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Evidence of post-formed structures during systemic *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* infection in resistant *Phaseolus vulgaris* genotypes

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Abstract

Bacterial wilt of the common bean caused by *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* results in economic losses. The aim of this study was to analyze the colonization of *C. flaccumfaciens* pv. *flaccumfaciens* in resistant, moderately resistant, and susceptible genotypes of common bean plants. The genotypes Ouro Branco and IPA 9 (resistant), Diacol Calima (moderately resistant), and CNFRS 11997 and CNFP 10429 (susceptible) were inoculated in the epicotyl, with 100 µL of bacterial suspension of the BRM 14933(*Cff25*). Disease severity was evaluated 21 days after inoculation (DAI), on a scale from 1 to 9. Plant samples were prepared for scanning electron microscopy analyses. Ouro Branco and IPA 9 (resistant) plants exhibited low colonization, the formation of filaments surrounding bacterial cells and vestures more developed in the pit the xylem vessels. Diacol Calima (moderately resistant) plants presented lower levels of colonization and filament formation than that of resistant cultivars. CNFC 10429 and CNFRS 11997 (susceptible) showed high levels of colonization in the xylem and vessel obstruction, preventing water and nutrient flow, which explains the symptoms of wilt and plant death. Thus, resistance to *C. flaccumfaciens* pv. *flaccumfaciens* can be explained by plant's capacity to limit pathogen propagation as a post-formed defense mechanism in this pathosystem.

Keywords: bacterial wilt; plant-pathogen interaction; susceptibility.

Evidência de estruturas pós-formadas na infecção sistêmica de *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* em genótipos resistentes de *Phaseolus vulgaris* L.

Resumo

Curtobacterium flaccumfaciens pv. *flaccumfaciens* é causadora da murcha-de-curtobacterium, responsável por perdas econômicas. O objetivo deste estudo foi analisar a colonização de *C. flaccumfaciens* pv. *flaccumfaciens* em genótipos de feijoeiro comum resistente, moderamente resistente e suscetível. Ouro Branco e IPA 9 (resistente), Diacol Calima (moderadamente resistente), CNFRS 11997 e CNFP 10429 (suscetíveis) foram inoculados, no epicótilo, com 100 µL de suspensão bacteriana do isolado BRM 14933(*Cff25*). A severidade da doença foi avaliada 21 dias após a inoculação, utilizando a escala de 1 a 9. As amostras para MEV foram desidratadas em série alcoólica, secas em ponto crítico com dióxido de carbono (CO_2), banhadas em ouro e analisadas em microscópio eletrônico de varredura. As plantas de Ouro Branco e IPA 9 (resistentes) exibiram baixa colonização, formação de filamentos envolvendo células bacterianas e guarnições mais desenvolvidas nas pontoações dos vasos do xilema. Diacol Calima (moderadamente resistente) apresentou menor colonização e formação de filamentos do que as cultivares resistentes. Os genótipos CNFC 10429 e CNFRS 11997 (suscetíveis) mostraram grande colonização no xilema, com vasos obstruídos, impedindo o fluxo de água e nutrientes, explicando os sintomas de murcha e morte da planta. Portanto, a resistência à *C. flaccumfaciens* pv. *flaccumfaciens* pode ser explicada pela capacidade da planta em limitar a multiplicação do patógeno como um mecanismo de defesa celular, sugerindo que este é um dos fatores de resistência estrutural pós-formado que ocorre nesse patossistema.

Palavras-chaves: murcha bacteriana; interação planta-patógeno; suscetibilidade.

1 Introduction

Bacterial wilt of the common bean plant (*Phaseolus vulgaris* L.), caused by *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (Cff), a vascular pathogen that occurs in the Americas (Brazil, Canada, and the USA), Australia, and Iran (OSDAGHI *et al.*, 2020). In Brazil, it has been reported in several producing regions, such as the South and Southeast, in Goias state, and in the Federal District (MARINGONI; ROSA, 1997; WENDLAND *et al.*, 2009; UESUGI *et al.*, 2003).

The disease causes economic losses in terms of yield and commercialization due to changes in the size, shape, and appearance of the seeds (HUANG *et al.*, 2009). In Brazil, the pathogen is disseminated in several bean producing regions, causing great economic losses, being considered a threat to the crop due to the occurrence of epidemics in the field resulting in productivity losses (PUIA *et al.*, 2021). In bean fields in Canada and research plots in Nebraska, up to 25% of bean seeds from infected plants showed symptoms (HARVESON *et al.*, 2006; 2015). Bacterial wilt control includes use of resistant cultivars, healthy seeds, and crop rotation (HALL, 1991; RAVA; COSTA, 2001; MARINGONI, 2002; OSDAGHI *et al.*, 2020).

C. flaccumfaciens pv. *flaccumfaciens* is an aerobic, gram-positive, mobile bacterium with straight or slightly curved rods. The pathogen survives in seeds and soil, infects through wounds and natural openings (SAETTLER, 1991; MARINGONI, 2002; OSDAGHI *et al.*, 2020) and colonizes the plant vascular system, resulting in darkening of the xylem vessels and obstruction of the vascular tissues. Symptoms of the disease are observed throughout the plant, including mosaic leaf patterns, flaccid leaves, wrinkled and burned leaf edges, dwarfing, wilt, and death (SAETTLER, 1991; WENDLAND *et al.*, 2009; MARINGONI *et al.*, 2015).

Analyzing pathogen colonization and location within specific plant tissues is critical for understanding plant-host interactions. High-resolution imaging, which allows researchers to clearly view the plant pathogen interacting with a specific plant cell, is needed to enhance our understanding of pathogen lifestyle and virulence mechanisms. However, it can be challenging to locate the pathogen on the plant surface or in a specific cell type (CALDWELL; IYER-PASCUZZI, 2019).

Multiplication of the bacteria in the xylem causes wilting, preventing the transport of water and nutrients to other plant tissues and organs. Cff cell masses are embedded in a matrix that absorbs water from surrounding cells, leading to water deficits in plant tissues (OSDAGHI *et al.*, 2020). The biofilm formed by the bacteria also plays an important role in the pathogenicity and aggressiveness of the pathogen, considering that its colonization completely obstructs the vessels, causing permanent wilting of the entire plant (HARDING *et al.*, 2019).

Interaction between common bean tissues and Cff can be analyzed by microscopy. For example, using optical microscopy, Maringoni *et al.* (2015) found that the colonization rate and percentage of primary xylem vessel obstruction were lower in resistant than susceptible cultivars. Additionally, Souza *et al.* (2006) used scanning electron microscopy (SEM) observed the activation of structural and biochemical defense mechanisms in resistant genotypes, as well as bacterial agglutination with filaments and tangled structures under punctuations of the xylem vessel.

Valdo *et al.* (2016) found that the genotypes IPA 9, Ouro Branco, Michelite, BRS Requinte and TU showed specific resistance to the major strains of Cff-induced wilt, whereas Coquinho, BRS Cometa, CNFP 10104, BRS Requinte, and A211 exhibited horizontal resistance. These results encouraged to research possible morphological and structural differences in the xylem of common bean genotypes differing in susceptibility to Cff.

In order to better understand plant-pathogen interactions, this study aimed to analyze the colonization of *C. flaccumfaciens* pv. *flaccumfaciens* in resistant, moderately resistant, and susceptible genotypes of common bean plants.

2 Material And Methods

2.1 Bacterial strains and genotypes

C. flaccumfaciens pv. *flaccumfaciens* BRM 14933 (*Cff25)* obtained from the Phytopathogenic and Multifunctional Microorganisms Collection of Embrapa Rice and Beans, was selected because it is the most aggressive strain on common bean according Valdo *et al.* (2016). The genotypes Ouro Branco and IPA 9 (resistant), Diacol Calima (moderately resistant), and CNFRS 11997 and CNFP 10429

(susceptible) were selected based on their response to Cff (WENDLAND *et al.*, 2012). They were obtained from the Common Bean Breeding Program and Genetic Germplasm Collection of Embrapa Rice and Beans.

2.2 Bacterial wilt evaluation

The experiment was conducted under controlled greenhouse conditions, using a completely randomized design with four plants. The genotypes were sown in 2-L vases with a 2:1 soil-to-substrate ratio. The strain was multiplied on nutrient agar medium and incubated at 28 °C for 48 h. The bacterial suspension was diluted in water and adjusted to a concentration of 10^8 ufc mL^{-1}. Inoculation was performed 10 days after sowing via two perforations in the epicotyl of each plant, with 100 µL of bacterial suspension. The control was inoculated with 100 µL of sterile distilled water. Disease severity was evaluated 21 days after inoculation (DAI), on a scale from 1 to 9, as proposed by Wendland *et al.* (2009), as follows: 1 – no symptoms; 2 – presence of mosaic on the leaves; 3 – leaf flaccidity; 4 – leaf flaccidity and mosaic; 5 – leaf flaccidity or mosaic, associated with burning or wrinkling of the leaf edges; 6 – dwarfism; 7 – dwarfism associated with other symptoms, except plant wilt; 8 – plant wilt associated with other symptoms; and 9 – plant death.

2.3 Scanning Electron Microscopy

Samples were collected 2 cm above and 2 cm below the inoculation site at 21 DAI (days after inoculation). The 0.1 cm-thick longitudinal sections were fixed in 2.5% glutaraldehyde (diluted in phosphate buffer solution – 0.2 M; pH 7.2) for 24 h. The samples were washed in cacodylate buffer (0.1 M; pH 7.0) three times for 10 min and dehydrated through a graded ethanol series. After drying in liquid CO_2 (Autosamdri®-815, Series A critical point dryer), the samples were covered with gold films (Denton Vacuum, LLC, Moorestown, NJ, USA). A JSM-6610 scanning electron microscope (JEOL Ltd, Tokyo, Japan) was used to generate the images at 4 kV. The analyzes were performed at the High-Resolution Microscopy Laboratory (LabMic) at the Physics Institute of the Federal University of Goias (UFG), in Goiânia, Goiás (GO), Brazil.

3 Results And Discussion

We used genotypes, previously evaluated by Valdo *et al.* (2016). The authors observed specific and non-specific interactions between Cff strains and common bean genotypes. In the present study, the same genotypes were used to analyze the xylem vessels colonized by Cff in resistant, moderately resistant, and susceptible genotypes by SEM.

IPA 9 and Ouro Branco plants exhibited flaccidity (score 3), Diacol Calima plants flaccidity, wrinkling, and burning at the leaf edge (score 5), and CNFRS 11997 and CNFP 101429 presented with dwarfism and wilt (score 8).

In the control plants, bacteria were not observed in the xylem vessels (helical and with vestured pits) (Figure 1A-F). Bacterial cells were present in the xylem of CNFC 10429, CNFRS 11997 (susceptible) and Diacol Calima (moderately resistant) (Figure 2A, F). These cells promoted the occlusion of helical vessels (Figure 2 A, C, E) and adhered to the vestured pits (Figure 2B, D, F). This explains the severe symptoms, such as leaf burning and wrinkling, wilt, and death in CNFC 10429. According to Maringoni (2002), in Carioquinha and IAC Carioca-Common genotypes (susceptible), bacteria colonize vessels without the development of structures related to resistance and multiplication. Fritschi (2008) analyzed the xylem vessels of grapevines and observed occlusions caused by another vascular pathogen, *Xylella fastidiosa,* which formed large aggregates of cells.

Figure 1. Micrographs of xylem vessels of common bean plant genotypes 20 days after inoculation with sterile distilled water (control plants). A – Ouro Branco. Bar = 20 µm. B – IPA 9. Bar = 2 µm. C – Diacol Calima. Bar = 5 µm. D – CNFRS 11997. Bar = 5 µm. E – CNFC 10429. Bar = 50 µm. F – Detail of the xylem of the CNFC 10429 lineage. Bar = 10 µm.

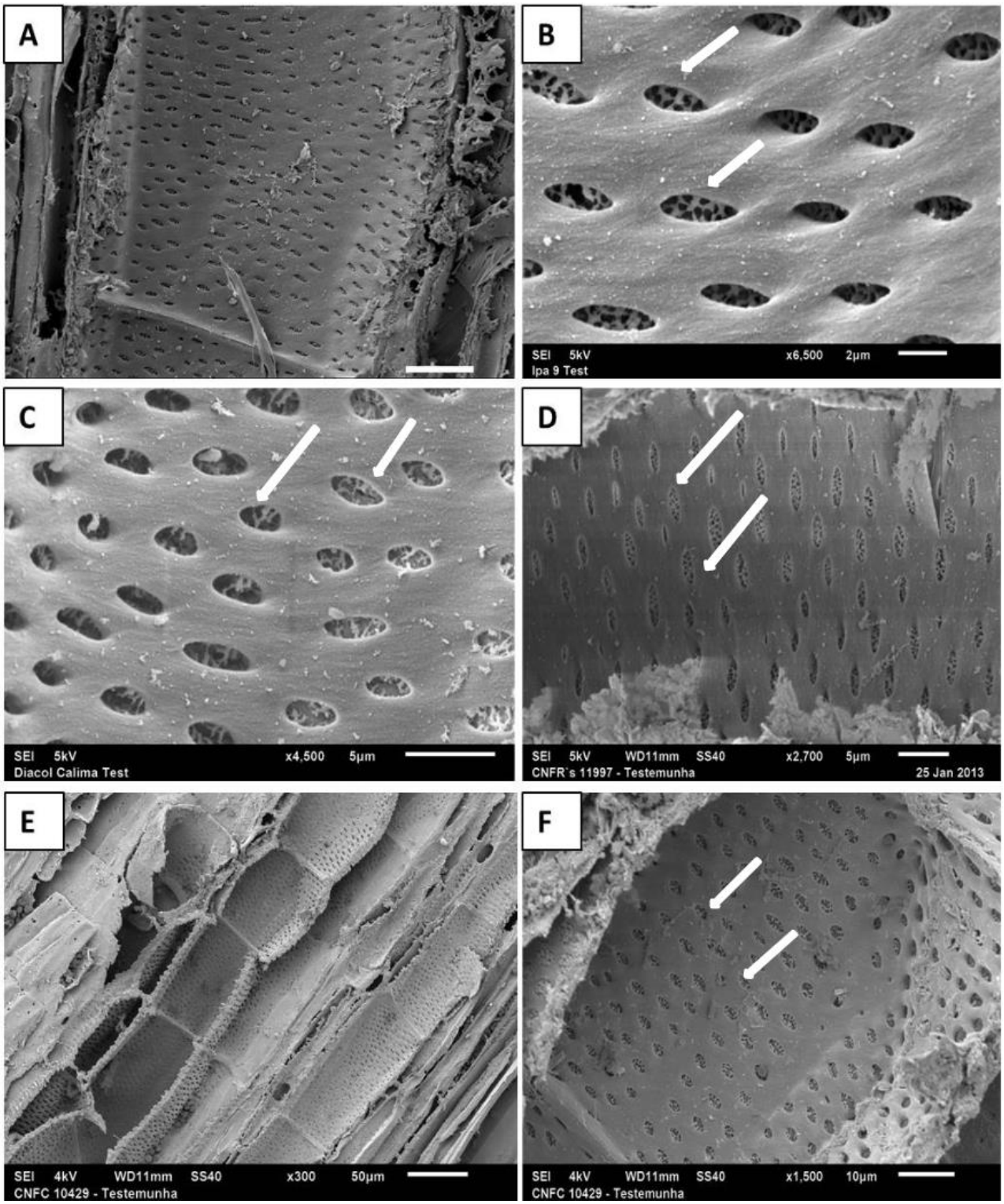
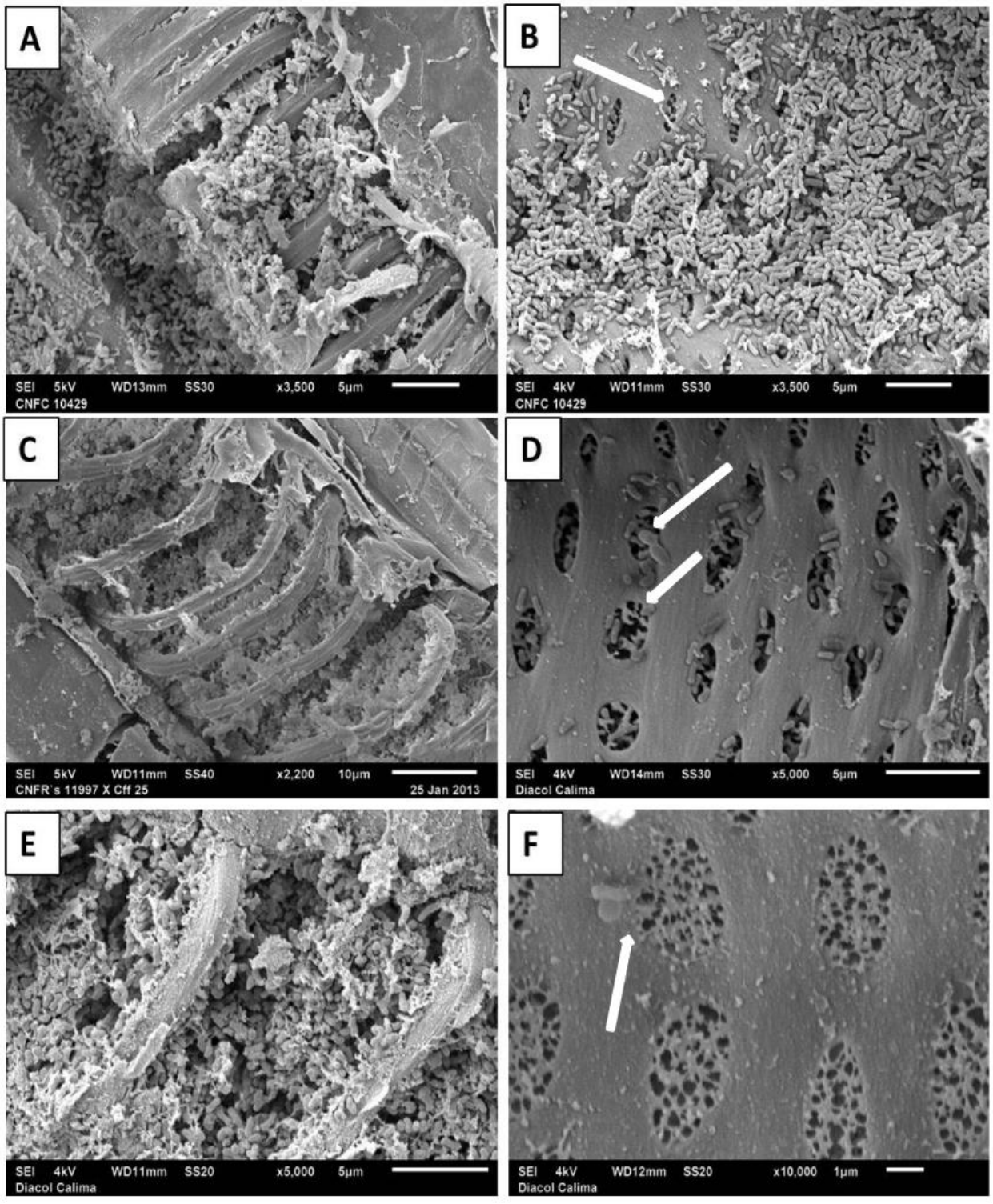


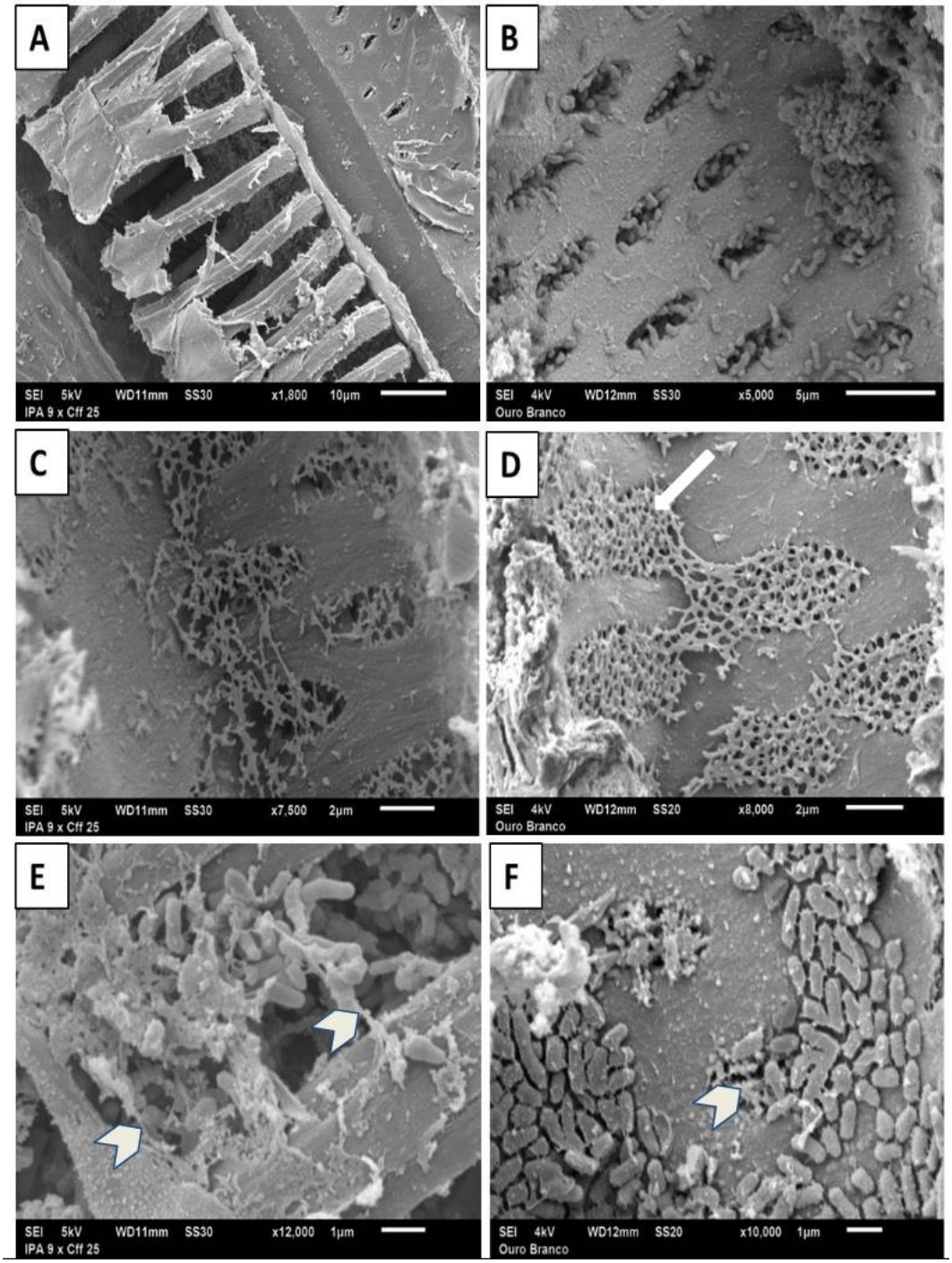
Figure 2. Micrographs of xylem vessels of common bean plant genotypes susceptible and moderately resistant to *C. flaccumfaciens* pv. *flaccumfaciens* inoculated with BRM 14933. A – Vessel with helical thickening of the CNFC 10429 genotype obstructed by bacterial cells. Bar = 5 µm. B – Vessel with vestured pits of the CNFC 10429 genotype with bacterial cells adhered to the wall. Bar = 5 µm. C –Vessel with helical thickening of the CNFRS 11997 genotype obstructed by bacterial cells. Bar = 10 µm. D – Details of the vessels of the Diacol Calima genotype with bacterial cells on the vestured pits. Bar = 5 µm. E – Detail of the bacterial cells connected by filaments (*) in the vessel with helical thickening of the Diacol Calima genotype. Bar = 5 µm. F – Detail of the vessel of the Diacol Calima genotype with few bacterial cells and more developed vestures in the pits. Bar = 1 µm. Arrow = vestured pits with bacteria.



In Diacol Calima, bacterial cells were observed in helical vessels and vestured pits. However, several vessels contained fewer bacterial cells (Figure 2D, F). Moreover, unlike the CNFC 10429 and CNFRS 11997 genotypes, Diacol Calima exhibited filaments associated with bacterial cells (Figure 2E). These filaments are of unknown origin that require further studies to investigate their function and nature.

IPA 9 and Ouro Branco (resistant) showed lower levels of colonization than the other genotypes, and no completely obstructed xylem vessels (Figures 3A, B). In these plants, more developed vestures in the pits were also observed, suggesting a plant defense mechanism to prevent the entry of bacteria (Figure 3C, D). The symptoms of the resistant genotypes were less severe possibly because these plants exhibited vestures more developed in the pit of the xylem vessels, a post-formed structural defense response that reduced the bacterial colonization beyond the infected area, evident in the flaccidity symptom (score 3) observed in Ouro Branco. Souza *et al.* (2006) also observed vestures more developed in the pit of the xylem vessels (called tangled structures) in the Ac-297, Ac-405, and Ac-592 cultivars of common bean plants, which are considered resistant to Cff. This defense mechanism prevents bacterial movement to other areas of the plant, thereby reducing the bacterial population (MARINGONI *et al.*, 2015). Pascholati and Leite (2011) also reported that resistance structures associated with the xylem may prevent movement of the pathogen to other plant parts. In the present study a greater response ability in resistant compared do susceptible genotypes was observed. In the interaction between *Xanthomonas axonopodis* pv. *phaseoli* and commom bean, bacterial cells were also observed in the intercellular spaces of moderately resistant genotypes (CONTRERAS, 2001). In the present study a greater response ability in resistant compared do susceptible genotypes was observed.

Figure 3. Micrographs of xylem vessels of common bean plant genotypes resistant to *C. flaccumfaciens* pv. *flaccumfaciens* inoculated with BRM 14933. A – Vessel with helical thickening of the IPA 9 genotype. Bar = 10 µm. B – Detail of vessels with vestured pits of the Ouro Branco genotype. Bar = 5 µm. C-D – Detail of vessels of the IPA 9 genotype and Ouro Branco genotype, with more developed vestures in the pits (arrow). Bar = 2 µm. E – Detail of the bacterial cells collected by filament in the vessel of the IPA 9 genotype (arrow head). Bar = 1 µm. F – Detail of the bacterial cells adhered to the wall and collected by filament in the vessel of the Ouro Branco genotype (arrow head). Bar = 1 µm.



Diacol Calima, IPA 9 and Ouro Branco plants also formed a larger number of narrow elongated filaments surrounding bacterial cells than the susceptible plants. However, the formation of filaments was more pronounced in the resistant IPA 9 and Ouro Branco genotypes, where they were interconnected with bacterial cells (Figure 3E, F). These filaments likely

hindered the proliferation of Cff from the inoculation site to other regions of the xylem. Additionally, the filaments adhered to the xylem vessels wall (Figure 3F), potentially preventing the multiplication and movement of the bacteria toward the surrounding regions and vessels, consequently reducing total bacterial population.

In common bean genotypes resistant to Cff studied here, filaments and vestures were more developed in the pit of the xylem vessels as a potential defense response to bacterial colonization. The SEM analyses provided information on pos-formed resistance structures to limit bacterial multiplication, confirming the occurrence of differential interactions between commom bean genotypes and Cff.

Cff-resistant genotypes can be used as sources of resistance in the common bean breeding program, being a strategy that can reduce the use of pesticides to control this disease. Future studies on other defense mechanisms such as biochemical can be performed. These studies show morphological differences involved in the response of the plant to the pathogen, interfering in the susceptibility according to the analyzed genotype. The structures can be identified and the selection of resistant cultivars can be selected based on markers that allow distinguishing this characteristic among common bean lines.

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Ethical Statement: The authors declare that the research was conducted in the absence of any conflict of interest.

Data availability statement: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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