NIKOLAS EMANUEL CHAVES SILVA

CONTRIBUTIONS TO THE UNDERSTANDING OF THE MECHANISMS UNDERLYING THE SUSCEPTIBILITY AND RESISTANCE OF COMMON BEAN TO *Xanthomonas citri* pv. *fuscans*

Dissertation submitted to the Plant Pathology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

Adviser: Jorge Luis Badel Pacheco

Co-adviser: Fabrício de Ávila Rodrigues

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“There are no facts, only interpretations. [...] *All things are subject to interpretation. Whichever interpretation prevails at a given time is a function of power and not truth”.*

(F.W. Nietzsche)

ABSTRACT

CHAVES-SILVA, Nikolas Emanuel, M.Sc., Universidade Federal de Viçosa, July, 2023. **Contributions to the understanding of the mechanisms underlying the susceptibility and resistance of common bean to *Xanthomonas citri* pv. *fuscans***. Adviser: Jorge Luis Badel Pacheco. Co-adviser: Fabrício de Ávila Rodrigues.

Common Bacterial Blight (CBB) is a disease of common bean (*Phaseolus vulgaris* L.) caused by *Xanthomonas citri* pv. *fuscans* (*Xcf*) and *X. phaseoli* pv. *phaseoli* with significant importance worldwide. The most recommended control method for the disease is the use of resistant cultivars. Several studies have shown that the resistance of common bean to CBB is primarily non-race-specific, quantitative and oligogenic. In previous studies conducted by the Laboratory of Molecular Phytobacteriology at the Universidade Federal de Vicosa, varieties resistant and susceptible to CBB were identified among a set of Brazilian varieties, but the mechanisms subjacent to these reactions during the common bean-*Xanthomonas* interactions are not fully understood. It is known that during the early stages of plant-bacteria interactions, plant hormones such as salicylic acid, jasmonic acid, and ethylene, accumulate in plant tissue concomitant with activation of other responses associated with the oxidative stress and key defense enzymes, such as phenylalanine ammonia-lyase. In order to subvert the plant defense response and to acquire nutrients, some phytopathogenic *Xanthomonas* species produce Transcriptional Activator-Like Effector (TALE) proteins, which are injected into the plant cell cytoplasm through a type III secretion system. Then, these TALE proteins reach the plant nucleus where they activate the expression of susceptibility genes. Identifying the repertoire of TALE proteins produced by *Xanthomonas* and their putative host target genes can provide important insights into the mechanisms that determine plant susceptibility. This Master's degree dissertation aimed to predict the TALE repertoire in a set of 39 *Xcf* strains and their *P. vulgaris* gene targets using genome sequences publicly available in GenBank (Chapter 1), and to assess differences in the activity of enzymes involved in plant defense and oxidative stress between *P. vulgaris* genotypes resistant (BC_3F_4 derived from a BRS Radiante × Carioca MG cross) and susceptible (Carioca MG) to *Xcf* (Chapter 2). A total of 56 different TALE genes were identified in the investigated *Xcf* strains, which were predicted to have six conserved binding sites in the *P. vulgaris* genome. Overall, higher defense-related enzyme activities were observed in the resistant genotype compared

to the susceptible one at 48 h post-inoculation with *Xcf*, which was consistent with a significant difference in bacterial population sizes in leaf tissue between the genotypes. These results provide important insights into the molecular mechanisms underlying the susceptibility and biochemical mechanisms subjacent to the resistance of *P. vulgaris* to *Xcf*.

Keywords: Common Bacterial Blight. Plant-defense enzymes. *Phaseolus vulgaris*. TALE.

RESUMO

CHAVES-SILVA, Nikolas Emanuel, M.Sc., Universidade Federal de Viçosa, julho de 2023. **Contribuições ao entendimento dos mecanismos relacionados à suscetibilidade e resistência do feijoeiro a *Xanthomonas citri* pv. *fuscans***. Orientador: Jorge Luis Badel Pacheco. Coorientador: Fabrício de Ávila Rodrigues.

O Crestamento Bacteriano Comum (CBC) é uma doença do feijoeiro (*Phaseolus vulgaris* L.) causada por *Xanthomonas citri* pv. *fuscans* (*Xcf*) e *X. phaseoli* pv. *phaseoli*, com grande importância mundialmente. O método de controle mais recomendado para a doença é o uso de cultivares resistentes. Vários estudos têm mostrado que a resistência do feijoeiro ao CBC é principalmente quantitativa e oligogênica. Em estudos anteriores conduzidos pelo Laboratório de Fitobacteriologia Molecular da Universidade Federal de Viçosa, foram identificadas variedades resistentes e suscetíveis ao CBC em um conjunto de variedades brasileiras, mas os mecanismos subjacentes a essas reações durante as interações entre o feijoeiro comum e *Xanthomonas* não estão completamente elucidados. Sabe-se que durante os estágios iniciais das interações planta-bactéria, hormônios vegetais, como ácido salicílico, ácido jasmônico e etileno, acumulam-se nos tecidos vegetais, juntamente com a ativação de outras respostas associadas ao estresse oxidativo e enzimas de defesa-chave, como fenilalanina amônia-liase. Para subverter a resposta de defesa da planta e adquirir nutrientes, algumas espécies fitopatogênicas de *Xanthomonas* produzem efetores similares a fatores de ativação de transcrição (*Transcriptional Activator-Like Effector;* TALEs), que são injetados no citoplasma da planta por meio do sistema de secreção tipo III. Em seguida, essas proteínas TALE alcançam o núcleo da planta, onde ativam a expressão de genes de suscetibilidade. Identificar o repertório de proteínas TALE produzidas por *Xanthomonas* e seus possíveis genes alvos no hospedeiro pode fornecer informações importantes sobre os mecanismos que determinam a suscetibilidade da planta. Esta dissertação de mestrado teve como objetivo predizer o repertório de TALEs em um conjunto de 38 estirpes de *Xcf* e seus alvos gênicos em *P. vulgaris* usando sequências genômicas disponíveis publicamente no GenBank (capítulo 1) e avaliar as diferenças na atividade de enzimas envolvidas na defesa das plantas e no estresse oxidativo entre genótipos de *P. vulgaris* resistente (BC_3F_4 derivada do cruzamento BRS Radiante × Carioca MG) e suscetível (Carioca MG) a *Xcf* (capítulo 2). Um total de 56 genes TALE diferentes foram identificados no

grupo de estirpes de *Xcf* estudado, os quais foram preditos como tendo seis sítios de ligação conservados no genoma de *P. vulgaris*. No geral, foram observadas atividades de enzimas relacionadas à defesa mais altas no genótipo resistente em comparação ao genótipo suscetível 48 h pós-inoculação com *Xcf*, o qual foi consistente com diferenças nos tamanhos das populações bacterianas nos tecidos foliares entre os genótipos. Esses resultados fornecem informações importantes sobre os mecanismos moleculares relacionados à suscetibilidade e os mecanismos bioquímicos subjacentes à resistência de *P. vulgaris* a *Xcf*.

Palavras-chave: Crestamento Bacteriano Comum. Enzimas de defesa vegetal. *Phaseolus vulgaris*. TALE.

SUMMARY

1 GENERAL INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is a highly important leguminous crop worldwide due to its nutritional value and socioeconomic significance (UEBERSAX et al., 2023). In Brazil, it ranks as the fourth most produced crop (CONAB, 2023) and holds cultural relevance as a traditional main dish for the population (MASSARANI et al., 2015; ANTUNES et al., 2021). Most of the common bean production is carried out by smallholder farmers (VIDAL et al., 2007; NAY et al., 2019; SANTOS et al., 2020), which directly influences the application of management practices to the crop. During the 2021/22 season, Brazil produced approximately 2,990 million tons of common bean, with domestic consumption accounting for 2,850 million tons (95.3% of total production).

Production of common bean is threatened by Common Bacterial Blight (CBB), a disease caused by the Gram-negative bacteria *Xanthomonas citri* pv. *fuscans (Xcf*) and *Xanthomonas phaseoli* pv. *phaseoli* (*Xpp*), which is considered the most damaging disease of the crop worldwide (WALLEN; JACKSON, 1975; GILLARD et al., 2009). The symptoms start as water-soaked lesions in the leaves that progressively become necrotic and surrounded by chlorotic haloes. The disease can also affect the stems, pods, and seeds (AKHAVAN et al., 2013). In Brazil, CBB is considered an important disease that is partially responsible for the low productivity of common bean in comparison with other countries. In the country, CBB is widely distributed and present in all production areas, such as in the states of Paraná, Minas Gerais, Goiás, and Rio Grande do Sul (DA SILVA JÚNIOR et al., 2022).

Susceptible plant genotypes exhibit considerable yield losses due to CBB depending on the prevailing environmental conditions (WALLEN; JACKSON, 1975; GILLARD et al., 2009). Although chemical compounds to control the disease are frequently recommended (DA SILVA JÚNIOR et al., 2022; MAPA), their efficacy in the field remains low (MARINGONI, 1990). Currently, the recommended strategies for CBB management are based on preventive techniques such as crop rotation and the use of pathogen-free seeds. The utilization of resistant common bean genotypes is highly desirable for disease management due to its effect in reducing pesticide use and its associated negative impact on human health, as well as on the environment. The use of resistant varieties also causes a reduction in production costs, improves the competitiveness of smallholder farmers, and is readily adopted by large-scale

producers (SINGH; SCHWARTZ, 2010; MIKLAS et al., 2017; DA SILVA JÚNIOR et al., 2022).

Plant resistance to pathogens has been traditionally categorized into two types: qualitative and quantitative (JONES; DANGL, 2006). Qualitative resistance is usually governed by a single dominant gene encoding a protein capable of directly or indirectly recognize the presence of a pathogen effector inside the plant tissues (MARONE et al., 2013). This recognition triggers a strong resistance response that halts pathogen colonization of plant tissue and disease symptom development, which contrasts with the different levels of disease severity observed in plant populations exhibiting quantitative resistance. In general, polygenes contribute together to the plant response observed in quantitative resistance (CORWIN; KLIEBENSTEIN, 2017).

Resistance to CBB has been reported in several *Phaseolus* species (SCHUSTER, 1955 apud HONMA, 1956; WELSH; GRAFTON, 2001). The nature of the resistance (qualitative or quantitative) and the number of genes involved are dependent on the plant genotype. For instance, the segregation of resistance in interspecific hybrids between a susceptible *P. vulgaris* genotype and a resistant *P. coccineous* genotype was better explained as being monogenic and recessive (WELSH; GRAFTON, 2001). Conversely, resistance of *Phaseolus acutifolius* in interspecific hybrids with *P. vulgaris* exhibited characteristics of being quantitative (HONMA, 1956). Similarly, the resistance of *P. vulgaris* varieties is commonly described as quantitative and polygenic, with several Quantitative Trait Loci (QTL) being already identified (SINGH; SCHWARTZ, 2010; VITERI et al., 2014; MIKLAS et al., 2017; ASSEFA et al., 2019; MONTERO et al., 2020; ALVES et al., unpublished data).

In previous studies conducted by the Laboratory of Molecular Phytobacteriology at the Universidade Federal de Vicosa, a collection of common bean varieties developed in different Brazilian institutions was evaluated for resistance to CBB through artificial inoculation with *Xcf* strain CFBM-UFV-0001 (MONTEIRO et al., 2020). The results indicated that cultivar BRS Radiante was highly resistant, while cultivar Carioca MG exhibited high susceptibility to the *Xcf* strain. Furthermore, the segregation of resistance to *Xcf* in the F_2 population of a BRS Radiante × Carioca MG cross supported the idea that this trait is quantitative and controlled by two dominant and complementary genes, with the additive action of polygenes (MONTEIRO et al., 2020).

Moreover, a Genome-Wide Association Study (GWAS) revealed the association of 10 Single-Nucleotide Polymorphisms (SNPs) with the resistance of *P. vulgaris* to *Xcf*, reinforcing the notion that it is a quantitative trait. Notoriously, the identification of genes coding for a putative ethylene-responsive protein kinase and a serine/threonine protein kinase in the proximity of some of the identified SNPs (MONTEIRO et al., 2021) suggests that the *P. vulgaris* quantitative resistance to *Xcf* involves defense-related pathways previously reported for other plant-bacteria interactions (FU et al., 2011; DEBIEU et al., 2016). Nonetheless, the mechanisms subjacent to the quantitative resistance of BRS Radiante to *Xcf* remain unclear.

It is known that during plant-bacteria interactions, transient accumulation of Reactive Oxygen Species (ROS) occurs as one of the first plant reactions elicited upon pathogen recognition. ROS molecules, such as superoxide anion ($O2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$), can function as antimicrobial agents, limiting the pathogen growth in plant tissues, and as signaling molecules to trigger the immune response (HANCOCK, 2016; CORWIN; KLIEBENSTEIN, 2017; GARCÍA-CAPARRÓS et al., 2021). Nonetheless, in order to protect its own tissues, the plant utilizes several enzymes to maintain the redox balance after ROS accumulation, including catalase (CAT), superoxide dismutase (SOD), peroxidase (POX), polyphenol oxidase (PPO), and ascorbate peroxidase (APX) (SALEEM; FARIDUDDIN; CASTROVERDE, 2021). A previous study reported an increase in the activities of APX, POX, and SOD in cultivars Ouro Negro (susceptible) and Diamante Negro (resistant) after infiltration with an *Xcf* strain, but no difference between the cultivars was observed (SILVA et al., 2020). In another study, no association between an increase in the activities of defense-related enzymes and a reduction of CBB severity was observed when cultivar Ouro Negro was treated with plant defense inducers (manganese and zinc phosphites) and inoculated with an *Xcf* strain (COSTA et al., 2020). Therefore, a possible involvement of the defense-related enzymes in the resistance response of common bean to *Xcf* remains inconclusive. Experiments comparing the response of resistant and susceptible genotypes whose genetic backgrounds (not related to resistance) are very similar have not been reported. In this regard, comparing the enzymatic response of an advanced backcross population derived from a BRS Radiante × Carioca MG cross with Carioca MG could provide clearer information about the biochemical response of *P. vulgaris* to *Xcf* .

A direct relationship between ROS and salicylic acid (SA) accumulation in plant tissue has been observed, consistent with reports indicating that accumulation of the phytohormone induces ROS production during the initial stages of the plant defense response (SALEEM; FARIDUDDIN; CASTROVERDE, 2021). Salicylic acid, jasmonic acid (JA), and ethylene (ET) are important phytohormones for the establishment of signaling pathways involved in plant immunity; for several plant species, while the SA pathway is important for plant resistance against biotrophic and hemibiotrophic pathogens, the JA/ET pathway is more relevant for plant resistance to necrotrophic pathogens. Furthermore, it has been demonstrated that the SA and JA/ET pathways are often (but not always) antagonistic (SHIGENAGA; ARGUESO, 2016).

The involvement of the SA and JA/ET pathways in the resistance response of common bean to *Xpp* has recently been revealed by the results of two different studies. A transcriptomic study comparing the gene expression profiles of cultivars BAT93 (resistant) and JaloEEP588 (susceptible) at 48 h post *Xpp* inoculation revealed the induction of genes associated with the SA pathway in resistant plants, while in susceptible plants an increase in the number of ET-related transcripts was detected (FOUCHER et al., 2020b). Similarly, Yang et al. (2022) reported a downregulation of ET-responsive genes in resistant *P. vulgaris* and *P. acutifolius* genotypes when compared with the gene expression profile of a susceptible *P. vulgaris* genotype after inoculation with *Xpp*. Nonetheless, similar studies inoculating common bean genotypes with *Xcf* strains have not been reported.

Accumulation of SA was previously associated with the increase in the activity of phenylalanine ammonia-lyase (PAL). This enzyme plays a key role during the plant response to biotic stress by converting phenylalanine to cinnamate, the precursor molecule for several defense-related pathways, such as the biosynthesis of flavonoids and SA (HE et al., 2020; YOU et al., 2020; SALEEM; FARIDUDDIN; CASTROVERDE, 2021). A transcriptomic study revealed the induction of genes related to the isoflavone biosynthesis pathway (*e.g.*, PAL genes) in a resistant *P. vulgaris* variety (an OAC Rex x OAC Seaforth hybrid) in response to *Xpp* inoculation (COX et al., 2021). Nonetheless, the involvement of the JA, SA, ET pathways, and PAL in the resistance response of *P. vulgaris* to *Xcf* remains to be investigated.

In order to subvert plant immunity, bacteria inject effector proteins through the type III secretion system directly into the plant cell cytoplasm (WHITE et al., 2009; BOCH; BONAS, 2010). These effectors have been shown to target different plant

functions to suppress the defense response (JONES; DANGL, 2006). Several *Xanthomonas* species inject into the plant cell cytoplasm a special family of type III effectors, the so-called Transcriptional Activator-Like Effectors (TALE) that modulate the expression of host plant susceptibility genes. These effector proteins possess N-terminal signals necessary for injection into the plant cell cytoplasm by the type III secretion system, Nuclear Localization Signals (NLS) that allow their import from the host cell cytoplasm to the nucleus by plant importins, and transcriptional activator domains for activation of host gene expression. In addition, the central portion of TALE proteins is constituted by tandem-arranges of quasi-identical repeats of 33-35 amino acids, where the residues of the 12 and 13 positions (referred to as Repetitive Variable Diresidue, RVD) are highly variable (BOCH; BONAS, 2010; BOCH; BONAS; LAHAYE, 2014).

The binding of the TALEs to specific nucleotide sequences in the promoters of plant susceptibility genes is determined by the composite RVD sequence of the internal repeats of the protein (BOCH et al., 2009; MOSCOU; BOGDANOVE, 2009). The deciphering of this code allowed the prediction of plant genes that could be targets of TALEs in diverse plant-bacteria interactions (DOYLE et al., 2012). In a study conducted by Ruh et al. (2017) with the genome sequences of a set of *Xcf* and *Xpp* strains, a total of 12 *P. vulgaris* genes were predicted to be targets of TALEs, some of them common for both bacterial pathogens. Nonetheless, since the repertoire of TALEs is dependent on the particular strain, further investigation on possible common bean gene targets using a larger set of *Xcf* isolates is required to gain a better understanding of the susceptibility.

This dissertation sought to shed some light on the molecular and biochemical mechanisms subjacent to the susceptibility and resistance reactions of common bean to *Xcf*. Specifically, the objectives were to predict the repertoire of TALEs in a large collection of *Xcf* strains and identify putative susceptibility genes in a *P. vulgaris* reference genome. Additionally, this research aimed to investigate alterations in the activity of defense-related enzymes in both susceptible (Carioca MG) and resistant (BC_3F_4) common bean genotypes during the early stages of the *P. vulgaris*-*Xcf* interaction.

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2 CHAPTER I: PREDICTION OF *Xanthomonas citri* pv. *fuscans* TRANSCRIPTIONAL ACTIVATOR-LIKE EFFECTORS AND THEIR GENE TARGETS IN THE *Phaseolus vulgaris* REFERENCE GENOME

2.1 ABSTRACT

Xanthomonas species are recognized by their production of Transcription Activator-Like Effectors (TALE) which modulate the plant host transcriptional machinery for their benefit. The repertoire of TALEs in *X. citri* pv. *fuscans* (*Xcf*), the causal agent of Common Bacterial Blight (CBB) in *Phaseolus vulgaris*, remains largely unknown. The aim of this work was to describe the repertoire of TALEs in a large number of *Xcf* strains and to describe host genes putatively induced by these effectors. For that, the TALEs were bioinformatically predicted in a group of 35 *Xcf* strains, based on their unique structural characteristics, including their internal tandem repeats sequences whose repeat variable di-residues (RVD) determine binding to host gene promoters. The composite RVDs sequences of each predicted TALE protein were used to predict putative binding sites in the *P. vulgaris* reference genome. A total of 56 TALE proteins were predicted among the *Xcf* analyzed genomes, carrying 26 distinct composite RDVs. In addition, six conserved sites in the *P. vulgaris* reference genome were predicted to be targeted by the repertoire of *Xcf* TALEs. Genes predicted to encode functions related to substance transporters, sugar metabolism, the ubiquitin-proteasome system, and the induction of the ethylene pathway were frequently found downstream from sites targeted by *Xcf* TALEs. These results provide important insights into the mechanisms underlying common bean susceptibility to *Xcf*.

2.2 INTRODUCTION

Plant pathogenic bacteria inject proteins called effectors into plant cells through the Type III Secretion System (T3SS) to evade the host immune system responses and enhance virulence (WHITE et al., 2009). *Xanthomonas* is an important genus of plant pathogenic bacteria that produces Transcription-Activator Like effectors (TALEs), which act as transcriptional inducers of specific host genes. TALE proteins are generally divided into four functional domains. The N-terminal portion carries the T3SS signal, required for the translocation of the effector protein directly from the bacterium into the host cell cytoplasm. The C-terminal region harbors a Nuclear

Localization Signal (NLS), which mediates the transport of the effector protein to the nucleus, and an Activation Factor Domain, which interacts with the plant machinery to induce the expression of targeted genes. In addition, the central portion of TALE proteins contains a DNA-Binding Domain, which confers binding specificity of the TALE to the host gene promoters (BOCH; BONAS; LAHAYE, 2014). Generally, these effectors bind to the promoter regions of susceptible genes, activating functions that favor bacterial development (BOCH et al., 2009; MOSCOU; BOGDANOVE, 2009).

The DNA-Binding Domain typically consists of 12.5-31.5 tandem-repeat sequences comprised of 34 quasi-conserved amino acids, although TALEs with lower numbers of repeats have been identified. In these repetitive sequences, positions 12 and 13 exhibit high variability, and thus, are referred to as Repetitive Variable Diresidues (RVDs). Specificity for binding to a nucleotide sequence in the host gene promoter is conferred by the RDVs (BOCH; BONAS, 2010). The binding specificity of RVDs has been elucidated, enabling the prediction of DNA target sites and susceptibility genes in host genomes (DOYLE et al., 2012; GRAU et al., 2016). Several host genes targeted by *Xanthomonas* TALEs have been predicted and experimentally validated, including those coding for membrane transporters, stress-response transcription factors, and proteins involved in phytohormone pathways (KAY; BONAS, 2009; HU et al., 2014; ÜSTÜN; BÖRNKE, 2014; ERKES et al., 2017; ZÁRATE-CHAVES et al., 2021).

Common Bacterial Blight (CBB) is the most significant bacterial disease affecting the common bean crop (*Phaseolus vulgaris* L.), causing significant losses in susceptible plant genotypes under conditions favourable for disease development (DA SILVA JÚNIOR et al., 2022). The disease is caused by two phytopathogenic *Xanthomonas* species, namely *X. citri* pv. *fuscans* (*Xcf*) and *X. phaseoli* pv. *phaseoli* (*Xpp*), which are distributed worldwide (WALLEN; JACKSON, 1975; GILLARD et al., 2009). The most effective management approach involves planting resistant cultivars, although resistance to the pathogens is often quantitative and complex (MIKLAS et al., 2017; MONTEIRO et al., 2020; 2021, ALVES et al. unpublished data).

Compared to other plant-*Xanthomonas* pathosystems, limited information is available regarding the repertoire of TALEs in the *Xcf* population. Darrasse et al. (2013) provided an initial identification of TALEs by studying a single *Xcf* strain carrying two different TALE genes. Subsequently, Ruh et al. (2017) further characterized these two TALEs and identified two additional ones in a set of 17 strains of both *Xcf* and *Xpp*. In

the study, the authors also predicted DNA-binding sites for the TALEs in the *P. vulgaris* reference genome, shedding light on putative mechanisms involved in CBB development. However, the mechanisms utilized by bacterial species to cause disease in their host plants are frequently determined in a strain-specific manner. Therefore, a more comprehensive analysis of a larger group of *Xcf* strains is needed to gain a better understanding of conserved and divergent mechanisms underlying TALE-triggered susceptibility in *P. vulgaris*.

A good understanding of the mechanisms underlying *Xcf* TALE function inside the common bean cell would help identify susceptibility factors and potentially facilitate the development of resistant cultivars by a susceptibility target avoidance mechanism. Hence, the objectives of this study were to predict the repertoire of TALEs in a diverse group of *Xcf* strains using the currently available bacterial genome sequences deposited in the GenBank, to identify their main target genes in a *P. vulgaris* reference genome, and to contribute to the understanding of common bean susceptibility to the pathogen.

2.3 MATERIALS AND METHODS

2.3.1 Genome assemblies

Thirty eight *Xcf* genome sequences obtained from GenBank (https://www.ncbi.nlm.nih.gov/datasets/taxonomy/366649/) were used for TALE prediction (Table 1). To confirm the taxonomic position of the strains whose genome sequences were included in the study, the Average Nucleotide Identity (ANI) of each genome sequence was compared to that of the *Xcf* NCPPB381 pathotype strain. Additionally, the quality of the genome assemblies was assessed using Benchmarking Universal Single-Copy Orthologs (BUSCO) version 5.4.6 (SIMÃO et al., 2015) with default parameters, specifically selecting the Xanthomonadales lineage for comparison. Assemblies with a completeness value lower than 95% were excluded from further analysis.

2.3.2 TALE and RVD Prediction

Prediction of TALEs was performed using AnnoTALE version 1.5 (GRAU et al., 2016) with default parameters. The resulting composite RVD sequences were obtained, and a minimum of 6.5 repeats was used as the functional cut-off value (BOCH et al., 2009). The amino acid sequences from functional TALE genes were

obtained using EMBOSS Transeq with default parameters and the bacterial codon table published by Madeira et al. (2022). To compare the sequences of genes predicted by AnnoTALE analysis with previously described TALE proteins, a Blastp (ALTSCHUL et al., 1997) analysis was performed. The composite RVD sequences of validated TALEs were classified using the current global database of TALE effectors from AnnoTALE.

2.3.3 Prediction of Plant Target Sites

Phaseolus vulgaris genes targeted by *Xcf* TALEs were predicted using Targeter 2.0 (DOYLE et al., 2012) with default parameters. Only predicted TALEs carrying at least 12.5 RVDs were considered for host target-site prediction (BOCH; BONAS, 2010). Matches between RVD sequences and target sites were identified in the reference genome of *P. vulgaris* G19833 (GenBank ID: ANNZ00000000.1) using the Target Finder tool (DOYLE et al., 2012). Putative binding-sites were classified according to the score value obtained from Target Finder based on binding affinity between the TALE protein and its predicted host target loci, in which lower scores mean a higher affinity and the best score possible means a putative perfect match. The loci in the *P. vulgaris* genome targeted by the top 20 matches per RVD were determined using the NCBI Sequence Viewer tool version 3.48.0 (https://www.ncbi.nlm.nih.gov/projects/sviewer/). Putative target genes were selected based on their distance from the predicted targeted loci; only genes located ≤ 3 kb downstream from the targeted locus were retained (RUH et al., 2017).

Table 1. *Xanthomonas citri* pv. *fuscans* genome sequences analyzed in this study.

Strain	NCBI accession number	Number of con-tigs.	Total length (bp)	G+C (%)	Contig N50	Contig L50	Geographic origin	Collection year
1008017	JAPEIR000000000.1	4	5,101,251	64.5	4,984,178	1	France	2010
4834-R	FO681494	4	5,088,683	64.7	4,981,995	1	France	1998
6165R Δ*tal22B*	CP072396	2	5,068,405	64.8	5,030,573	1	Canada	1957
BRM48906	CP110298	3	5,230,237	64.6	5,126,023	1	Brazil	2014
CFBP1815	LT961741	106	4,948,046	64.5	89,106	18	Greece	1978
CFBP4884	JPHG01000001	203	5,003,118	64.5	79,889	18	France	1998
CFBP6166	LT960820	112	4,978,387	64.5	81,069	19	South Africa	1963
CFBP6533	CP110301	2	5,154,616	64.6	5,097,546	1	Tunisia	1989
CFBP6960	LT960964	108	4,982,831	64.8	93,163	18	Reunion Island, France	2000
CFBP6970	LT961161	106	4,997,456	64.9	99,739	18	United States	1990
CFBP6988	CP026331	8	5,131,789	64.6	1,199,983	2	Reunion Island, France	2000
CFBP6990	LT960951	50	5,121,453	64.6	199,405	8	Reunion Island, France	2000
CFBP6992	LT961733	78	5,517,999	64.4	155,216	13	Reunion Island, France	2000
CFBP6994	OCZE01000583	583	5,134,490	64.5	16,119	98	Tanzania	1990
CFBP6996	AVET02000001	10	5,093,707	64.5	1,179,078	2	Reunion Island, France	2000
CFBP7766	LT961254	127	5,175,291	64.6	79,558	20	Cameroon	2009
CFBP7767	LT961058	120	5,107,388	64.7	81,069	19	Cameroon	2009
FH61	CP029270	3	5,285,875	64.5	5,187,120	1	United States	

G55	JAJITO010000097	123	5,055,235	64.5	93,986	18		
ISO118C1	CP012053	2	5,312,763	64.5	5,357,611	1	Canada	2013
ISO118C5	CP012051	2	5,357,613	64.5	5,312,765	1	Canada	2013
ISO12C3	CP012055	2	5,357,175	64.5	5,312,327	1	Canada	2013
LMG826	NZ_JPYF01000000	1,314	4,857,009	64.9	8,953	165	Belgium	2014
LNPV30.30	CP110296	2	5,156,952	64.6	5,099,883	1	China	2006
M12	CP029267	3	5,141,064	64.8	5,050,918	1	Malawi	
NCPPB1056	JSEV02000055	134	5,150,070	64.8	75,549	20	United Kingdom	1961
NCPPB1058	JSEY02000057	168	5,180,142	64.7	71,334	25	Ethiopia	1961
NCPPB1402	JSEW02000064	240	5,342,561	64.7	39,404	43	Uganda	1962
NCPPB1433	JSBT02000062	154	5,044,382	64.5	73,844	20	Hungary	1956
NCPPB1654	JSBR02000052	134	5,032,953	64.5	81,530	16	South Africa	1963
NCPPB2665	JSBQ02000055	154	5,092,836	64.5	71,115	19	Italy	1973
NCPPB3660	JSEX02000039	183	5,153,912	64.5	73,151	23	Brazil	1975
NCPPB381 ★	NZ_JTKK02000049	136	4,955,361	64.5	74,010	21	Canada	1957
NCPPB670	JRRE02000088	152	5,233,004	64.7	71,533	22	Uganda	1958
PR8F	CP029264	3	5,302,232	64.6	5,208,226	1	Puerto Rico	
X621	JXHS03000046	135	5,073,693	64.5	99,856	16	South Africa	1995
XCP631	JXLW02000061	189	5,152,698	64.5	61,459	26	Colombia	2004
Xff49	CP023294	81	5,279,077	64.8	112,211	15	Brazil	2017

★ Pathotype strain

2.4 RESULTS

2.4.1 Quality of genome assemblies

All the genome sequences retrieved from GenBank showed ANI values higher than 98% when compared with that of the *Xcf* pathotype strain. Most of the genome sequences had at least 98.7% completeness (C) (Table 2). The genome sequences of strains CFBP6994 and LMG826 were excluded from further analysis due to their exhibiting completeness values lower than 95%. Additionally, the genome sequence of strain 6165R Δ*tal22B* was excluded because it has already been reported that it is a derivative from the wild-type strain in which the only TALE gene identified was deleted (FOUCHER et al., 2022). The number of complete and single-copy BUSCOs in the retained genomes was between 1105 and 1150. The number of complete and duplicated BUSCOs was ≤ 3 for most genomes, except CFBP6992 and Xff49 whose numbers were 16 and 32, respectively. For most genomes, the number of fragmented BUSCOs was ≤ 8, except NCPPB1402 which had 16, and the number of missing BUSCOs was ≤ 9. These results indicated that the genome assemblies were suitable for reliable TALE gene predictions.

Table 2. Similarity to the pathotype strain genome sequence and completeness of *Xanthomonas citri* pv. *fuscans* genome sequences investigated in this study.

Strain	ANI (%)[1]	Completeness (C)	Complete and single copy (S)	Complete and duplicated (D)	Fragmented (F)	Missing (M)
1008017	99.82	99.5	1146	0	2	4
4834-R	99.81	99.4	1145	0	3	4
6165R Δtal22B	99.99	99.5	1144	2	2	4
BRM48906	98.79	99.0	1140	0	7	5
CFBP1815	99.84	99.7	1148	0	0	4
CFBP4884	99.82	99.5	1146	0	2	4
CFBP6166	99.85	99.5	1146	0	1	5
CFBP6533	98.82	99.7	1148	0	1	3
CFBP6960	99.79	99.7	1148	0	0	4
CFBP6970	99.86	99.7	1146	2	0	4
CFBP6988	98.78	99.8	1150	0	0	2
CFBP6990	98.78	99.8	1150	0	0	2
CFBP6992	98.35	99.8	1133	16	1	2
CFBP6994	98.44	94.6	1087	2	41	22
CFBP6996	98.46	99.7	1146	2	1	3
CFBP7766	99.77	99.7	1148	0	0	4
CFBP7767	99.75	99.4	1145	0	1	6
FH61	99.84	99.6	1145	2	1	4

G55	98.42	99.5	1145	1	3	3
ISO118C1	99.80	99.6	1145	2	1	4
ISO118C5	99.80	99.6	1145	2	1	4
ISO12C3	99.80	99.6	1145	2	1	4
LMG826	99.97	88.0	1013	1	73	65
LNPV30.30	98.80	99.7	1149	0	1	2
M12	99.86	99.6	1146	1	1	4
NCPPB1056	99.81	99.4	1143	2	2	5
NCPPB1058	99.75	99.5	1144	2	1	5
NCPPB1402	99.82	97.9	1124	3	16	9
NCPPB1433	99.83	99.4	1145	0	3	4
NCPPB1654	99.82	99.5	1146	0	2	4
NCPPB2665	99.82	99.2	1143	0	4	5
NCPPB3660	99.83	99.4	1143	2	2	5
NCPPB381 ★	-	99.5	1144	2	2	4
NCPPB670	99.86	99.2	1142	1	4	5
PR8F	99.80	99.5	1145	1	2	4
X621	99.87	99.5	1146	0	2	4
XCP631	99.78	99.2	1142	1	3	6
Xff49	99.83	98.7	1105	32	8	7

[1] Comparison with the pathotype *X. citri* pv. *fuscans* strain NCPPB381

★ Pathotype strain of *X. citri* pv. *fuscans*

2.4.2 Predicted TALE proteins and their RVD sequences

AnnoTALE predicted a total of 56 TALE gene sequences from the 35 curated *Xcf* genomes, some of which were shared among different strains. TALE genes were shared among strains regardless of whether they were located in plasmids (TALE6, TALE16, and TALE17) or in the chromosome (TALE7, TALE9, and TALE38) (Table 3). Among the predicted TALE genes, 22 code for proteins with less than 6.5 repeats in the DNA-binding domain. BlastP searches against the NCBI database of the amino acid sequences coded by the TALEs selected after applying the functional cut-off repeat number revealed that 27 of them matched previously known TALEs of the AvrBs3 family, and 7 matched to proteins annotated as TALE repeat-containing protein. No TALE sequences were found in the genomes of strains CFBP1815, CFBP6166, CFBP6960, CFBP6970, CFBP6990, CFBP7766, CFBP7767, and G55. The lack of prediction of TALEs was not related to genome completeness assessed by BUSCO.

Table 3. Predicted TALE genes in the genomes of *Xanthomonas citri* pv. *fuscans* strains and their matching sequences by Blastp.

TALE	Strains	Description	NCBI accession number	E-value	Identity (%)	Query cover (%)
1	4834-R (pla)^{p}	XfuTAL1 (AvrBs3)	CDF63714.1	0.0	100	94.0
2	4834-R (plc)^{p}	XfuTAL2 (AvrBs3)	CDF63772.1	0.0	100	91.0
3	1008017 (pC) ^{p}	AvrBs3	WP_099800837.1	0.0	98.5	96.0
4	1008017 (pA)^{p}	TALE23A (AvrBs3)	WP_099800836.1	0.0	100	99.0
5	BRM48906	AvrBs3	WP_264687394.1	0.0	100	99.0
6	BRM48906 (pA)^{p} LNPV30.30 (pA)^{p} M12 (unnamedA)^{p}	TALE23A (AvrBs3)	WP_099802904.1	0.0	100	99.0
7	BRM48906 CFBP6533 LNPV30.30	TALE18G (AvrBs3)	WP_099802044.1	0.0	100	99.0
8	CFBP4884	-	-	-	-	-
9	CFBP6533 LNPV30.30	AvrBs3	WP_215812027.1	0.0	100	99.0
10	CFBP6533 (pA)^{p}	AvrBs3	WP_099866758.1	0.0	96.6	99.0
11	CFBP6988	-	-	-	-	-
12	CFBP6992	-	-	-	-	-
13	CFBP6996	-	-	-	-	-
14	FH61 (unnamedA)^{p}	AvrBs3	WP_215825281.1	0.0	100	99.0

15	FH61 (unnamedC)[p]	AvrBs3	WP_215825279.1	0.0	100	99.0
16	ISO118C1 (Xff45)[p] ISO118C5 (Xff45)[p] ISO12C3 (Xff45)[p]	TALE18H (AvrBs3)	WP_099868344.1	0.0	100	99.0
17	M12 (unnamedC)[p] PR8F (unnamedC)[p]	TALE18H (AvrBs3)	WP_099770699.1	0.0	100	99.0
18	NCPPB381 ★	-	-	-	-	-
19	NCPPB381 ★	TALE repeat-containing protein	WP_046832489.1	0.0	97.8	100
20	NCPPB 670	-	-	-	-	-
21	NCPPB 670	TALE repeat-containing protein	WP_046833138.1	0.0	100	100
22	NCPPB 670	AvrBs3	WP_099802904.1	0.0	100	100
23	NCPPB 670	-	-	-	-	-
24	NCPPB1056	TALE repeat-containing protein	WP_198527881.1	0.0	100	99.0
25	NCPPB1056	NY95_22110 (AvrBs3)	KKW49061.1	0.0	100	100
26	NCPPB1056	-	-	-	-	-
27	NCPPB1058	NY98_24300 (AvrBs3)	KKW48552.1	0.0	100	100
28	NCPPB1058	NY98_21590 (AvrBs3)	KGU49669.2	0.0	100	100
29	NCPPB1058	-	-	-	-	-
30	NCPPB1058	NY98_24300 (AvrBs3)	KKW48552.1	0.0	100.	99.4
31	NCPPB1058	-	-	-	-	-

32	NCPPB1058	-	-	-	-	-
33	NCPPB1402	-	-	-	-	-
34	NCPPB1402	XfuTAL2 (AvrBs3)	CDF63772.1	0.0	100	99.0
35	NCPPB1402	AvrBs3	WP_046832139.1	0.0	100	100
36	NCPPB1433	-	-	-	-	-
37	NCPPB1433	TALE repeat-containing protein	WP_046832575.1	0.0	100	100
38	NCPPB1433 NCPPB2665	-	-	-	-	-
39	NCPPB1433	AvrBs3	WP_099800837.1	0.0	100	99.0
40	NCPPB1654	-	-	-	-	-
41	NCPPB1654	-	-	-	-	-
42	NCPPB1654	-	-	-	-	-
43	NCPPB1654	AvrBs3	WP_099800837.1	0.0	100	100
44	NCPPB2665	-	-	-	-	-
45	NCPPB2665	AvrBs3	WP_099800837.1	0.0	100	100
46	NCPPB3660	TALE repeat-containing protein	WP_080949312.1	0.0	100	99.0
47	NCPPB3660	AvrBs3	WP_046831802.1	0.0	100	99.0
48	PR8F (unnamedA)^{p}	TALE23A (AvrBs3)	WP_099800836.1	0.0	100	99.0
49	X621	AvrBs3	WP_099770699.1	0.0	100	99.0

50	X621	TALE repeat-containing protein	WP_047138409.1	0.0	100	100
51	X621	-	-	-	-	-
52	XCP631	TALE repeat-containing protein	WP_082334236.1	0.0	100	99.0
53	XCP631	-	-	-	-	-
54	Xff49 (plC)[p]	AvrBs3	WP_099770699.1	0.0	100	99.0
55	Xff49 (plA)[p]	-	-	-	-	-
56	Xff49 (plA)[p]	-	-	-	-	-

[p] TALE gene is located in a plasmid (names in parentheses)
★ Pathotype strain
- indicates TALE with less than 6.5 internal repeats

Three synonymous substitution events were found when comparing the sequences of TALE4, located in "plasmid A" of strain 1008017, and TALE48, located in "plasmid unnamed A" of strain PR8F (Figure 1). These mutations were found in the DNA sequences coding for amino acids near the RVDs of the 2nd, 5th, and 11th internal repeats of the predicted effector proteins.

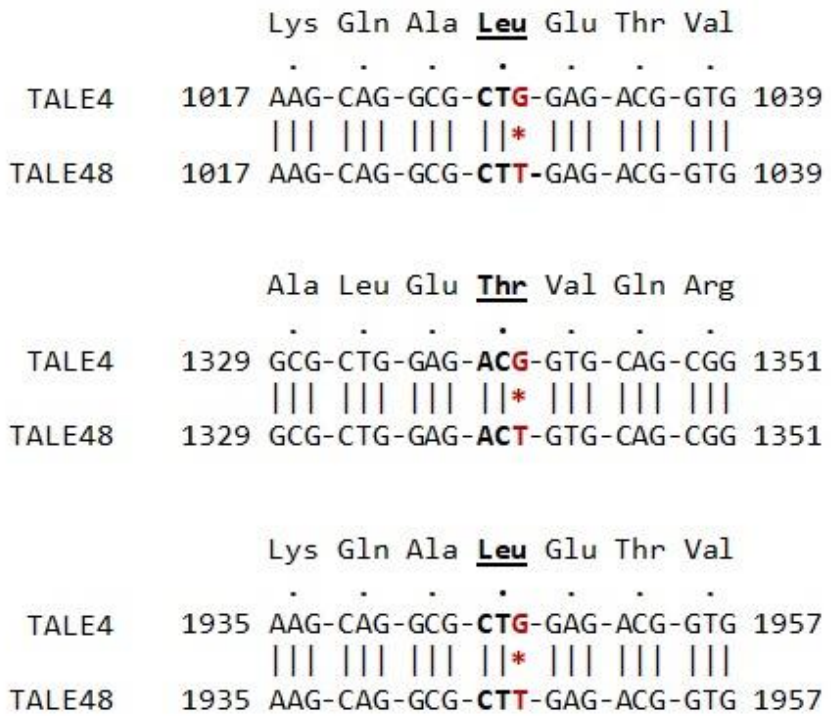

Figure 1. Synonymous substitutions in internal repeats of predicted *Xanthomonas citri* pv. *fuscans* effectors TALE4 and TALE48. Top: second repeat, middle: fifth repeat, and bottom: eleventh repeat of the effector protein.

2.4.3 Composite RVD groups

A total of 26 different composite RVDs were predicted when considering a functional cut-off value of 6.5 repeats. Redundancy among composite RVD sequences of different TALE genes was evident. For instance, predicted TALE3, TALE15, TALE16, TALE17, TALE49, and TALE54 shared the same composite RVD. The same occurred with predicted TALE4, TALE6, TALE14, and TALE48, as well as with TALE7 and TALE9. The TALE Class Builder tool separated the composite RVD sequences into six different groups according to their similarity, albeit with some variations within groups (Table 4).

Table 4. Composite RDV groups of *Xanthomonas citri* pv. *fuscans* predicted TALE proteins.

TALE Groups	
Group I	
HD HY NN N* HD HY NN HD NG	TALE46
-- -- NG NI HD HY NN HD NG	TALE24
-- -- NI NI NN NG NN HD NI	TALE19
Most likely common binding sequence:	
TC T A A C T G C T	
Group II	
NN HD HD NG HD NI NG NI HD -- -- -- -- -- -- -- -- --	TALE21
NI NG HD NG HD NN N* NI -- -- -- -- -- -- -- -- -- --	TALE30
NI NG HD NG HD NI -- -- -- -- -- -- -- -- -- -- -- --	TALE28
NI NG HD NG HD NI NG NI -- -- -- -- -- -- -- -- -- --	TALE27
NI NG HD NG HD NI NG NI HD -- -- -- -- -- -- -- -- --	TALE39
NI NG HD NG HD NI NG NI HD HY -- -- -- -- -- -- -- --	TALE45
NI NG HD NG HD NI NG NI HD HY NN N* HD -- -- -- -- --	TALE43
NI NG HD NG HD NI NG NI HD HY NN N* HD NG HY NN HD NG	TALE54
NI NG HD NG HD NI NG NI HD HY NN N* HD NG HY NN HD NG	TALE49
NI NG HD NG HD NI NG NI HD HY NN N* HD NG HY NN HD NG	TALE15
NI NG HD NG HD NI NG NI HD HY NN N* HD NG HY NN HD NG	TALE3
NI NG HD NG HD NI NG NI HD HY NN N* HD NG HY NN HD NG	TALE16
NI NG HD NG HD NI NG NI HD HY NN N* HD NG HY NN HD NG	TALE17
-- -- -- -- -- -- -- -- -- -- NN N* HD NG HY NN HD NG	TALE52
-- -- -- -- -- -- -- -- -- -- -- N* HD NG HY NN HD NG	TALE34
-- NI NG HD NG HD NI NG NI HY NN N* HD NG HY NN HD NG	TALE2
Most likely common binding sequence:	
TA T C T C A T A C T G C C T A G C T	

Group III

```
NI NG NI NG NI NG NI NN HD HD NN NN HD NI HD NI HD HD HD HD HD NG NG  TALE10
NI NG NI NG NI NN NG NN HD HD NN NN HD NI HD NI HD HD HD HD HD NG NG  TALE6
NI NG NI NG NI NN NG NN HD HD NN NN HD NI HD NI HD HD HD HD HD NG NG  TALE4
NI NG NI NG NI NN NG NN HD HD NN NN HD NI HD NI HD HD HD HD HD NG NG  TALE14
NI NG NI NG NI NN NG NN HD HD NN NN HD NI HD NI HD HD HD HD HD NG NG  TALE48
NI NG NI NG NI NN NG NN HD NN NN -- -- -- -- -- -- -- -- -- -- -- --  TALE50
NI NG NI NG NI NN NG NN HD -- -- -- -- -- -- -- -- -- -- -- -- -- --  TALE22
NI NG NI NG NI NN NG -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- --  TALE25
NI NG NI NG NI NG NN HD HD HD -- -- -- -- -- -- -- -- -- -- -- -- --  TALE35
```

Most likely common binding sequence:

```
TA  T  A  T  A  G  T  G  C  C  G  G  C  A  C  A  C  C  C  C  C  T  T
```

Group IV

```
NG NN HD HD NN NN HD NI HD NI HD TALE37
```

Most likely common binding sequence:

```
TT  G  C  C  G  G  C  A  C  A  C
```

Group V

```
-- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- HD HD HD HD HD HD HD NG NG  TALE47
NI NN NN HD NI HD NI HD HD HD HD HD NI NG NI NG NI NN NG NN HD HD NF HD NI HD HD HD HD HD NG NG  TALE1
```

Most likely common binding sequence:

```
TA  G  G  C  A  C  A  C  C  C  C  C  A  T  A  T  A  G  T  G  C  C  T  C  C  C  C  C  C  C  T  T
```

Group VI

N* HD HD NG NG NI NG NI NI NN NI HD HD HD NI HD NK NG TALE9
N* HD HD NG NG NI NG NI NI NN NI HD HD HD NI HD NK NG TALE7
N* HD HD NG NG NI NG NI HD NN NI HD HD HD NI HD NK NG TALE5

Most likely common binding sequence:

TC C C T T A T A A G A C C C A C G T

2.4.4 Common bean genes targeted by *Xcf* TALE proteins

The composite RVD sequences of 17 TALE proteins were used to search for their putative target loci in the *P. vulgaris* reference genome G19833, some of which showed sequence redundancy. Next, the top 20 targeted loci were positioned in the common bean genome, and it was determined if they laid in promoter regions of host genes. No gene whose promoter potentially carried the target site of the TALE1 was identified, while diverse plant functions were predicted to be targeted by the other TALE proteins of the *Xcf* strains (Table 5). Remarkably, genes involved in the transport of substances, sugar metabolism, protein degradation, and the ethylene response were commonly identified.

Table 5. *Phaseolus vulgaris* G19833 genes predicted to be induced by *Xanthomonas citri* pv. *fuscans* TALE proteins.

TALE[1]	TALE group	Strain	Plant locus	Score[2]	Plant-induced genes ID	Annotation[3]
1	V	4834-R				
2	II	4834-R	NC_023753.1 + 244549	8.23	*PHAVU_007G003700g*	Myo-inositol transporter
			NC_023752.1 + 52158941	9.61	*PHAVU_008G209900g*	Abscisic-aldehyde oxidase
			NC_023756.1 + 18713032	9.81	*PHAVU_004G088300g*	GIGANTEA
			NC_023757.1 + 38338119	9.82	*PHAVU_003G172400g*	F-box/LRR-repeat protein 14
			NC_023757.1 + 11194096	9.85	*PHAVU_003G073000g*	AP-3 complex subunit sigma
			NC_023752.1 + 20410075	10.01	*PHAVU_008G131500g*	Ethylene-responsive transcription factor
			NC_023752.1 + 11169746	10.01	*PHAVU_008G102100g*	E3 ubiquitin-protein ligase PRT6
			NC_023754.1 + 31020637	10.07	*PHAVU_006G207900g*	Chloroplast-targeted copper chaperone
			NC_023757.1 + 29139555	10.24	*PHAVU_003G116600g*	GPN-loop GTPase
3 15 16 17 49 54	II	1008017 FH61 ISO118C1 ISO118C5 ISO12C3 M12 PR8F X621 Xff49	NC_023751.1 + 21753884	8.39	*PHAVU_009G148800g*	Xylan alpha-glucuronosyltransferase
			NC_023754.1 + 29191735	9.45	*PHAVU_006G182900g*	Nuclear fusion defective 6
			NC_023756.1 + 45047217	11.25	*PHAVU_004G168800g*	Casein kinase 1
			NC_023753.1 + 3613244	11.28	*PHAVU_007G045600g*	ADP-ribosylation factor-like protein 8
4 6 14 48	III	1008017 BRM48906 LNPV30.30 M12 FH61 PR8F	NC_023750.1 + 40030862	13.87	*PHAVU_010G130200g*	Ethylene-responsive transcription factor
			NC_023759.1 + 1124206	16.25	*PHAVU_001G013100g*	Uncharacterized sugar kinase
			NC_023751.1 + 36134728	17.37	*PHAVU_009G248400g*	ABC transporter C
			NC_023753.1 + 8531062	17.45	*PHAVU_007G085700g*	Cleavage stimulating factor 64
5	VI	BRM48906	NC_023759.1 + 50509430	8.77	*PHAVU_001G245900g*	Endo-beta-N-acetylglucosaminidase
			NC_023755.1 + 36557469	9.72	*PHAVU_005G137300g*	LOB domain-containing protein 1
			NC_023749.1 + 28379554	10.25	*PHAVU_011G128400g*	Sec1 family domain-containing protein
			NC_023750.1 + 40079257	11.83	*PHAVU_010G130600g*	Transcription factor WER

7 9	VI	BRM48906 CFBP6533 LNPV30.30	NC_023755.1 + 36557469 NC_023750.1 + 1486044 NC_023759.1 + 50509430 NC_023757.1 + 39679407 NC_023758.1 + 47178311 NC_023751.1 + 26127805	7.41 10.5 10.83 11.03 11.22 11.78	*PHAVU_005G137300g* *PHAVU_010G009400g* *PHAVU_001G245900g* *PHAVU_003G185000g* *PHAVU_002G310200g* *PHAVU_009G178400g*	LOB domain-containing protein 1 MFS transporter Endo-beta-N-acetylglucosaminidase DNA polymerase kappa Ethylene-responsive transcription factor TINY serine-protein kinase ATM
10	III	CFBP6533	NC_023750.1 + 40030862 NC_023753.1 + 8531062 NC_023751.1 + 23937892 NC_023753.1 + 2858208 NC_023759.1 + 42391449 NC_023758.1 + 1789712 NC_023754.1 + 31863272 NC_023759.1 + 48969195	13.01 14.79 15.75 16.29 17.28 17.49 17.49 17.6	*PHAVU_010G130200g* *PHAVU_007G085700g* *PHAVU_009G164300g* *PHAVU_007G035500g* *PHAVU_001G162900g* *PHAVU_002G016300g* *PHAVU_006G220000g* *PHAVU_001G228500g*	Ethylene-responsive transcription factor Cleavage stimulating factor 64 Transcription factor bHLH137 ATP-dependent Clp protease Embryo Sac development arrest protein SUMO-conjugating enzyme SCE1 Uncharacterized protein U-box domain-containing protein 21
43	II	NCPPB1654	NC_023750.1 + 38253314 NC_023757.1 + 11670450 NC_023755.1 + 31695651 NC_023751.1 + 21753884 NC_023749.1 + 2799132 NC_023759.1 + 35343683	4.84 5.04 5.26 5.26 5.26 5.46	*PHAVU_010G115900g* *PHAVU_003G075400g* *PHAVU_005G108400g* *PHAVU_009G148800g* *PHAVU_011G032200g* *PHAVU_001G125800g*	Protein Yippee-like At4g27745 Non-lysosomal glucosylceramidase Wound-responsive family protein Xylan alpha-glucuronosyltransferase RING/U-box superfamily protein Two-component response regulator ARR9

[1] TALE proteins carrying redundant RVD sequences
[2] Scores of RVD-nucleotide binding affinity. Best score possible for RVD1: 9.34; RVD2: 7.61; RVD3: 7.76; RVD4: 6.96; RVD5: 4.44; RVD7: 4.46; RVD10: 6.30; and RVD43: 4.84
[3] Annotation by functional KEGG orthologs

2.5 DISCUSSION

In this study, we set out to predict the TALE proteins in a group of 38 *Xcf* strains and to determine their common bean gene targets using genome sequences of the pathogen and a reference host genome available in the GenBank. First, the taxonomic position of the strains whose genome sequences were utilized in the analysis was confirmed by paired ANI comparisons with the *Xcf* pathotype strain. Then, since the quality of genome sequences is essential for the accurate prediction of TALE proteins, the genome sequences were subjected to BUSCO. High completeness values were shown by BUSCO for most of the strains when compared to the Xanthomonadales lineage, indicating they were suitable for TALE predictions.

The results indicated that the genomes of individual *Xcf* strains encode between 0 and 3 functional TALE proteins. Interestingly, no TALE genes (or pseudogenes) were predicted in the genomes of 8 out of 35 strains. AnnoTALE predicts TALE genes based on the gene sequence of at least one conserved tandem repeat and validates this prediction (or lack thereof, in the case of pseudogenes) by the association of C- and N-terminal regions of the TALE protein (GRAU et al., 2016). The software did not identify any single repeat in the genomes of nine strains, suggesting that no TALE gene was present in the genomes of these strains and raising the hypothesis that TALEs may not be essential for *Xcf* pathogenicity. This idea was experimentally verified by Foucher et al. (2022) through the inoculation of an *Xcf* TALE-mutant strain in susceptible common bean plants. The absence of TALE genes drastically reduced the virulence of the mutant strain, although visible disease symptoms were still observed in the leaves 14 days after inoculation. Similarly, a study on the contributions of TALEs to the virulence of *X. oryzae* pv. *oryzae* on rice plants showed that a TALE-mutant strain continued to cause symptoms four weeks after inoculation but did not colonize the leaf veins and systemically spread in the leaf tissue (YU et al., 2011). These observations suggest TALEs could be qualitatively involved in specific stages of disease development during the plant-bacteria interaction. As for the *P. vulgaris-Xcf* interaction, no comparison of the virulence of isogenic strains expressing and not expressing particular TALEs has been reported. Additionally, no information was found regarding the roles of TALEs in the late stages of CBB development, such as systemic and seed colonization through the plant vessels.

A repertoire of 56 different TALE genes was predicted for the set of *Xcf* strains analyzed. Protein BLAST analysis revealed that most predicted TALE gene sequences matched those of TALEs previously reported, mostly effectors of the AvrBs3 family. These matches validate the prediction of TALE genes in the *Xcf* genomes by AnnoTALE conducted in this study. Moreover, some of the proteins that matched the effectors predicted here were also previously predicted for the *Xcf* 4834-R strain, namely *XfuTAL1* and *XfuTAL2* (DARRASSE et al. 2013). Later, Ruh et al. (2017) reported the sequences of four TALEs: *TAL23A* (same as *XfuTAL1*), *TAL18H* (same as *XfuTAL2*), and *TAL18G*, which were also found here. Up until now, these two works were the basis of TALE predictions in the *P. vulgaris-Xanthomonas* pathosystems.

In this study, new TALE proteins were identified in a larger number of *Xcf* genomes and their sequence similarity to previously reported TALEs is described. We found sequence similarity between the *TALE17* described here and *TAL18H_CFBP6164* and *TAL18H_CFBP6546R* found in different bacterial species; while *TALE17* was predicted here in *Xcf* strains M12 and PR8F, the TALEs reported by Ruh et al. (2017) were predicted in strains of *Xpp*. Since no DNA sequences were provided by Ruh et al. (2017), it was not possible to identify additional allelic variants between predicted TALE proteins. Furthermore, some TALE genes were shared between different *Xcf* strains (located either in plasmids or chromosomes). This finding is in line with Ruh et al. (2017), who hypothesized horizontal gene transfer events (mediated by plasmid conjugation and transposons) related to TALE genes between *Xanthomonas* strains causing CBB. Additional mechanisms may shape the evolution of TALEs and *Xcf* pathogenicity. For instance, three synonymous substitutions were identified when comparing the DNA sequences of *TALE4* and *TALE48*, all of them located in the binding domain of the effector protein near the RVDs. Nonetheless, no nonsynonymous substitutions were identified in this study.

The RVD sequence of TALE proteins is crucial to the specificity of binding to target DNA sites in the host genome (MOSCOU; BOGDANOVE, 2009). Here, sequence redundancy among RVDs of different TALE proteins was found, suggesting the presence of gene variants with the same binding specificity in the plant genome. Nonetheless, experimental evidence suggests different TALE proteins with the same RVD sequence contribute differently to bacterial virulence. For example, Foucher (2020) inoculated a susceptible common bean genotype with several *Xcf* strains

carrying mutations in RVD-redundant *XfuTAL1* homologs and observed differences in disease severity and in the expression of a susceptibility gene, but not in the bacterial population in the inoculated plants at 14 days post-inoculation. These findings suggest other domains of the TALEs, in addition to the composite RVD sequence, contribute quantitatively to disease development and expression of targeted genes. In this regard, it is tempting to hypothesize that the strength with which the activation domains interact with the host transcription machinery could play an important role.

Sequence similarity analysis of TALE RVDs and their classification in groups is a useful approach to assessing their evolution and the commonality of their host target sites (ERKES et al., 2017). Our analysis revealed six host loci targeted by the repertoire of TALEs predicted in the set of *Xcf* genomes investigated. The majority of predicted RVDs of the TALE proteins were placed in group II. Yet, TALE effectors from the same group could target different genes in the *P. vulgaris* genome due to slight sequence differences within the group. In general, four main general cellular functions of common bean are predicted to be influenced by the repertoire of *Xcf* TALE proteins.

Some of the most characterized susceptibility genes induced by *Xanthomonas* TALEs in their hosts encode SWEET proteins, membrane carbohydrate transporters that take sugars from the host cytoplasm to the apoplast, favoring the bacterial growth. Studies have described the induction of *SWEET* gene transcription by TALEs in the citrus-*X. citri* pv. *citri* and in cassava-*X. phaseoli* pv. *manihotis* pathosystems; both pathogens are phylogenetically closely related to *Xanthomonas* causing CBB (HU et al., 2014; ZARATE-CHAVES et al., 2021). In our study, no evidence of common bean *SWEET* gene induction by *Xcf* TALE proteins was found, albeit a transcriptomics study revealed the induction and repression of a *SWEET* gene in susceptible and resistant genotypes, respectively (FOUCHER et al., 2020).

In our prediction of host gene targets, we observed the recurrence of transport-related genes (*PHAVU_007G003700g*, myo-inositol transporter; *PHAVU_009G248400g*, ABC transporter; and *PHAVU_010G009400g*, MFS transporter). Besides carbohydrates, ABC and MFS transporters are involved in the transport of a wide range of molecules in plant cells such as peptides, nitrate, and phytohormones (DEVANNA et al., 2021). Furthermore, myo-inositol has been associated with hormone balance, cell signaling, and responses to biotic and abiotic stresses in plant cells (BRUGGEMAN et al., 2020). We also identified genes related to

intracellular vesicle trafficking, cytoskeleton reorganization, and secretion (*PHAVU_003G073000g*, AP-3 complex subunit sigma; *PHAVU_007G045600g*, ADP-ribosylation factor 8; and *PHAVU_011G128400g*, sec1 family domain-containing protein), which have not been previously associated with other plant-*Xanthomonas* pathosystems (MEMON, 2004; ZWIEWKA et al., 2011; KARNAHL et al., 2018). Additionally, genes related to plant sugar metabolism were identified among the common target genes of *Xcf* TALEs (*PHAVU_009G148800g*, Xylan alpha-glucuronosyltransferase; *PHAVU_001G013100g*, sugar kinase; and *PHAVU_001G245900g*, Endo-beta-N-acetylglucosaminidase). Altogether, these results suggest the induction of degradation of sugar molecules and their release from the plant cell cytoplasm to bacterial cells in the apoplast could be a common bean susceptibility factor exploited by *Xcf*; as already been shown for plant *SWEET* genes induced by TALEs of *Xanthomonas* that infect other plant species, such as rice and citrus (VERDIER et al., 2012; STREUBEL et al., 2013; COX et al., 2017).

Another biochemical function associated with the predicted host genes induced by *Xcf* TALEs was protein degradation via the ubiquitin-proteasome system (UPS; *PHAVU_003G172400g*, F-box/LRR-repeat protein 14; *PHAVU_008G102100g*, E3 ubiquitin-protein ligase PRT6; *PHAVU_002G016300g*, SUMO-conjugating enzyme SCE1; *PHAVU_001G228500g*, U-box domain-containing protein 21; and *PHAVU_011G032200g*, RING/U-box superfamily protein). In addition to protein degradation, the UPS is related to plant cell senescence and induction of the jasmonic acid pathway (RAAB et al., 2009; ZHANG; XU; GUO, 2020). The UPS pathway has been associated with the function of the non-TALE protein XopD from the pepper and tomato pathogen *X. euvesicatoria* which enters the plant nucleus and induces the ubiquitination of certain resistance-associated transcription factors (KAY; BONAS, 2009). Also, the non-TALE XopAE, described in *X. euvesicatoria*, was found to interact with a plant E3 ubiquitin ligase, enhancing bacterial virulence in *Arabidopsis thaliana* by inhibiting the defense response (POPOV; MAJHI; SESSA, 2018). Conversely, a previous genetic mapping study identified a gene encoding an E3 ubiquitin-protein ligase in a locus that contributes to the quantitative resistance of common bean genotypes against CBB (ZHU et al., 2016). Our work presents the first prediction of the *P. vulgaris* ubiquitin-proteasome system as a potential target of *Xcf* TALEs, but further functional analysis is required to elucidate whether UPS induction is TALE-specific and contributes to *Xcf* virulence in common bean.

Plant hormone balance plays a key role during plant-pathogen interactions. Our results suggest the repertoire of candidate *Xcf* TALEs interferes with the hormonal balance of common bean plants. We consistently observed genes related to the induction of the ethylene (ET) pathway (*PHAVU_008G131500g*, ethylene-responsive transcription factor At4g13040; *PHAVU_010G130200g*, ethylene-responsive transcription factor AIL1; *PHAVU_002G310200g*, ethylene-responsive transcription factor TINY; and *PHAVU_005G108400g*, wound-responsive family protein). These genes are responsible for inducing or participating in the ET-dependent metabolic pathway that controls plant resistance responses to necrotrophic pathogens, insects, and abiotic stresses such as wounds (KIM; BOVE; ASSMANN, 2008; LICAUSI; OHME-TAKAGI; PERATA, 2013; CATINOT et al., 2015; DOWD; JOHNSON, 2020). These findings are in line with the results of a transcriptomics study that revealed the induction of the ethylene-related pathways in inoculated susceptible bean plants, while in the resistant genotype, the ET pathway was repressed and the salicylic acid pathway was induced (FOUCHER et al., 2020). Furthermore, a recent study provided a comprehensive characterization of the induction of the ethylene-responsive transcription factor AIL1 gene in common bean susceptible plants by the *Xcf* XfuTAL1 effector (FOUCHER, 2020), confirming our hypothesis that the induction of the ET pathway contributes to the susceptibility of common bean to *Xcf*. Here, we predicted three additional ET-related genes in the *P. vulgaris* genome that are potentially induced by TALE candidates of the *Xcf* strains investigated.

It is worth considering that previous studies have highlighted the influence of sequencing technology on TALE predictions due to the repetitive nature of the internal tandem-repeat portions of these proteins. Technologies that provide short-length reads (*e.g.*, Illumina) can pose difficulties in correct sequencing or assembly of the internal repeats of TALE genes when compared to technologies that release longer reads (*e.g.*, PacBio) (GRAU et al., 2016; PENG et al., 2016; RUH et al., 2017). In this study, 22 out of 56 predicted TALE genes were excluded from the complete analysis due to their short composite RVD sequences, which were below the functional cut-off proposed by Boch et al. (2009). Furthermore, we observed sequence identity between some short and long composite RVD sequences within the TALE groups, suggesting overlapping or redundancy in host gene targets. We cannot conclusively rule out the possibility that the short composite RDVs resulted from sequencing or assembly errors.

Taken together, these considerations suggest that the repertoire of TALEs in the diversity of *Xcf* strains analyzed in this study and their *P. vulgaris* gene targets may have been underestimated. In any case, the results of this study shed light on the mechanisms underlying the susceptibility of *P. vulgaris* to *Xcf*. They indicate that *Xcf* may manipulate the transport of carbohydrates and other substances, protein degradation, and the ethylene signaling pathway to favor the infection of common bean plants.

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3 CHAPTER II: DEFENSE-RELATED ENZYMATIC ACTITIVIES IN SUSCEPTIBLE AND RESISTANT *Phaseolus vulgaris* GENOTYPES IN RESPONSE TO *Xanthomonas citri* pv. *fuscans*

3.1 ABSTRACT

Common Bacterial Blight (CBB) is an important disease that compromises common bean production worldwide. It is caused by the Gram-negative bacteria *Xanthomonas citri* pv. *fuscans* (*Xcf*) and *X. phaseoli* pv. *phaseoli*. Resistance to CBB is the best strategy for CBB management, however, the underlying mechanisms of common bean resistance to the disease (*e.g.*, the activity of defense-related enzymes) are not fully understood. The aim of this study was to compare the activities of β-1,3-glucanase, phenylalanine ammonia-lyase, peroxidase, polyphenol oxidase, and lipoxygenase in leaf tissue of BC_3F_4 (resistant) common bean plants derived from a BRS Radiante (resistant) × Carioca MG (susceptible) cross with those of the cultivar Carioca MG with or without the inoculation of *Xcf*. A significant increase in the activity of all enzymes was observed in the BC_3F_4 plants compared to Carioca MG at 48 h post-inoculation. Furthermore, these increases in enzyme activities were associated with reductions of bacterial populations in leaf tissue also at 48 h post-inoculation and the severity of disease symptoms. These results contribute to the elucidation of the biochemical mechanisms involved in the resistance response of *P. vulgaris* to *Xcf*.

3.2 INTRODUCTION

Common Bacterial Blight (CBB) is an important disease that affects the productivity of the common bean (*Phaseolus vulgaris* L.) crop, causing significant yield losses, particularly in tropical regions (WALLEN; JACKSON, 1975; GILLARD et al., 2009; DA SILVA JÚNIOR et al., 2022). CBB is caused by two distinct Gram-negative bacteria, *Xanthomonas citri* pv. *fuscans* (*Xcf*) and *X. phaseoli* pv. *phaseoli* (*Xpp*), which can occur singly or together in the field (DA SILVA JÚNIOR et al., 2022). Chemical treatments have proven ineffective in controlling the disease, making the use of resistant cultivars the most recommended method (MARINGONI, 1990). Consequently, researchers worldwide have made considerable efforts in trying to identify sources of resistance and to understand the genetics and inheritance behind the resistance phenotype (SINGH; MUÑOZ, 1999; MIKLAS et al., 2006; VITERI et al., 2014; ZHU et al., 2016; MONTEIRO et al., 2020).

Several studies have investigated the resistance of common bean and other *Phaseolus* species to CBB and described it as being a quantitative trait controlled by polygenes, as well as being cultivar- and strain-dependent (SINGH; SCHWARTZ, 2010; MIKLAS et al., 2017; MONTEIRO et al., 2021; ALVES et al., unpublished data). The Laboratory of Molecular Phytobacteriology at the Universidade Federal de Viçosa conducted phenotyping to assess the reactions of a set of Brazilian cultivars to inoculation with an *Xcf* strain (MONTEIRO et al., 2020). Cultivar BRS Radiante was classified as highly resistant, while Carioca MG was classified as highly susceptible to the bacterium. Inheritance studies demonstrated that the F_2 population derived from a cross between these two cultivars segregated in a proportion consistent with the action of two dominant genes and additional polygenic effects. Next, the crosses were continued until generating the BC_3F_3 population (MONTEIRO et al., unpublished data).

The mechanisms underlying the resistance of common bean to CBB have not been fully elucidated. However, some genes, such as those coding for lipoxygenase, oxygenase, protein kinases, phosphatases, and Nucleotide Binding site - Leucine-Rich Repeat (NBS-LRR) proteins, have been found near to Single-Nucleotide Polymorphisms (SNPs) associated with resistance (WU et al., 2017; MONTEIRO et al., 2021; BARBOSA et al., 2022). Additionally, transcriptomic analyses have revealed the upregulation of genes associated with the phenylpropanoid and salicylic acid pathways, isoflavone and phenylalanine biosynthesis as well as Reactive Oxygen Species (ROS) balance in resistant common bean genotypes (FOUCHER et al., 2020; COX et al., 2021; YANG et al., 2022). Nonetheless, to the best of our knowledge, a functional demonstration of the participation of these genes in the resistance of common bean to *Xcf* has not been reported.

Plant biochemical alterations, including the activity of defense-related enzymes, have been studied in the context of quantitative resistance in plants against pathogen attack (CORWIN; KLIEBENSTEIN, 2017; GOU et al., 2023). However, in the common bean-*Xanthomonas* pathosystems, the role of these enzymes has not been sufficiently studied. Some studies conducted with *Xpp* strains have reported increases in the activities of β-1,3-glucanase, peroxidase, polyphenol oxidase, and phenylalanine ammonia-lyase in common bean plants treated with different resistance inducers (FARAHANI; TAGHAVI; AFSHARIFAR, 2016; TOILLIER et al., 2010; QUEIROZ, 2019; COSTA et al., 2020). Only a single study compared the response of resistant (Diamante Negro) and susceptible (Ouro Negro) common bean cultivars to an *Xpp*

strain and described high peroxidase activity in resistant plants (SILVA et al., 2020). Nonetheless, alterations in the activity of other enzymes involved in the common bean plant response to *Xcf* have not been conclusively unveiled. Therefore, the objective of this study was to determine the effect of *Xcf* infection on the activity of *P. vulgaris* defense-related enzymes by comparing the resistant common bean inbred line BC_3F_4 (derived from a cross between BRS Radiante and Carioca MG) and its susceptible parental Carioca MG.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial strain

The strain CFBM-UFV-0001 of *Xcf* was used for plant inoculations. Previous studies demonstrated that cultivars BRS Radiante and Carioca MG are resistant and susceptible to this strain, respectively (MONTEIRO et al., 2020). To conduct the experiments, the strain was retrieved from stocks in 30% glycerol maintained at -80 ºC in the culture collection of the Laboratory of Molecular Phytobacteriology of the Department of Plant Pathology at Universidade Federal de Vicosa, Brazil (CFBM-UFV), and grown on solid 523 medium (KADO; HESKETT, 1970) at 28 °C for 48 h.

3.3.2 Plant genotypes and maintenance

Seeds of the common bean cultivar Carioca MG were kindly provided by Professor José Eustáquio de S. Carneiro (Department of Agronomy, Universidade Federal de Vicosa, MG, Brazil). This cultivar was used as the susceptible genotype in the biochemical experiments. Plants of the BC_3F_4 generation derived from a cross BRS Radiante × Carioca MG (MONTEIRO et al., unpublished data) were used as the resistant genotype in the same experiments. To obtain the BC_3F_4 population, BC_3F_3 seeds (MONTEIRO et al., unpublished data) were sown in 1-l pots containing a 1:1 mixture of soil and plant substrate Tropstrato (Vida Verde, Mogi Mirim, SP, Brazil). Plants were maintained in a greenhouse under environmental conditions (Vicosa, MG, Brazil; 20.7549° S, 42.8786° W), fertilized every 15 d with Niphokam (Fênix-Agro-Pecus Industrial Ltda, Tietê, SP, Brazil) and irrigated every other day until pod development was complete. Plants of Carioca MG and the BC_3F_4 population were grown as indicated above and maintained in a growth chamber at 28 °C with a 12-h photoperiod until bacterial inoculation. The plants were fertilized every 15 d with

Niphokam (Fênix-Agro-Pecus Industrial Ltda, Tietê, SP, Brazil) and irrigated according to their needs.

3.3.3 Plant inoculation and disease evaluation

Plants at the V3 growth stage were placed in a mist chamber (relative humidity ≥ 90%) 24 h before bacterial inoculation. For inoculum preparation, colonies of *Xcf* CFBM-UFV-0001 were recovered from cultures grown on solid 523 medium for 48 h and resuspended in 10 mM $MgCl_2$. The optical density at 600 nm of the bacterial suspension was adjusted to 0.1 (~2×10^7 Colony-Forming Units (CFU) ml^{-1}), and a drop of Tween-80 was added. The bacterial suspension was sprayed onto the plant leaves using an atomizer (Jet Master, Schulz S.A., Joinville, SC, Brazil) until runoff. The plants were returned to the mist chamber for 24 h and subsequently transferred to the growth chamber at 28 °C for disease development. Fifteen days after inoculation (DAI), disease severity was evaluated on inoculated trifoliate leaves according to a diagrammatic scale ranging from 1 to 9 (SCHOONHOVEN; PASTOR-CORRALES, 1987), where: 1 = absence of visible symptoms and 9 = highly severe symptoms.

3.3.4 Quantification of the bacterial population *in planta*

The *Xcf* population in the leaf tissue of inoculated plants was estimated at 0, 1, 2, and 9 DAI. To this end, trifoliate leaves were collected and placed in an ice bucket. Five leaf discs (diameter = 7 mm) were taken from five standardized locations on the three leaflets and macerated in 1 ml of sterile water in a 1.5 ml microfuge tube. The macerate was subjected to 1:10 serial dilutions in sterile water, and 20 µl of each dilution (10^0 to 10^8) were dropped onto the surface of solid 523 medium amended with 100 µg ml^{-1} rifampicin and 50 µg ml^{-1} cycloheximide. The plates were incubated at 28 °C for 48 h and the number of CFU was counted. The bacterial population sizes were expressed in CFU cm^{-2} of leaf tissue.

3.3.5 Enzymatic analysis

At 24 and 48 h after bacterial inoculation, trifoliate leaves were removed from inoculated plants and immediately placed in liquid nitrogen. The leaves were macerated with a mortar and pestle under liquid nitrogen until reaching a powdery texture, and the powder was stored at -80 °C. A total of 100 mg of leaf sample was used for enzyme extraction. For that, the leaf powder was resuspended in 1 ml of extraction buffer (50 mM potassium phosphate, 1 mM ethylenediaminetetraacetic acid,

and 1 mM phenylmethylsulphonyl fluoride) and centrifuged at 13,000 × *g* at 4 °C for 15 min. The supernatant was stored at 4 °C and used for the subsequent enzymatic activity assays.

The activities of β-1,3-glucanase (GLU; EC 3.2.1.39), lipoxygenase (LOX; EC 1.13.11.12), polyphenol oxidase (PPO; EC 1.10.3.1), and phenylalanine ammonia-lyase (PAL; EC 4.3.1.24) were determined according to the protocols described by Fortunato et al. (2015), and the activity of peroxidase (POX; EC 1.11.1.7) was determined according to the protocol described by Debona et al. (2012); both adjusted to a final volume of 250 µl to be conducted in microplate wells.

3.3.6 Experimental design and data analysis

Data from disease evaluation and enzymatic analysis were submitted to homoscedasticity and normality tests. Data adjusted to parametric tests were analyzed according to F-test and grouped with Tukey's test ($p \leq 0.05$). The data from disease severity assays were analyzed based on a completely randomized design considering the variations between the two plant genotypes. The enzymatic activity and bacterial population size data were analyzed considering a triple-factor, determining the interactions between different plant genotypes, times of evaluation, and bacterial inoculation. Data were analyzed according to Kruskal-Wallis one-way analysis of variance ($p \leq 0.05$) when not normal or homoscedastic. All data analysis and graphical constructions were carried out in R (R CORE TEAM, 2022) using the packages lmtest (ZEILEIS; HOTHORN, 2002), ExpDes (FERREIRA; CAVALCANTI; NOGUEIRA, 2014), and ggplot2 (WICKHAM, 2016).

3.4 RESULTS

3.4.1 Disease severity

The two common bean genotypes showed contrasting reactions to inoculation with *Xcf* at 15 DAI (Figure 1A). Typical necrotic lesions surrounded by chlorotic haloes were observed in the trifoliate leaves of Carioca MG plants whereas no visible disease symptoms were distinguished on leaves of BC_3F_4 plants. In this way, CBB severity was different between the analyzed plant genotypes (Appendix Table A1). The average severity score was 5.7 for Carioca MG and 1.0 for BC_3F_4 plants (Figure 1B).

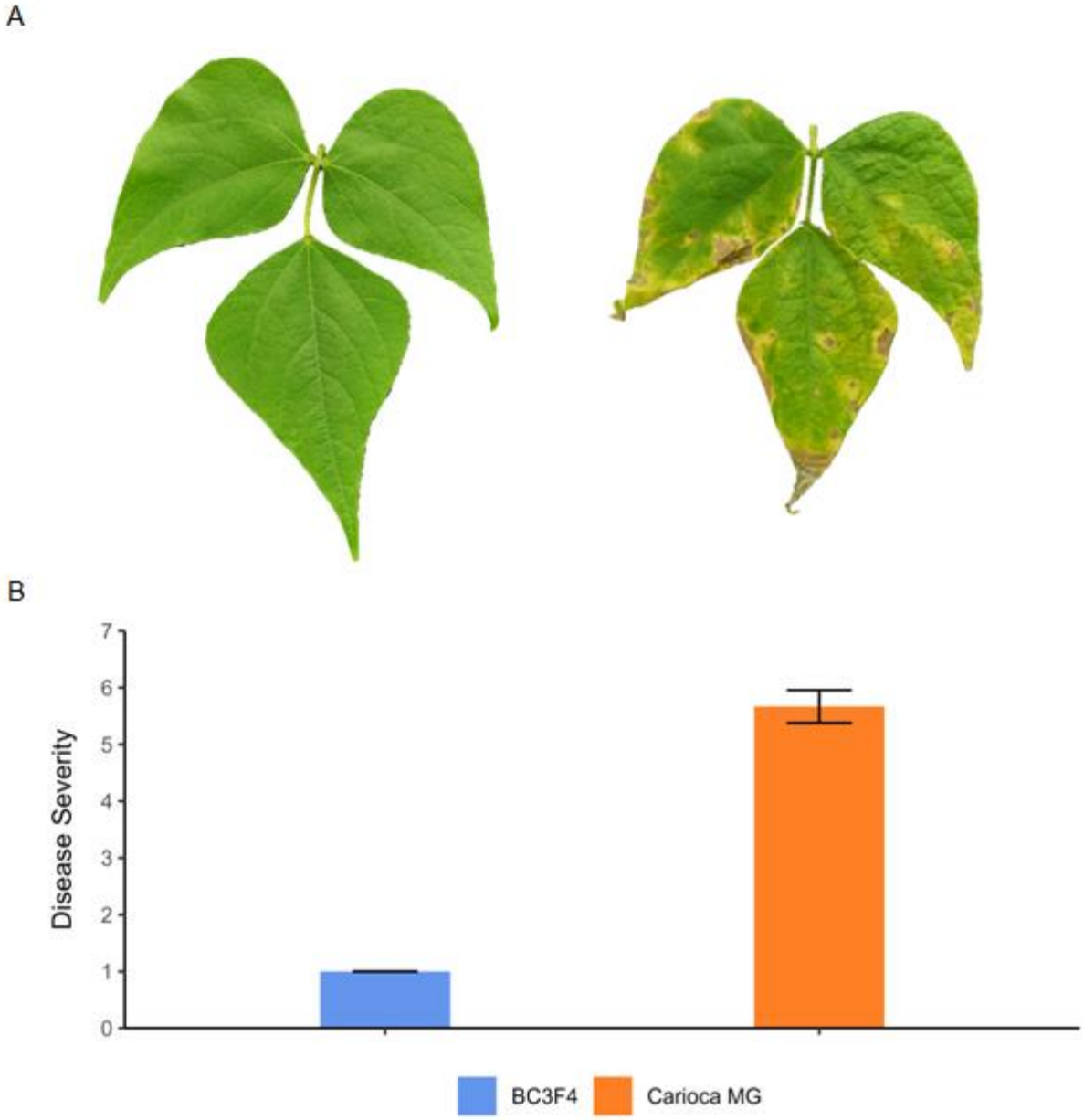

Figure 1. Response of common bean Carioca MG and BC_3F_4 plants to inoculation with *Xanthomonas citri* pv. *fuscans* at 15 days after inoculation. (A) representative trifoliate leaves of inoculated BC_3F_4 (left) and Carioca MG (right) plants exhibiting disease symptoms; images are shown in the same scale. (B) Disease severity rate.

3.4.2 Bacterial population *in planta*

Quantification of the *Xcf* population in leaf tissue of the two genotypes was conducted to gain insights into the dynamics of the bacterial growth during various stages of the common bean-*Xcf* interaction, ranging from the early stages (0-2 DAI) to symptom induction (9 DAI). There was no significant difference in the *Xcf* population sizes between the two plant genotypes until the first DAI. However, different population

dynamics were evident after this time. The pathogen population in BC_3F_4 plants decreased between the first and second DAI, but remained relatively stable in Carioca MG plants. Conversely, an increase in the bacterial population was observed in Carioca MG plants from the second to the ninth DAI, coinciding with the appearance of the first disease symptoms. In contrast, the *Xcf* population in BC_3F_4 plants decreased during the same period (Appendix Table A2). Moreover, the pathogen population in leaf tissue of Carioca MG leaves was 1.8 and 2.5 times higher than that in BC_3F_4 leaves at 2 and 9 DAI, respectively. The *Xcf* population was around 10^5 to 10^6 CFU cm^{-2} in Carioca MG leaf tissue at the time of symptom appearance (9 DAI), while in BC_3F_4 plants it was between 10^3 and 10^4 CFU cm^{-2} at the same evaluation time (Figure 2).

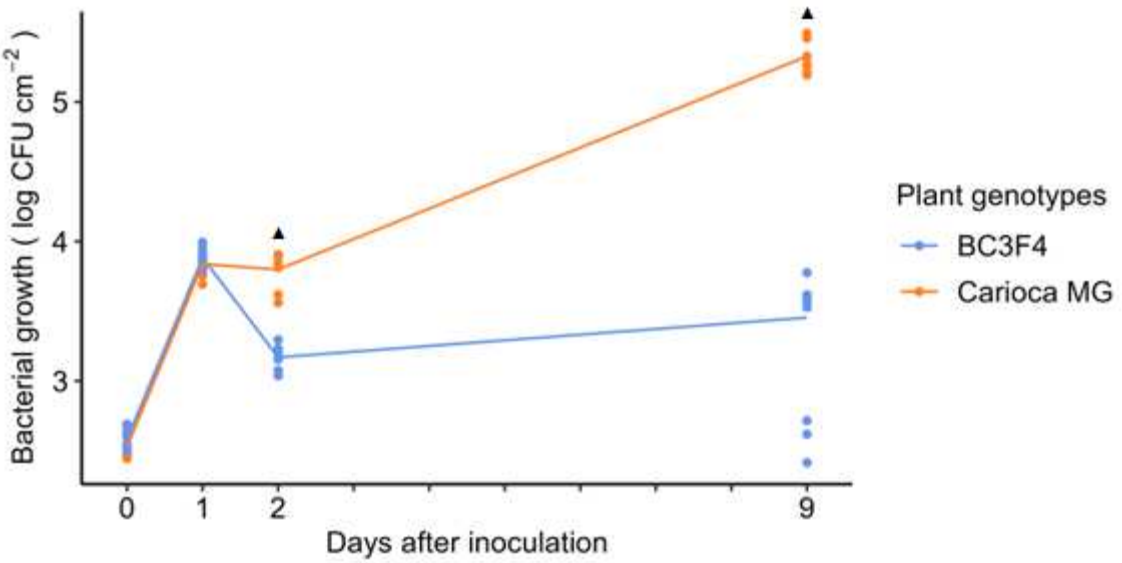

Figure 2. Population sizes of *Xanthomonas citri* pv. *fuscans* in leaf tissue of common bean BC_3F_4 and Carioca MG plants. The black triangle (▲) indicates a significant difference between genotypes within a particular evaluation time (Kruskal-Wallis rank sum test; $p \leq 0.05$)

3.4.3 Defense-Related Enzyme Activities

The results revealed significant effects of and interactions between plant genotype, time of evaluation, and plant inoculation (Appendix Table A3) on enzymatic activity. Higher enzymatic activities were observed in the resistant genotype (BC_3F_4) compared to the susceptible one (Carioca MG) at 48 h post inoculation with *Xcf* for GLU, PAL, PPO, and LOX (Figure 3). The activity of GLU in inoculated plants was reduced by 35% from 24 h to 48 h post-inoculation in Carioca MG plants and increased by 51% in BC_3F_4 plants, which also showed a reduction in activity when non-inoculated

with *Xcf*. At 48 h post-inoculation, GLU activity in BC_3F_4 plants was higher than in Carioca MG plants (Figure 3A).

A similar response was noted for PAL. It was observed that at 48 h post-inoculation, PAL activity was 1.9 times higher than at 24 h for BC_3F_4 inoculated plants, while Carioca MG plants maintained the mean level of enzyme activity. The PAL activity for the genotypes at 48 h was significantly different; BC_3F_4 plants exhibited PAL activity 2.2 times higher than Carioca MG plants. Furthermore, the inoculation experimental factor significantly reduced and increased the PAL activity in the susceptible and resistant genotypes, respectively. An increase in PAL activity at 48 h in non-inoculated Carioca MG plants was observed (Figure 3B).

Both common bean genotypes showed a decrease in PPO activity in the absence of *Xcf*. On the other hand, upon bacterial inoculation, the BC_3F_4 plants maintained the PPO activity level while in Carioca MG it was reduced by 50.7% from 24 to 48 h. At 48 h, PAL activity was 47% lower in Carioca MG than in BC_3F_4 plants (Figure 3C).

The activity of LOX was reduced from 24 h to 48 h in non-inoculated BC_3F_4 plants. Conversely, LOX activity significantly increased during the same period in the BC_3F_4 plants inoculated with *Xcf*. At 48 h post-inoculation, LOX activity in BC_3F_4 plants was 40% higher than in Carioca MG plants. No other signficicant differences in LOX activity were observed among the treatments (Figure 3D).

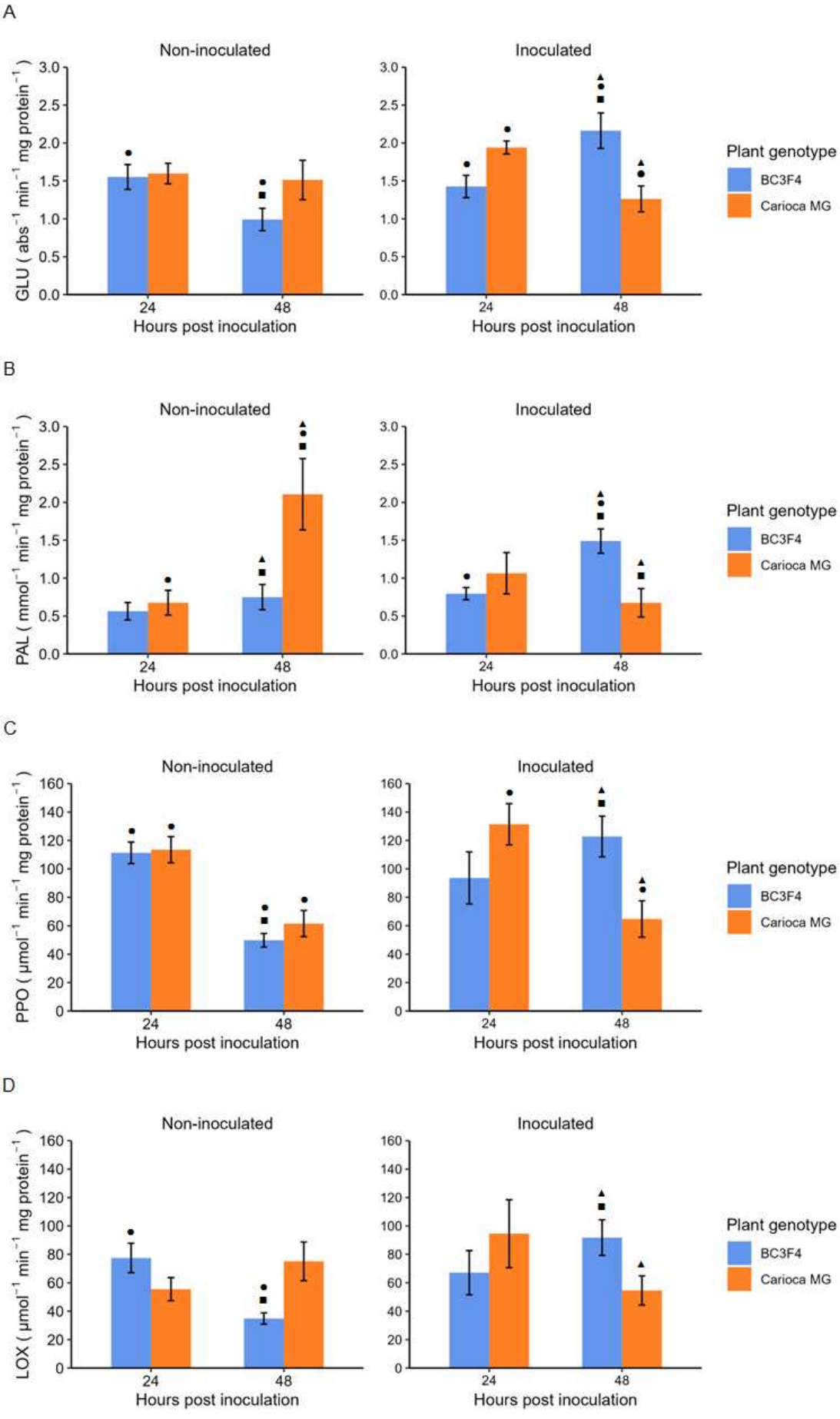
A
Non-inoculated
Inoculated
GLU (abs⁻¹ min⁻¹ mg protein⁻¹)
Hours post inoculation
B
PAL (mmol⁻¹ min⁻¹ mg protein⁻¹)
C
PPO (µmol⁻¹ min⁻¹ mg protein⁻¹)
D
LOX (µmol⁻¹ min⁻¹ mg protein⁻¹)
Plant genotype
BC3F4
Carioca MG

Figure 3. Activities of defense-related enzymes in leaf tissue of resistant (BC_3F_4) and susceptible (Carioca MG) plants non-inoculated or inoculated with *Xanthomonas citri* pv. *fuscans*. Bars represent means and vertical lines depict the standard errors of the means. Symbols above the bars represent significant differences (Tukey's test; $p \leq 0.05$) between means. Triangles (▲) indicate differences between genotypes within a particular evaluation time; circles (●) indicate differences between times of evaluation for a particular genotype and within non-inoculated or inoculated plants. Squares (■) represent differences between non-inoculated and inoculated plants for each genotype.

The activity of POX was similar in non-inoculated plants of both genotypes regardless of the time of evaluation. The enzyme activity in inoculated Carioca MG plants was reduced from 24 h to 48 h post inoculation, while in BC_3F_4 plants no significant difference was observed during the same evaluation period. POX activity at 48 h was 1.7-fold higher in BC_3F_4 plants than in Carioca MG plants inoculated with *Xcf* (Appendix Table A4) (Figure 4).

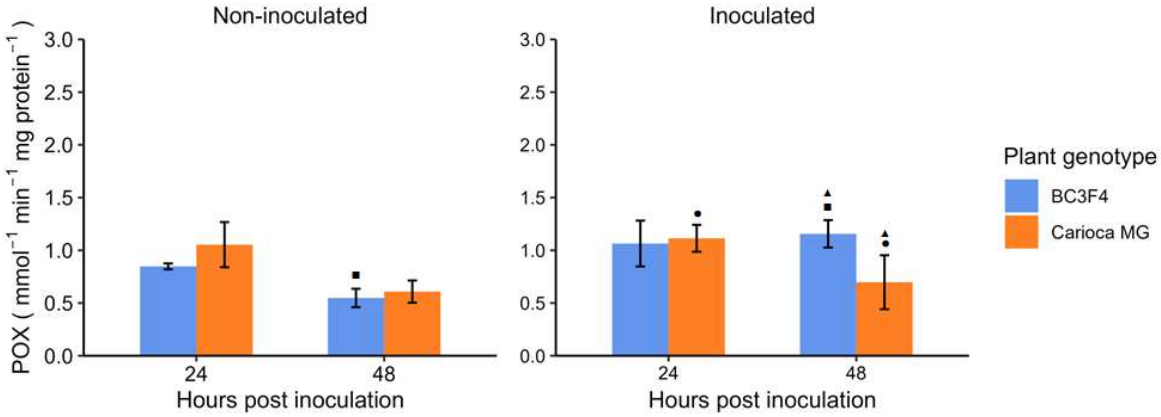

Figure 4. Peroxidase activity in leaf tissue of resistant (BC_3F_4 population) and susceptible (Carioca MG) plants non-inoculated or inoculated with *Xanthomonas citri* pv. *fuscans*. Bars represent means and vertical lines depict the standard errors of the means. Symbols above the bars indicate significant differences (Kruskal-Wallis rank sum test; $p \leq 0.05$) between means. Triangles (▲) indicate differences between genotypes within a particular evaluation time; circles (●) indicate differences between times of evaluation for a particular genotype and within non-inoculated or inoculated plants. Squares (■) represent differences between non-inoculated and inoculated plants for each plant genotype.

3.5 DISCUSSION

This study aimed to characterize the enzymatic responses of a common bean genotype resistant to CBB and compare them with those of a susceptible one. We investigated the reactions of the two genotypes (Carioca MG and BC_3F_4 plants) with respect to the time of appearance and severity of disease symptoms, sizes of bacterial populations in infected tissue and activity of defense-related enzymes (GLU, PAL, PPO, LOX, and POX), upon inoculation (or not) with *Xcf* strain CFBM-UFV-0001.

At 15 DAI, the BC_3F_4 plants exhibited little or no disease symptoms, whereas Carioca MG plants showed a higher disease severity, around 5-6 on the

severity scale, confirming the selection of resistant plants of the segregant F_2 population obtained from the Carioca MG x BRS Radiante cross (MONTEIRO et al., 2020; MONTEIRO et al., unpublished data). Also, a higher *Xcf* population size was recovered from Carioca MG than from BC_3F_4 beginning at 2 DAI. Yet, the bacterium was also recovered from the resistant genotype at all times evaluated. This limitation of bacterial growth in plant tissues supports the notion that common bean resistance to CBB is quantitative (SINGH; SCHWARTZ, 2010; MIKLAS et al., 2017; MONTEIRO et al., 2021; MONTEIRO et al., 2020; ALVES et al., unpublished data). In comparison, Foucher et al. (2020) showed differences in *Xpp* population sizes between susceptible (JaloEEP558) and resistant (BAT93) common bean genotypes at 5 DAI (three days later than in this study), supporting the idea that activation of resistance is dependent on the plant genotype and bacterial strain. On the other hand, previous studies observed increases in *Xcf* and *Xpp* populations in susceptible common bean cultivars until around 6 to 10 DAI when the population seems to stabilize (GABRIEL et al., 1989; JACQUES et al., 2005), which is consistent with our results.

Quantitative resistance to pathogens is a complex plant response involving various mechanisms, including the modulation of defense-related pathways and the activity of some key enzymes (POLAND et al., 2009; CORWIN; KLIEBENSTEIN, 2017; GOU et al., 2023). In this study, we observed distinct enzymatic profiles between the two genotypes in response to *Xcf* inoculation. The activities of GLU, PAL, PPO, LOX, and POX were significantly higher in the resistant plants compared to the susceptible ones at 48 h post-inoculation, but not at 24 h. These results align with previous studies reporting that transcriptional responses of defense-related pathways in common bean plants start at 24 h (FARAHANI; TAGHAVI; AFSHARIFAR, 2016) or 48 h after inoculation with *Xpp* (FOUCHER et al., 2020; COX et al., 2021). However, to the best of our knowledge, similar studies have not been reported for *Xcf*.

Several previous studies have evaluated the activity of defense-related enzymes in common bean genotypes in response to *Xpp* infection over time, albeit with the purpose to investigate the effectiveness of compounds in controlling CBB. For instance, Queiroz (2019) showed that PAL and POX activity was higher in plants pretreated with zinc-amino acid chelate compounds compared to non-treated plants three days after *Xpp* inoculation, which correlated with lower disease severity. Similarly, Toillier et al. (2010) observed an increase in POX activity six days after inoculation but increases in GLU and PAL activities were only observed on the ninth

day in common bean plants treated with a resistant inducer based on the Basidiomycete *Pycnoporus sanguineus*. In the context of innate plant resistance, Silva et al. (2020) reported that POX activity differed between susceptible and resistant common bean plants after nine days post *Xpp* inoculation. In the current study, earlier activation of enzymatic activities in response to *Xcf* inoculation was observed, suggesting different timings of defense induction depending on the plant genotype or the *Xanthomonas* species. Nonetheless, further investigation is required to confirm these hypotheses.

Transcriptional activation of PR proteins, such as PR-2 (GLU) is associated with the plant response to pathogen recognition (FORTUNATO et al., 2015; JAIN; KHURANA, 2018; GUPTA et al., 2022). In this study, in the susceptible plants, GLU activity decreased from 24 h to 48 h after inoculation, while an increase was observed in the resistant plants during the same period. GLU activity has been previously linked to plant resistance to other *Xanthomonas* (BABU et al., 2003; GUPTA et al., 2022). Transcriptomic analysis of common bean plants inoculated with *Xpp* demonstrated similar patterns, with the repression in susceptible and upregulation in resistant cultivars of transcription of GLU gene homologs at 48 h post-inoculation (FOUCHER et al., 2020).

Phenylalanine ammonia-lyase is a key enzyme involved in the phenylpropanoid pathway, tissue lignification, as well as biosynthesis of flavonoids and phytoalexins, all of which contribute to the defense responses of plants against several pathogens (YOU et al., 2020; GOU et al., 2023). PAL activity has been associated with induced resistance of common bean to *Xpp* (TOILLIER et al., 2010; QUEIROZ, 2019; COSTA et al., 2020). Also, transcriptomic analyses of susceptible and resistant common bean plants have shown an increase in PAL gene transcription, along with other genes involved in the phenylpropanoid pathway only in the resistant genotype (COX et al., 2021; YANG et al., 2022). In our study, PAL activity increased from 24 h to 48 h after *Xcf* inoculation only in the BC_3F_4 plants, supporting the notion of the involvement of PAL in CBB resistance in different common bean genotypes and *Xanthomonas* species.

Lipoxygenase is an important enzyme involved in plant defense against pathogens that mediates the production of defense-related compounds during plant-bacteria interactions, which in common bean has been shown to be activated during the Hypersensitive Response (HR) elicited by *Pseudomonas savastanoi* pv.

phaseolicola (CROFT; JUTTNER; SLUSARENKO, 1993). LOX genes have been found near Single-Nucleotide Polymorphisms (SNPs) associated with common bean resistance to *Xpp* (ZHU et al., 2016; SIMONS et al., 2021), and downregulation of LOX transcripts was observed in *Xpp*-inoculated plants compared to non-inoculated plants (COX et al., 2021). In our study, a significant increase in LOX activity was observed in leaf tissue of the resistant genotype at 48 h after *Xcf* inoculation and when comparing it with the susceptible genotype, which is consistent with previous reports.

Both POX and PPO are enzymes involved in maintaining the balance of ROS and cellular homeostasis in plant cells after the oxidative burst (CAMEJO et al., 2019; GARCÍA-CAPARRÓS et al., 2021; SALEEM; FARIDUDDIN; CASTROVERDE, 2021). Here, a reduction in POX and PPO activity in the susceptible genotype in the presence of *Xcf* is shown, while the activity in the resistant genotype remained relatively stable from 24 h to 48 h. Consistently, previous studies have reported increases in POX and PPO activities in susceptible common bean genotypes treated with inducers of resistance in the presence of *Xpp* (TOILLIER et al., 2010; QUEIROZ, 2019; COSTA et al., 2020). Conversely, the transcription of POX genes was found to be higher in a resistant common bean genotype compared to that of a susceptible genotype at 48 h after *Xpp* inoculation (COX et al., 2021). Again, further investigation is required to sort out these contrasting results suggesting the common bean response may be dependent on the *Xanthomonas* species.

Overall, this study provides insights into some of the biochemical mechanisms subjacent to the resistant response of common bean to *Xcf*. The significant differences observed in the activities of GLU, PAL, PPO, LOX, and POX, concomitant with reduced severity of CBB symptoms and bacterial population sizes *in planta*, when comparing resistant and susceptible genotypes, suggest their potential involvement in the defense response of common bean against *Xcf*. Our findings contribute to the better understanding of the biochemical mechanisms underlying the resistance of *P. vulgaris* to *Xcf* and open avenues for further research in this host-pathogen interaction.

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4 GENERAL CONCLUSIONS

Contributing to the knowledge about the mechanisms underlying the interaction between common bean and *Xanthomonas citri* pv. *fucans*, it is concluded that:

- Some strains of *Xcf* seem not to carry TALE genes;
- The TALE repertoire of *Xcf* has at least six conserved binding sites in the *P. vulgaris* genome sequence;
- The *Xcf* TALEs putatively induce the transcription of *P. vulgaris* genes related to sugar metabolism, cellular transporters, the ubiquitin-proteasome system, and ethylene-responsive pathways;
- The BC_3F_4 population derived from Carioca MG x BRS Radiante is highly resistant to *Xcf* infection and possesses mechanisms to limit *Xcf* growth in plant tissue;
- The BC_3F_4 plants limit the *Xcf* growth beginning between the 24 h and 48 h post-inoculation;
- The activities of GLU, PAL, LOX, PPO, and POX are higher in BC_3F_4 plants than in the Carioca MG plants at 48 h post-inoculation with *Xcf*;
- The difference in induction of defense-related enzyme activities between *P. vulgaris* genotypes resistant and susceptible to *Xcf* as well as the limitation of bacterial growth at 48 h post-inoculation observed in this study, suggest the resistance response is activated between the 24 h and 48 h post-inoculation.

APPENDIX

Table A1. Summary statistics of disease severity of common bacterial blight in Carioca MG and BC_3F_4 plants at 15 d after inoculation with *Xanthomonas citri* pv. *fuscans*

Treatment group	N	Mean rank
Carioca MG	15	5.7 a[1]
BC_3F_4	21	1.0 b
Total	36	

$\chi^2 = 32.049$
df = 1

[1] Means followed by the same letter do not differ significantly according the Kruskal-Wallis rank sums test (*p*-value = 1.503×10^{-8}).
N, number of repetitions.
df, degrees of freedom.

Table A2. Results of one-way ANOVA on *Xanthomonas citri* pv. *fuscans* population sizes in Carioca MG and BC_3F_4 leaf tissue

Treatment group	N	Mean rank
Carioca MG at 9 DAI	9	68.0 a[1]
BC_3F_4 at 1 DAI	9	52.5 b
Carioca MG at 1 DAI	9	48.9 b
Carioca MG at 2 DAI	9	47.3 b
BC_3F_4 at 9 DAI	9	27.8 c
BC_3F_4 at 2 DAI	9	26.0 c
BC_3F_4 at 0 DAI	9	13.1 d
Carioca MG at 0 DAI	9	8.4 d
Total	72	

$\chi^2 = 62.596$
df = 7

[1] Means followed by the same letter do not differ significantly according the Kruskal-Wallis rank sums test (*p*-value = 4.567×10^{-11}).
N, number of repetitions.
df, degrees of freedom.

Table A3. Results of Analysis of Variance on different enzymatic activities in Carioca MG and BC_3F_4 plants at 15 days after inoculation with *Xanthomonas citri* pv. *fuscans*

GLUCANASE

	DF	SS	MS	*F*-value	*p*-value
Genotype	1	0.01982	0.01982	0.1398	ns[1]
Inoculation	1	0.84879	0.84879	5.9866	0.0207
Time	1	0.14741	0.14741	1.0397	ns
Genotype*Inoculation	1	0.51738	0.51738	3.6491	ns

Genotype*Time	1	0.47096	0.47096	3.3217	ns
Inoculation*Time	1	0.43258	0.43258	3.051	ns
Genotype*Inoculation*Time	1	2.04273	2.04273	14.4077	7.00E^{-04}
Residuals	29	4.11165	0.14178		
Total	36	8.59132			

CV = 24.22%

PHENYLALANINE AMMONIA-LYASE

	DF	**SS**	**MS**	***F*-value**	***p*-value**
Genotype	1	0.04973	0.04973	1.0869	ns
Inoculation	1	0.03822	0.03822	0.8354	ns
Time	1	0.43271	0.43271	9.4582	0.0046
Genotype*Inoculation	1	0.4449	0.4449	9.7246	0.0041
Genotype*Time	1	0.00028	0.00028	0.006	ns
Inoculation*Time	1	0.15674	0.15674	3.4261	0.0744
Genotype*Inoculation*Time	1	0.62754	0.62754	13.7169	9.00E^{-04}
Residuals	29	1.32674	0.04575		
Total	36	3.07686			

CV = 22.42%

POLYPHENOL OXYDASE

	DF	**SS**	**MS**	***F*-value**	***p*-value**
Genotype	1	1.26275	1.26275	0.0019	ns
Inoculation	1	3155.27831	3155.27831	4.662	0.0392
Time	1	11963.3066	11963.3066	17.6762	2.00E^{-04}
Genotype*Inoculation	1	740.76368	740.76368	1.0945	ns
Genotype*Time	1	4187.50365	4187.50365	6.1872	0.0189
Inoculation*Time	1	4169.08002	4169.08002	6.16	0.0191
Genotype*Inoculation*Time	1	6334.37396	6334.37396	9.3593	0.0047
Residuals	29	19627.27471	676.80258		
Total	36	50178.84369			

CV = 27.62%

LIPOXYGENASE

	DF	**SS**	**MS**	***F*-value**	***p*-value**
Genotype	1	0.01806	0.01806	0.1285	ns
Inoculation	1	0.55493	0.55493	3.9504	ns
Time	1	0.20793	0.20793	1.4802	ns
Genotype*Inoculation	1	0.17114	0.17114	1.2183	ns
Genotype*Time	1	0.03183	0.03183	0.2266	ns
Inoculation*Time	1	0.1833	0.1833	1.3049	ns
Genotype*Inoculation*Time	1	2.20375	2.20375	15.688	4.00E^{-04}
Residuals	29	4.07374	0.14047		
Total	36	7.44468			

CV = 9.08%

PEROXIDASE					
	DF	**SS**	**MS**	***F*-value**	***p*-value**
Genotype	1	0	0	1.1526	ns
Inoculation	1	0	0	0.8692	ns
Time	1	0	0	0.826	ns
Genotype*Inoculation	1	0	0	0.9766	ns
Genotype*Time	1	0	0	0.9195	ns
Inoculation*Time	1	0	0	0.6977	ns
Genotype*Inoculation*Time	1	0	0	0.8286	ns
Residuals	29	0	0		
Total	36	0			
CV = 18.55%					

CV, Coefficient of Varianc; DF, Degrees of Freedom; SS, Sum of Squares; MS, Mean Squares;
[1] non-significant, *p*-value > 0.05

Table A4. Results of Analysis of Variance on different enzymatic activities in Carioca MG and BC_3F_4 plants at 15 days after inoculation with *Xanthomonas citri* pv. *fuscans*

PEROXIDASE		
Treatment group	**N**	**Mean rank**
Inoculated BC_3F_4 at 48 HPI	5	27.8 a[1]
Inoculated Carioca MG at 24 HPI	5	26.7 a
Non-inoculated Carioca MG at 24 HPI	4	23.6 ab
Inoculated BC_3F_4 at 24 HPI	5	21.2 abc
Non-inoculated BC_3F_4 at 24 HPI	5	19.6 abcd
Inoculated Carioca MG at 48 HPI	4	12.0 bcd
Non-inoculated Carioca MG at 48 HPI	4	10.7 cd
Non-inoculated BC_3F_4 at 48 HPI	5	8.8 d
Total	37	
$\chi^2 = 14.92$		
df = 7		

[1] Means followed by the same letter do not differ significantly according the Kruskal-Wallis rank sums test (*p*-value = 0.0371).
HPI, hours post inoculation.
N, number of repetitions.
df, degrees of freedom.