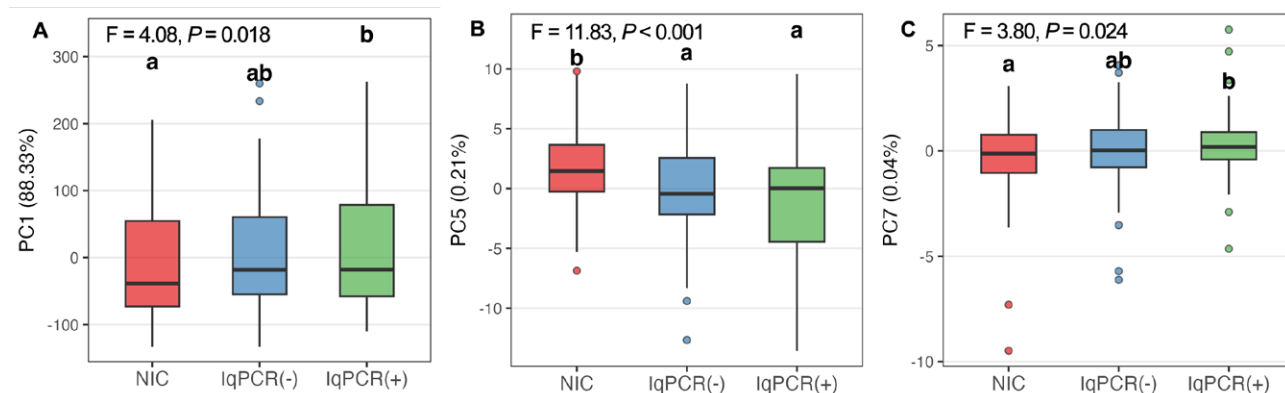


Figure 5. Box plots of principal component scores by treatment group for the three principal components (PCs), showing statistically significant differences among treatments (NIC = non-inoculated plants; IqPCR(-) = *Xylella fastidiosa* inoculated and qPCR negative plants; IqPCR(+) = *X. fastidiosa* inoculated and qPCR positive plants). ANOVAs were carried out for the first ten PCs, using olive cultivar as a blocking factor. PC1 (A, 88.33%), PC5 (B, 0.21%) and PC7 (C, 0.04%) were the only components yielding significant ($P < 0.05$) treatment effects. Differences between group means were assessed by Tukey's *post hoc* test. Letters above the boxes indicate statistically homogeneous groups ($P < 0.05$).

in reflectance, especially in this region (Figures 4 and 5 A). PC7 showed contrasting loadings between the green (515–575 nm, negative) and red (662–685 nm, positive) regions, although the treatment differences were small (Figure 5 C).

At the multivariate level, PERMANOVA showed a statistically significant effect of treatment on the overall spectral dissimilarity matrix ($R^2 = 0.005$, $F = 0.75$, $P = 0.014$). Pairwise comparisons confirmed that non-inoculated plants differed significantly from the inoculated qPCR-positive plants ($P = 0.027$) and the inoculated qPCR-negative plants ($P = 0.030$), while no difference ($P = 0.498$) was found between the two inoculated groups. Homogeneity of multivariate dispersion among the treatment groups was confirmed by a betadisper test ($F = 0.04$, $P = 0.960$), indicating that the differences detected by PERMANOVA reflected true location effects rather than differences in within-group variance.

The spectral differences observed between non-inoculated and inoculated plants are in line with previous evidence of physiological alterations caused by *X. fastidiosa* in olive. In particular, the greater reflectance in the blue and red-edge regions captured by PC5 was previously detected at canopy scale in *X. fastidiosa*-infected olive trees before visible symptoms appeared (Zarco-Tejada *et al.*, 2018; Poblete *et al.*, 2021). This pattern was shared by qPCR-negative and qPCR-positive inoculated plants, indicating that the inoculation process, even in the absence of confirmed systemic infections, may have induced pre-symptomatic physiological plant responses detectable at the spectral level. In contrast, the additional separation of non-inoculated and qPCR-positive

plants along PC1 and PC7, associated with near-infrared and chlorophyll-related wavelengths, indicated stronger infection-associated spectral alterations in plants where the pathogen was successfully detected by qPCR. Nevertheless, inoculated plants with negative qPCR results also differed significantly from non-inoculated controls in PC5, indicating that infection-related spectral responses may occur even when the pathogen is not detected in sampled tissue. This pattern is consistent with a gradient response, in which systemic infections in plants with molecularly confirmed infection induces broader physiological changes, potentially linked to modifications in leaf structure and pigment disruption, as previously reported in olive plants infected by *X. fastidiosa* (Román-Écija *et al.*, 2026). However, the relatively small magnitude of these differences, particularly for PC7, suggests that their biological significance should be interpreted with caution and further analyses are required to clarify their relevance.

The strong genotype effect in modulating the spectral expression of infection highlights the importance of incorporating genotype-specific information into spectral classification models and cautions against assuming uniform spectral responses across olive germplasm. Overall, despite the low proportions of variance explained by the discriminant components, the consistency of treatment effects across univariate and multivariate analyses supports the existence of subtle, but real, specific spectral signatures associated with *X. fastidiosa* inoculation in olive under controlled conditions. Precise hyperspectral image segmentation and automated extraction of pure leaf pixels were essential for reliably

capturing these subtle spectral differences, avoiding spectral mixing effects that could otherwise mask early plant physiological alterations associated with infections.

The high-quality spectral data generated by the developed phenotyping platform provide a valuable resource for future integration with automated pre-symptomatic detection models for *X. fastidiosa* infections, with potential applicability beyond olive to other woody host plants and crops. Such approaches may improve detection of infections at asymptomatic stages, and facilitate detailed characterization of plant physiological responses associated with vascular pathogens. In addition, the platform developed in the present study offers a robust tool for high-throughput plant phenotyping, enabling rapid evaluation of olive genotype responses to infection. Implementation of robust early-detection and phenotyping methods for assessments of economically important crops could strengthen *X. fastidiosa* surveillance and improve disease management strategies, while supporting plant breeding programmes aimed at identifying and developing resistant cultivars.

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