Evaluation of defense gene expression and the virulence factor *Cac1* in the interaction between *Phaseolus vulgaris* and *Colletotrichum lindemuthianum*

Evaluación de la expresión de genes de defensa y del factor de virulencia Cac1 en la interacción *Phaseolus vulgaris* y *Colletotrichum lindemuthianum*

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ABSTRACT

Anthracnose is one of the most limiting diseases in bean cultivation, leading to decreased yield. Mechanisms associated with the induction of the bean defense response during the interaction with *Colletotrichum lindemuthianum* have been studied, but little is known about the expression of certain virulence factors of this fungus during the infection process. The aim of this study was to evaluate specific molecular determinants triggered during the interaction between *C. lindemuthianum* and bean plants. For this purpose, qPCR was used to evaluate changes in the expression of the virulence factor *Cac1* in two isolates of *C. lindemuthianum* (Cl(a) and Cl(b)) with contrasting virulence profiles, and to correlate them with the expression of plant defense genes *PR1*, *PR3*, *PR4*, and *POD* during the early stages post-infection. Molecular ITS analysis showed that both isolates belonged to the Orbiculare clade; however, they clustered differently, a characteristic associated with their distinct virulence profiles. When they were inoculated in bean plants, the Cl(a) isolate was more virulent than the Cl(b) isolate, generating the highest severity value. The Cl(b) isolate induced higher expression of the evaluated plant defense genes than the Cl(a) isolate. However, the virulence factor *Cac1* of *C. lindemuthianum* showed significantly higher expression in Cl(a) than in Cl(b). These results suggest that the Sutagao bean cultivar exhibits a lower expression of defense genes exposed to an isolate of *C. lindemuthianum* expressing the virulence factor *Cac1* in the initial stages of infection.

Key words: Sutagao bean cultivar, anthracnose, *Cac1* gene, plant defense, Orbiculare clade.

RESUMEN

La antracnosis es una de las enfermedades más limitantes del cultivo de frijol, ocasionando disminución del rendimiento. Se han estudiado mecanismos asociados con la inducción de la respuesta de defensa del frijol durante la interacción con *Colletotrichum lindemuthianum*, pero poco se conoce sobre la expresión de algunos factores de virulencia de este hongo durante el proceso de infección. El objetivo de este estudio fue evaluar determinantes moleculares desencadenados durante la interacción entre *C. lindemuthianum* y frijol. Para ello, mediante qPCR se evaluaron cambios en la expresión del factor de virulencia *Cac1* en dos aislados de *C. lindemuthianum* (Cl(a) y Cl(b)) con perfiles de virulencia contrastantes, y se correlacionaron con la expresión de los genes de la planta *PR1*, *PR3*, *PR4* y *POD*, durante estadios tempranos de la infección. Los análisis ITS mostraron que ambos aislados pertenecían al clado Orbiculare, aunque se agruparon de manera diferente, característica asociada con sus diferentes perfiles de virulencia. Al inocularse en plantas de frijol, el aislamiento Cl(a) resultó ser más virulento que el Cl(b), generando la mayor severidad; pero Cl(b) indujo una mayor expresión de los genes de defensa de la planta en comparación con Cl(a). Sin embargo, el factor de virulencia *Cac1* de *C. lindemuthianum* mostró una expresión significativamente mayor en Cl(a) que en Cl(b). Estos resultados sugieren que el cultivo de frijol Sutagao presenta una menor expresión de los genes de defensa cuando se enfrenta a un aislado de *C. lindemuthianum* que expresa el factor de virulencia *Cac1* en las etapas iniciales de la infección.

Palabras clave: cultivar de frijol Sutagao, antracnosis, gen *Cac1*, defensa de plantas, clado Orbiculare.

Introduction

Beans, which are grain legumes, are an important source of protein, especially for populations in the tropical areas of Latin America and East Africa (Schwartz & Pastor-Corrales, 2005). In Colombia, beans are of great importance in the peasant economy because they are traditionally cultivated by small and medium-family producers in the Andean zone and constitute an important source of protein for these populations (Ospina Parra *et al*., 2020). Anthracnose caused by the fungus, *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara in beans (*Phaseolus vulgaris* L.) is considered the most limiting disease in this cultivated species generating substantial losses in yield and a significant reduction in seed quality. Environmental conditions such as air humidity greater than 80% favor the development of infection, especially in susceptible cultivars. The symptoms of this disease in

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bean plants are characterized by necrotic lesions on leaf veins, petioles, stems, and pods (Alvarez-Diaz *et al*., 2022). In leaves, the disease initially appears as necrotic spots on the veins, which extend to form large necrotic lesions that may be accompanied by leaf chlorosis and finally cause leaf death. In pods, lesions are depressed cankers that generate salmon-colored centers, which correspond to the reproductive structures of the pathogen (Pedroza *et al*., 2022).

The infection process of *C. lindemuthianum*, a hemibiotrophic fungus, has been well characterized and divided into an initial biotrophic phase followed by a necrotrophic phase. The biotrophic stage begins 24 h after the arrival of the conidia, the formation of the germ tube and the appressorium, and continues at 48 h with the formation of the vesicle and primary hyphae inside the plant cell (Nabi *et al*., 2024). The transition from the biotrophic to necrotrophic phase occurs at 72 h and is characterized by a morphological change from primary hyphae to secondary hyphae that favors the colonization of the pathogen (Romero *et al*., 2024).

The use of resistant genotypes is the most reliable and cost-effective management strategy for the control of *C. lindemuthianum* because it can reduce yield losses without the negative environmental impact of fungicide application (Alvarez-Diaz *et al*., 2022). However, the wide diversity in virulence associated with the various races of this fungus is the most important reason for the absence of durable resistance to anthracnose in beans (Costa *et al*., 2021). The genetics of this resistance have been studied, revealing that the plant-pathogen interaction between *C. lindemuthianum* and Sutagao cultivar of *P. vulgaris* is specific at race-cultivar level. The race specific resistance is conferred by the presence of resistance genes called *Co* genes that are unique to each genetic pool (Campa *et al*., 2017).

Similarly, studies on the mechanisms associated with the induction of bean plant defense responses during the interaction with *C. lindemuthianum* have demonstrated the importance of pathogenesis-related proteins (PRs) and the activation of salicylic acid (SA) hormone pathways, where *PR1* serving as a marker for the biosynthesis of this hormone. However, the induction of defense mechanisms dependent on jasmonic acid (JA) has also been reported (Alvarez-Diaz *et al*., 2022; Pedroza *et al*., 2022; Shams *et al*., 2020).

The virulence factors of *C. lindemuthianum* in this pathosystem remain largely unexplored. In the genus *Colletotrichum*, the expression of genes involved in the production of cyclic AMP (cAMP) plays an important role in virulence. For instance, *Cac1* codes for an adenylate cyclase in species belonging to the clade Orbiculare and its homologs *CgRhoB* in the Gloeosporioides clade, as well as *ChRgf* and *ChCdc25* in the Higginsianum clade. This adenylate cyclase is involved in the reaction to produce cAMP, which is essential for the activation of protein kinases that regulate conidial germination, appressoria penetration, and invasive fungal growth, which are key determinants of the infection process (Jiang *et al*., 2021). In addition to *Cac1*, annotation of the secretome of the *C. lindemuthianum* infection process in bean revealed the expression of Carbohydrate-Active enzymes (CAZymes), membrane transporters, Candidates to Secreted Effector Proteins (CSEPs), and some extracellular membrane (CFEM) domain proteins implicated in virulence (Romero *et al*., 2024).

Therefore, the aim of this study was to evaluate the expression of some bean defense genes to two different isolates of *C. lindemuthianum* and to evaluate the expression of the virulence factor *Cac1* involved in the kinase-like signaling cascade that regulates fungal morphogenesis and pathogenesis.

Materials and methods

Obtaining isolates of *C. lindemuthianum*

The two isolates of *C. lindemuthianum* were obtained from the Alliance Bioversity International-CIAT microorganism bank and reactivated in Petri dishes containing PDA medium. One of them was named isolate Cl(a) and the other was named isolate Cl(b). Subsequently, a replicate of each was made from hyphal transfer on PDA, which was incubated at 24°C in the dark. To ensure genetic uniformity of the fungal isolates, monosporic cultures were prepared (Ortiz *et al*., 2011).

DNA extraction

Each isolate was grown in Sabouraud Dextrose Broth medium and incubated at 24°C for 21 d in dark conditions. After this time, the grown mycelium was recovered and macerated with liquid nitrogen to subsequently carry out the DNA extraction using the DNeasy Plant Mini Kit from Qiagen®, following the manufacturer's protocol.

Sequencing of the ITS region

To confirm the genus and species of the isolates obtained, amplification of the ITS region was performed using the primers ITS-4 (TCCTCCGCTTATTGATATATGC) and ITS-5 (GGAAGTAAAAGTCGTAACAAGG) (Irinyi *et al*., 2015). Reactions for PCR were brought to a final volume of 20 µl containing 2.0 µl of 10 X Buffer, 0.8 µl of $MgCl_2$ at

50 mM, 0.4 µl of dNTPs at 10 mM, 0.4 µl of each primer at 10 µM, 1 U of Taq Polymerase, 2.0 µl of DNA from each isolate, and 13.8 µl of Milli-Q water. The parameters used for the PCR included a pre-incubation step at 95°C for 2 min, followed by 40 cycles of amplification under a thermal profile of 95°C for 30 s, 54°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 5 min. Amplification reactions were run on a Bio-Rad Thermal Cycler C1000. The PCR products were purified and sequenced in the Sequencing and Molecular Analysis Service (SSiGMoL) from the Universidad Nacional de Colombia. The sequences obtained were compared to entries in the NCBI public database using the BLASTn algorithm.

Phylogenetic analysis

Sequences obtained from the ITS region were aligned using the ClustalW algorithm in the CLC Main Workbench v5.5 program (CLCBio). A set of 14 reference sequences of *C. lindemuthianum*, one of *C. orbiculare*, and one of *C. trifolii* were used to represent diversity within the Orbiculare clade (Tab. 1) according to Guevara-Suarez *et al.* (2022).

TABLE 1. GenBank accession numbers of the nucleotide sequence of the ITS region of the ribosomal RNA of *C. lindemuthianum* isolates used for phylogenetic analysis.

***Colletotrichum* species**	**Strain/Culture collection**	**GenBank accession number**
C. lindemuthianum Cl(a)	Cl2a	PP545307 (this work)
C. lindemuthianum Cl(b)	Cl2b	PP545302 (this work)
C. lindemuthianum	L79	KJ956028
C. lindemuthianum	L85	KJ956029
C. lindemuthianum	L83	KJ956030
C. lindemuthianum	L70	KJ956031
C. lindemuthianum	K29	KJ956032
C. lindemuthianum	CBS 144.31	MH855161
C. lindemuthianum	CBS 132.57	JX546806
C. lindemuthianum	CBS 143.31	JX546808
C. lindemuthianum	CBS 152.28	JX546813
C. lindemuthianum	CBS 131.57	JX546805
C. lindemuthianum	CBS 147.31	JX546810
C. lindemuthianum	CBS 150.28	JX546811
C. lindemuthianum	CBS 151.56	JX546812
C. lindemuthianum	CBS 153.28	JX546814
C. orbiculare	CBS 570.97	NR152271
C. trifolii	CBS 158.83	NR152275
C. acutatum	CBS 112996	NR144794
C. gloeosporioides	IMI 356878	NR150754
C. boninense	ICMP 17904 / MAFF 305972	NR165949

Additionally, the ITS sequences of other *Colletotrichum* clades, such as Acutatum, Gloeosporioides, and Boninense (Tab. 1), were selected as outgroups. Subsequently, Gblocks was performed removing the uninformative ends of the sequences and concatenating the alignments. To construct the phylogenetic tree, the maximum likelihood (ML) method was used together with the Kimura evolution model with gamma distribution (K2+G), and 1,000 bootstrap replicates were considered. The evolution model and tree generation were determined using MEGA v.7.0.

Morphological characterization

From each culture of *C. lindemuthianum* grown in PDA, a mycelial disk of 5 mm diameter was transferred to a new culture medium with a punch and incubated at 24°C in the dark for 21 d. After this time, the macroscopic and microscopic characteristics of each of them were evaluated, and aspects such as colony color according to the Pantone® scale colony appearance and presence of sporulation were described. In addition, mycelial growth was measured and the growth rate was calculated with the equation:

$$\text{Mycelial growth rate (mm d}^{-1}\text{)} = \frac{MG_2 - MG}{t_2 - t_1} \quad (1)$$

where MG corresponds to mycelial growth and t to time in days.

The assay was conducted twice, with three biological replicates per isolate. The growth rate data were analyzed under the RStudio program (RSTUDIO-2023.09.1-494.EXE) and a t-Student test was performed to establish significant statistical differences between the two isolates.

Plant material and establishment of the experiment

Bean seeds of the Sutagao cultivar were supplied by the legume germplasm bank of the Faculty of Agricultural Sciences at the Universidad Nacional de Colombia. This cultivar results from a cross between the parents G2333 (Mesoamerican origin and resistance differential) and Cabrera (Andean origin). The seeds were placed in growth trays, sown in MKS1 peat substrate, and maintained in the plant propagation greenhouse, at an average temperature in the range of 18-25°C, with a relative humidity of 60-80% and a natural photoperiod of 12 h. In addition, the irrigation of the plants was ensured by applying 10 ml of water per plant two to three times per week.

Fungal biomass propagation

Inoculum growth of each monosporic isolate of *C. lindemuthianum* was carried out following the methodology proposed by Castellanos *et al.* (2011). For this purpose,

previously sterilized fresh bean pods (*Phaseolus vulgaris*) were inoculated with fragments of the fungi grown on PDA for over two weeks to promote the formation of acervuli and conidia, which represent the infective structures of the pathogen. The two isolates, Cl(a) and Cl(b), were incubated on this substrate for 21 d at 24°C in the dark.

Pathogenicity tests

To prepare the inoculum, increased fungal biomass along with the bean pods were macerated and filtered. The conidial suspension obtained was counted in a Neubauer chamber and adjusted to a concentration of 1×10^7 conidia ml^{-1}. Then, 14-d-old bean plants were inoculated by brushing the conidial suspension over both the upper and lower sides of the leaves to ensure that they were completely covered (Pedroza *et al*., 2022). Plants were maintained in relative humidity conditions above 80% to favor the infection process. To assess the pathogenicity potential of the Cl strains, three treatments were implemented: control plants inoculated with sterile distilled water, plants inoculated with Cl(a) isolate, and plants inoculated with Cl(b) isolate. The assay was conducted twice, with 15 biological replicates for each treatment.

Disease severity evaluation

Severity was assessed using the ordinal scale proposed by Van Schoonhoven and Corrales (1987), which assigns scores from one to nine according to the symptoms severity with one indicating no disease and nine indicating leaf death. This evaluation was performed every 4 d, and photographs of disease progression were taken at each evaluation up to 14 dpi. The area under the disease progress curve (AUDPC) was calculated from the severity data using the equation described by Shaner and Finney (1977).

AUDPC data were analyzed using the RStudio program (RSTUDIO-2023.09.1-494.EXE). Analysis of variance (ANOVA) of repeated measures was performed, followed by Tukey's test to determine significant statistical differences between the treatments.

RNA extraction and cDNA synthesis

RNA was extracted from 14-d-old bean plants inoculated using the brushing method with the same conidial suspension used for the pathogenicity test. The infection test was conducted twice with 15 biological replicates. Uninfected bean plants of the same age were used as controls. Three leaves from each test were collected at the early stages of the infection at 24, 48, 72, and 96 h post-inoculation (hpi). Subsequently, RNA pools were created from the extraction of each sample time series. Total RNA was extracted using a CTAB-based protocol with LiCl. Plant tissue was macerated with liquid nitrogen in a sterile mortar until reduced to a fine powder, to which approximately 3.0 ml of extraction buffer (CTAB 2%, Tris-HCl pH 8.0, 100 mM, NaCl 1.4 M, Na_2SO_3 1%, Polyvinylpyrrolidone PVP-40 2%, β-Mercaptoethanol 2% and EDTA pH 8.0, 20 mM) was added. One ml of the homogenized mixture was transferred to a 2 ml tube and placed in a water bath at 65°C for 15 min. Subsequently, after incubation, 1 ml of chloroform-isoamyl alcohol (24:1) was added, mixed, and centrifuged at 10,000 rpm for 20 min at 4°C. After the time elapsed, the aqueous phase (approximately 800 µl) was transferred to a new 2 ml tube, to which 1 ml of chloroform-isoamyl alcohol (24:1) was added. The samples were mixed and centrifuged at 10,000 rpm for 20 min at 4°C. Once the aqueous phase was generated, 800 µl was added to a sterile 1.5 ml tube. The RNA precipitation was performed using 1 ml of 4 M LiCl overnight at 4°C. Afterwards, the samples were centrifuged at 10,000 rpm for 40 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 500 µl of pre-warmed TE-SDS buffer (Tris-HCl pH 8.0, 10 mM; EDTA pH 8.0, 1 mM; SDS 1%) at 37°C. Next, 700 µl pure isopropanol and 200 µl 5 M NaCl were added to each tube. They were mixed by inversion several times and incubated for 1 h at -20°C. Then, they were centrifuged at 10,000 rpm for 15 min at 4°C, the supernatant was discarded, and the pellet was washed with 500 µl of 70% ethanol. Final centrifugation was performed for 5 min at 10,000 rpm at 4°C. The supernatant was discarded, and the pellet was dried for 1 h at room temperature. Finally, it was resuspended in 50 µl of TE buffer (Tris-HCl pH 8.0, 10 mM; EDTA pH 8.0, 1 mM) and quantified using a Thermo Scientific NanoDrop™ Spectrophotometer.

Subsequently, RNA from each treatment was treated with ThermoFisher® DNase I, following the manufacturer's protocol. To verify the absence of DNA in these samples, PCR amplification of the elongation factor (EF1α) of *P. vulgaris* was performed and the absence of amplification of this gene was verified. For this, PCR reactions were brought to a final volume of 20 µl containing 2.0 µl of 10 X Buffer, 0.8 µl of $MgCl_2$ at 50 mM, 0.4 µl of dNTPs at 10 mM, 0.4 µl of each primer (Tab. 2) at 10 µM, 1 U of Taq Polymerase, 2.0 µl of DNase-treated RNA, and 13.8 µl of Milli-Q water. The temperature profile used was 95°C for 10 min, followed by 35 amplification cycles under a thermal profile of 95°C for 1 min, 60°C for 30 s, and 72°C for 2 min. From this treated RNA, cDNA synthesis was performed using ThermoFischer® M-MLV Reverse Transcriptase according to the manufacturer's protocol.

Determination of primer efficiency by qPCR

To determine the amplification efficiency of the bean genes *EF1-α* (elongation factor), *POD* (peroxidase), *PR-1*, *PR-3*, and *PR-4* (antifungal compounds and chitinases) (Tab. 2), serial dilutions of *P. vulgaris* DNA were prepared. The starting concentration was 7.5 ng μl^{-1} leading to dilutions of 0.75, 0.075, 0.0075, 0.00075, 0.000075, and 0.0000075 ng μl^{-1}. Three replicates per dilution were performed. Reactions for qPCR were brought to a final volume of 10 µl containing 5 µl of BlasTaqTM 2 X qPCR Master Mix, 0.2 µl of each primer (Tab. 2) at 10 µM, and 3 µl of *P. vulgaris* DNA. The reactions were performed with a Jena Analytik qTOWER3 thermal cycler with the following parameters used for qPCR: pre-incubation at 95°C for 10 min, followed by 40 cycles of amplification under a thermal profile of 95°C for 1 min, 60°C for 30 s, and 72°C for 2 min. Additionally, one cycle for the dissociation curve (melting curve) was included, increasing the temperature from 60°C to 95°C in 15 s. Three replicates per dilution were performed and SYBR Green was used for detection.

Likewise, serial dilutions of DNA from the two isolates, Cl(a) and Cl(b), were carried out to determine the amplification efficiency of the *C. lindemuthianum clrRNA* (mRNA) and *Cac1* (adenylate cyclase) genes (Tab. 3). The starting concentration was 10.6 ng μl^{-1} leading to dilutions of 1.06, 0.106, 0.0106, 0.00106, 0.000106, and 0.0000106 ng μl^{-1}. Three replicates per dilution were performed. Reactions for qPCR were brought to a final volume of 10 µl containing 5 µl of BlasTaqTM 2 X qPCR Master Mix, 0.2 µl of each primer (Tab. 3) at 10 µM, and 3 µl of *C. lindemuthianum* DNA. The reactions were performed on the Jena Analytik qTOWER3 thermal cycler with the following parameters for qPCR: pre-incubation at 95°C for 2 min, followed by 40 cycles of amplification under a thermal profile of 95°C for 1 min, 60°C for 30 s, and 72°C for 2 min. Additionally, a cycle for the dissociation curve (melting curve) was included, with the temperature increasing from 60 to 95°C in 15 s. Three replicates per dilution were performed, SYBR Green was used for detection.

The efficiency of each primer was calculated using the three Ct values obtained for each dilution of *P. vulgaris* and *C. lindemuthianum* DNA with the following equation:

$$E = \sqrt[-\text{slope}]{\text{logarithm index}} \quad (2)$$

Differential expression analysis

Three replicates of the cDNA were used for each treatment at 24, 48, 48, 72, and 96 hpi for the differential expression analysis of the *POD*, *PR-1*, *PR-3*, and *PR-4* genes, which are related to the plant defense response, using qPCR with the relative quantification method. For this analysis, the crossing point (CP) values of the reference gene (housekeeping gene) *EF1-α* and each of the evaluated

TABLE 2. Sequences of primers for amplification of *EF1-α*, *PR-1*, *PR-3*, *PR-4,* and *POD* genes of *Phaseolus vulgaris*.

Gen	Function	Sequence (5' - 3')	Reference
EF1-α	Elongation factor I	CGGGTATGCTGGTGACTTTT CACGCTTGAGATCCTTGACA	(Mayo *et al*., 2015)
POD	Peroxidase II	TCCTTTTCAGCACTTTCACT AGAAAGCAGTGTTCTTGTGG	(Oliveira *et al*., 2015)
PR-1	Antifungal compound	TGGTCCTAACGGAGGATCAC TGGCTTTTCCAGCTTTGAGT	(Mayo *et al*., 2015)
PR-3	Chitinase	ATTGTTGTGCCAATCCCTTT CACCGCCATACAGTTCAAAA	(Oliveira *et al*., 2015)
PR-4	Chitinase	CGCAGTGAGTGCATATTGT TGTTTGTCACCCTCAAGCAC	(Mayo *et al*., 2015)

TABLE 3. Sequences of primers for amplification of *clrRNA* and *Cac1* genes of *C. lindemuthianum.*

Gen	Function	Sequence (5' - 3')	Reference
clrRNA	rRNA	CCTGTTCGAGCGTCATTTCA CCGGTGCGAGGTGGTATG	(Fontenelle *et al*., 2017)
Cac1	Adenylate cyclase	GCGAGCATAGGTGAAACGTT ATGTTGTTCCTCCGCACGTC	(Romero *et al*., 2024)

defense genes were compared. The qPCR reactions were run to a final volume of 10 μ*l* containing 5 μ*l* of BlasTaqTM 2 X qPCR Master Mix, 0.2 μ*l* of each primer at 10 μ*M* (Tab. 2), and 3 μ*l* of cDNA. The reactions were performed on the Jena Analytik qTOWER3 thermal cycler with the following parameters used for qPCR: pre-incubation at 95°C for 10 min, followed by 40 amplification cycles under a thermal profile of 95°C for 1 min, 60°C for 30 s, and 72°C for 2 min. SYBR Green was used for detection. One cycle for the dissociation curve (melting curve) was included, with the temperature increasing from 60°C to 95°C in 15 s.

Likewise, three replicates of the cDNA were used for each treatment and differential expression analysis of the *C. lindemuthianum* virulence factor *Cac1*. For this analysis, the CP values of the housekeeping genes, *clrRNA*, and *Cac1* were compared. Reactions for qPCR were brought to a final volume of 10 μ*l* containing 5 μ*l* of BlasTaqTM 2 X qPCR Master Mix, 0.2 μ*l* of each primer at 10 μ*M* (Tab. 3), and 3 μ*l* of cDNA. The reactions were performed on the Jena Analytik qTOWER3 thermal cycler with the following parameters used for qPCR: pre-incubation at 95°C for 2 min, followed by 40 amplification cycles under a thermal profile of 95°C for 1 min, 51°C for 30 s, and 72°C for 2 min. SYBR Green was used for detection. One cycle for the dissociation curve (melting curve) was included, with the temperature increasing from 60°C to 95°C in 15 s.

The Pfaffl comparative method (Pfaffl, 2001) was used to calculate the relative expression for each gene evaluated using three repetitions of Ct values obtained for each treatment. The data were analyzed using the RStudio program (RSTUDIO-2023.09.1-494.exe). An analysis of variance (ANOVA) of repeated measures was performed, followed by Tukey's test to establish significant statistical differences between the treatments.

Results

Sequencing of the ITS region

The homology comparison of the ITS region of *C. lindemuthianum* isolates Cl(a) and Cl(b) sequences against the GenBank database showed an identity and coverage percentage greater than 99% with sequences of *C. lindemuthianum* (Tab. 4). Additionally, the results of the phylogenetic analysis performed with the ITS sequences of pathogenic *Colleotrichum* spp. strains from the Orbiculare clade (Guevara-Suarez *et al*., 2022) showed that Cl(a) and Cl(b) isolates grouped differentially in this clade. Isolate Cl(a) was placed in the *C. lindemuthianum* third group reported by Guevara-Suarez *et al*. (2022), unlike isolate Cl(b), which was located in the second group (Damm *et al*., 2013; Guevara-Suarez *et al*., 2022) (Fig. 1).

Morphological characterization of the isolates of *C. lindemuthianum*

The two isolates of *C. lindemuthianum* showed similarities in mycelial color, appearance, and colony shape, with concentric rings on the PDA medium (Tab. 5 and Fig. 2A). However, a distinguishing feature was observed in each, setting them apart. Seven days after sowing, isolate Cl(a) acquired a salmon coloration (Fig. 2A), which correlated with the production of acervuli and conidia, which was confirmed by microscopic visualization (Fig. 2B). In contrast, isolate Cl(b) did not sporulate on the PDA medium, showing only mycelial growth (Fig. 2C). Similarly, isolate Cl(b) formed a colony with radial streaks, which were not observed in isolate Cl(a) (Fig. 2A). The results of mycelial growth rate analysis revealed that the Cl(b) exhibited a significantly higher rate than Cl(a) (Tab. 5). However, the behavior of both isolates Cl(a) and Cl(b) in the bean medium used for the inoculum increase was similar, and both isolates sporulated 7 d after sowing.

TABLE 4. Nucleotide sequence homology of the ITS of Cl(a) and Cl(b) isolates of *C. lindemuthianum*.

Isolate	GenBank accession number	Identity (%)	Coverage (%)	Homology GenBank accession number
Cl(a)	PP545307	99.82	99.00	*C. lindemuthianum* KR012909
Cl(b)	PP545302	99.82	99.00	*C. lindemuthianum* JX546805

TABLE 5. Macroscopic and microscopic characteristics of *C. lindemuthianum* isolates grown on different media and mycelial growth rates.

Isolate	Colony color	Colony aspect	Presence of radial streaks	Sporulation in PDA medium	Sporulation in bean	Mycelial growth rate (mm d^{-1})
Cl(a)	Grey-brown	Cottony	No	Yes	Yes	2.7 a
Cl(b)	Grey-brown	Cottony	Yes	No	Yes	3.9 b

Different letters represent values with significant differences ($P<0.05$) according to the Tukey´s test.

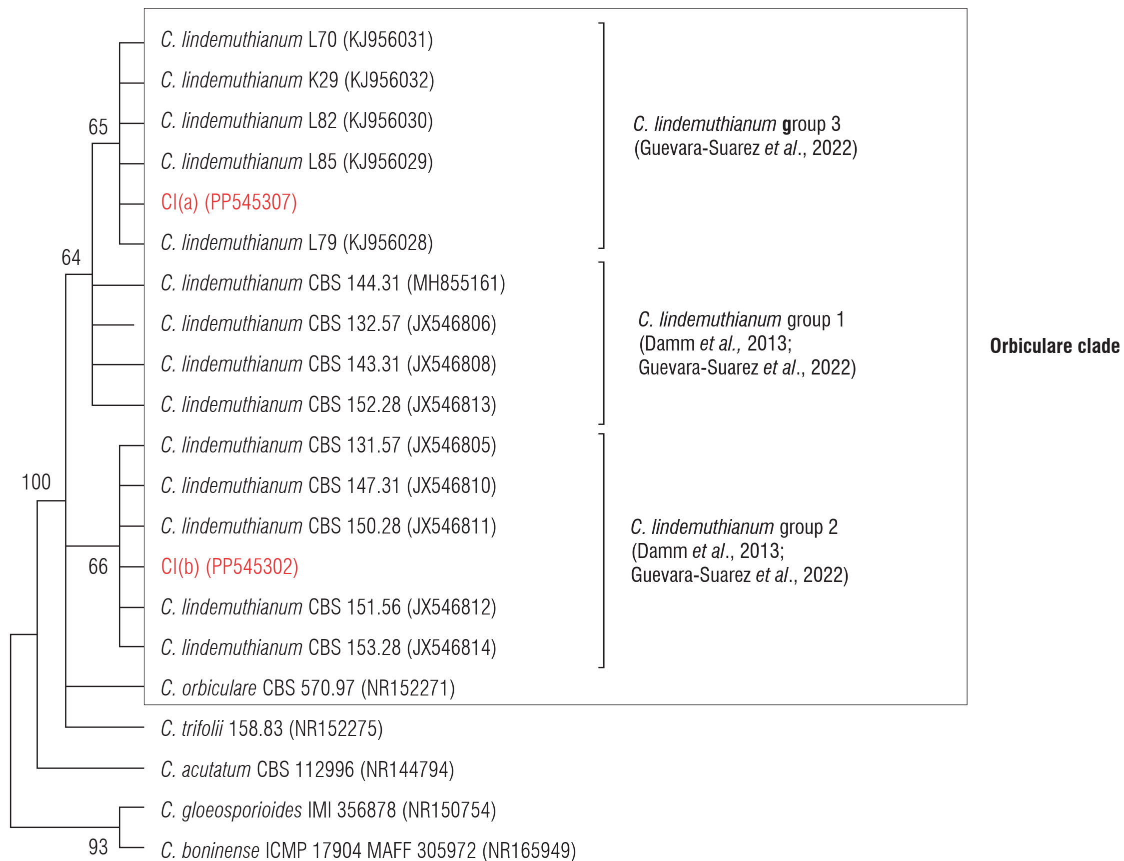

FIGURE 1. Phylogenetic tree based on the ITS region of *C. lindemuthianum* isolates. The maximum likelihood Kimura model with gamma distribution was used, with 1,000 bootstrap replicates. The culture collections of the Orbiculare clade are delimited by the black box. The Orbiculare clade comprises the three groups of *C. lindemuthianum* (Guevara-Suarez *et al*., 2022) as well as strains from *C. orbiculare* and *C. trifolii*. Sequences from specimens belonging to the Acutatum, Gleosporioides, and Boninense clades were used as outgroups. The GenBank accession numbers of the isolates are indicated in the brackets.

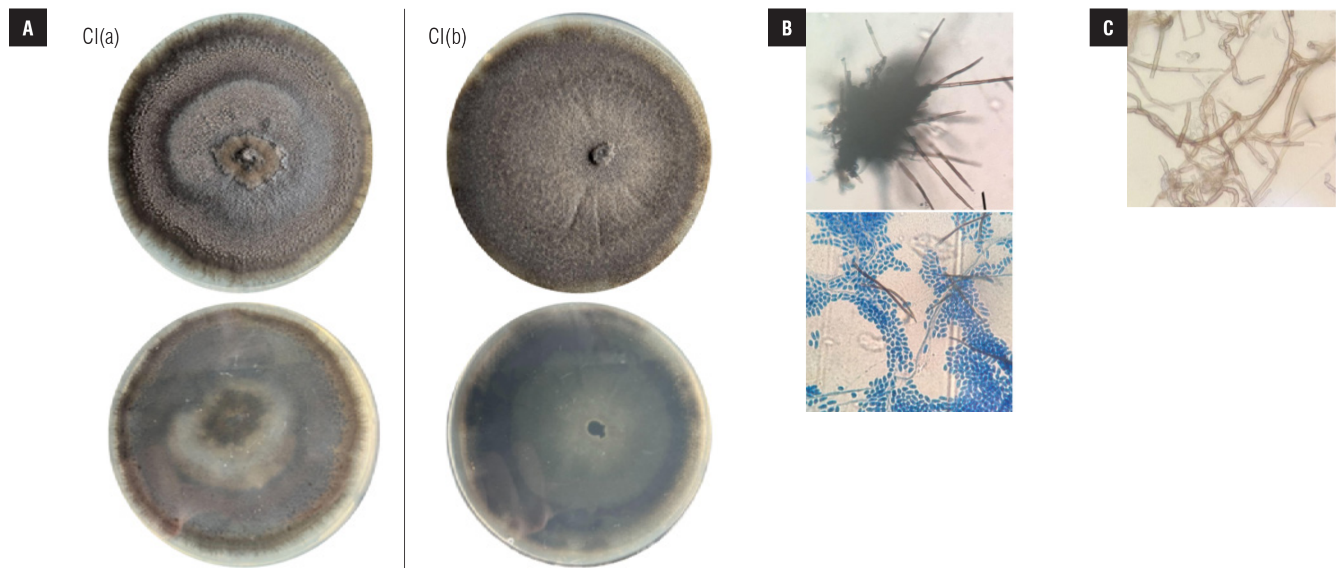

FIGURE 2. Macroscopic and microscopic characteristics of Cl(a) and Cl(b) isolates of *C. lindemuthianum*. A) Colonies of Cl(a) and Cl(b) observed 21 d after culture on PDA medium; the images on the left correspond to the front of the colony, and those on the right correspond to the back of the colony; B) Acervuli and conidia of Cl(a) observed at 40 X seven days after culture on PDA medium; C) Characteristics of the mycelium of Cl(b) observed at 40 X seven days after culture on PDA medium. A Canon EOS Rebel T5 was used to take the photos.

Evaluation of disease severity

The evaluation of disease progression showed that bean plants of the Sutagao cultivar inoculated with isolate Cl(a) had a severity score of 9 (the highest value on the scale) at 14 dpi. The symptoms included severe necrosis and leaf death, which classified the plants as susceptible to this isolate (Fig. 3A). In contrast, plants inoculated with isolate Cl(b) showed a severity score of 4 (intermediate on the scale) and exhibited small necrotic lesions on the underside of the leaf, evident on secondary and primary veins, classifying the plants as having intermediate resistance to this isolate (Fig. 3B).

The plants inoculated with isolate Cl(a) presented significantly higher AUDPC values than the plants inoculated with Cl(b), indicating that isolate Cl(a) is more virulent and produces a greater amount of disease than Cl(b) (Fig. 4). From the early days after inoculation (4 dpi), significant differences were found between the two isolates, which

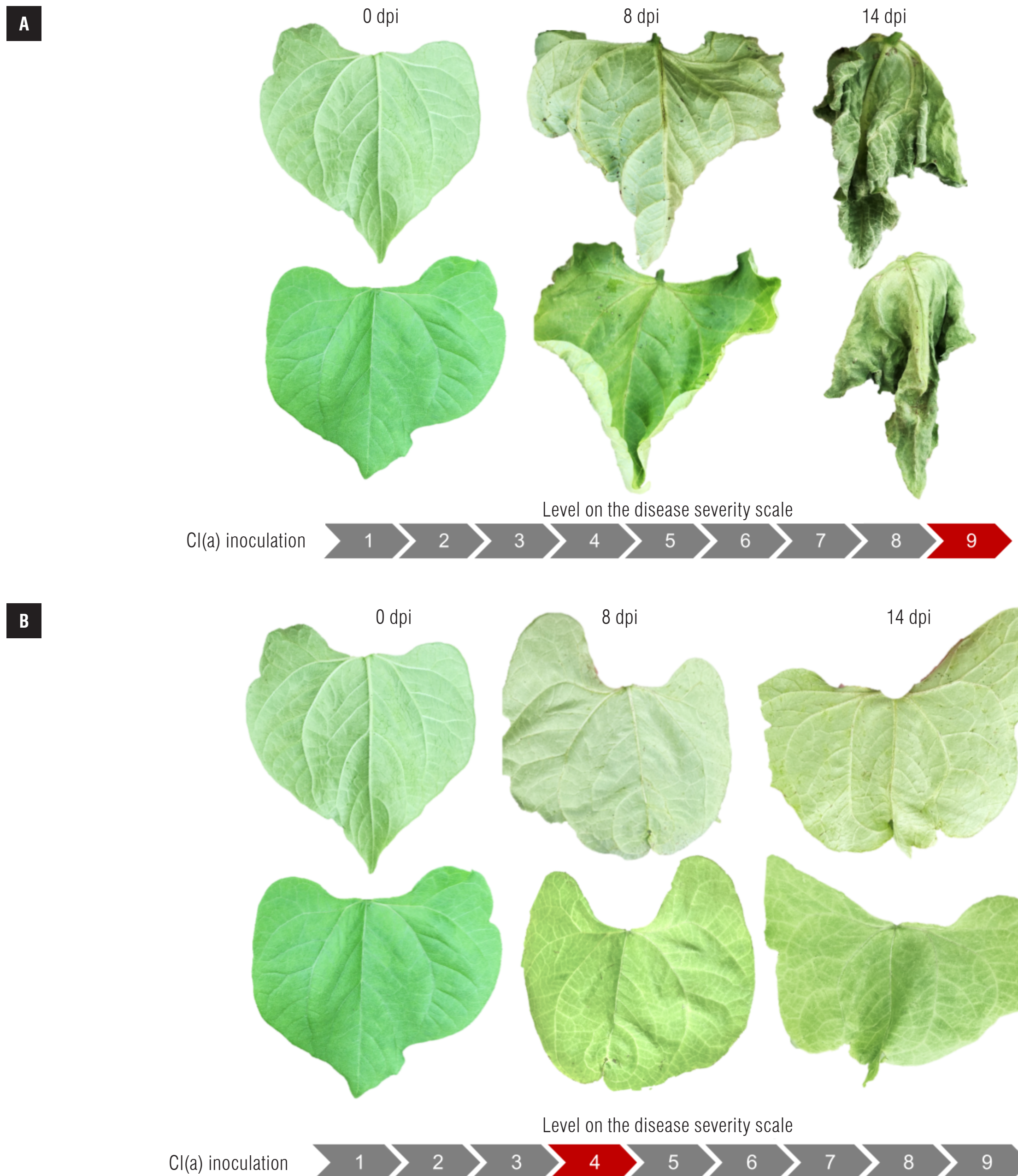

FIGURE 3. Evaluation of symptomatology caused by two isolates of *C. lindemuthianum* on the Sutagao bean cultivar at 0, 8, and 14 dpi. A) Plants inoculated with Cl(a); B) Plants inoculated with Cl(b). The upper images correspond to the underside of the bean leaf, and the lower images show the leaf blades. A Canon EOS Rebel T5 was used to take the photos.

were maintained until the end of the evaluation of disease progression (Fig. 4).

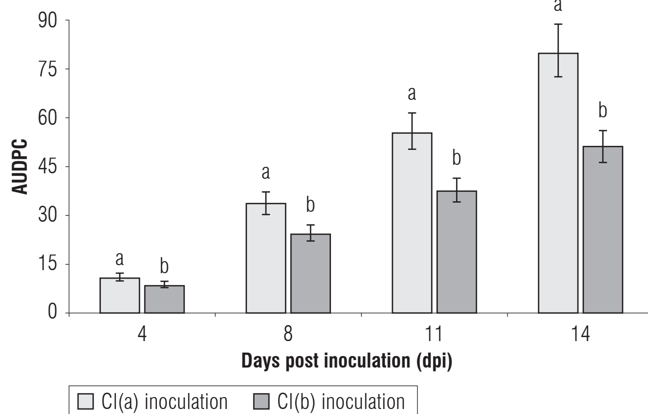

FIGURE 4. Area under the disease progress curve (AUDPC) obtained from inoculation with both isolates of *C. lindemuthianum* Cl(a) and Cl(b) on 14-d-old plants of the Sutagao bean cultivar at different evaluation times. Analysis of variance (ANOVA) of repeated measures was performed followed by the Tukey's test. Bars show the standard error of the mean calculated from 30 individual plants. Different letters represent values with significant differences between treatments ($P<0.05$).

Differential expression analysis associated with plant defense genes

Changes in the expression of bean *POD*, *PR1*, *PR3*, and *PR4* genes showed significant differences between plants inoculated with Cl(a) and Cl(b). Plants inoculated with Cl(b) exhibited significantly higher expression of these genes than plants inoculated with Cl(a) (Fig. 5). The early expression of *PR1* and *PR4* was evident in plants infected with both isolates at 24 hpi (Fig. 5A-B), whereas the expression of *PR3* and *POD* increased later at 48 and 72 hpi (Fig. 5C-D).

Differential expression analysis of *Cac1* gene of *C. lindemuthianum*

The change in the expression of the *C. lindemuthianum Cac1* gene in Sutagao bean plants inoculated with Cl(a) and Cl(b) showed significant differences between the two isolates. Virulent isolate Cl(a) showed a significantly higher expression of this fungal virulence factor than the hypovirulent Cl(b) isolate. From the early hours after inoculation (24 hpi), significant differences were found between

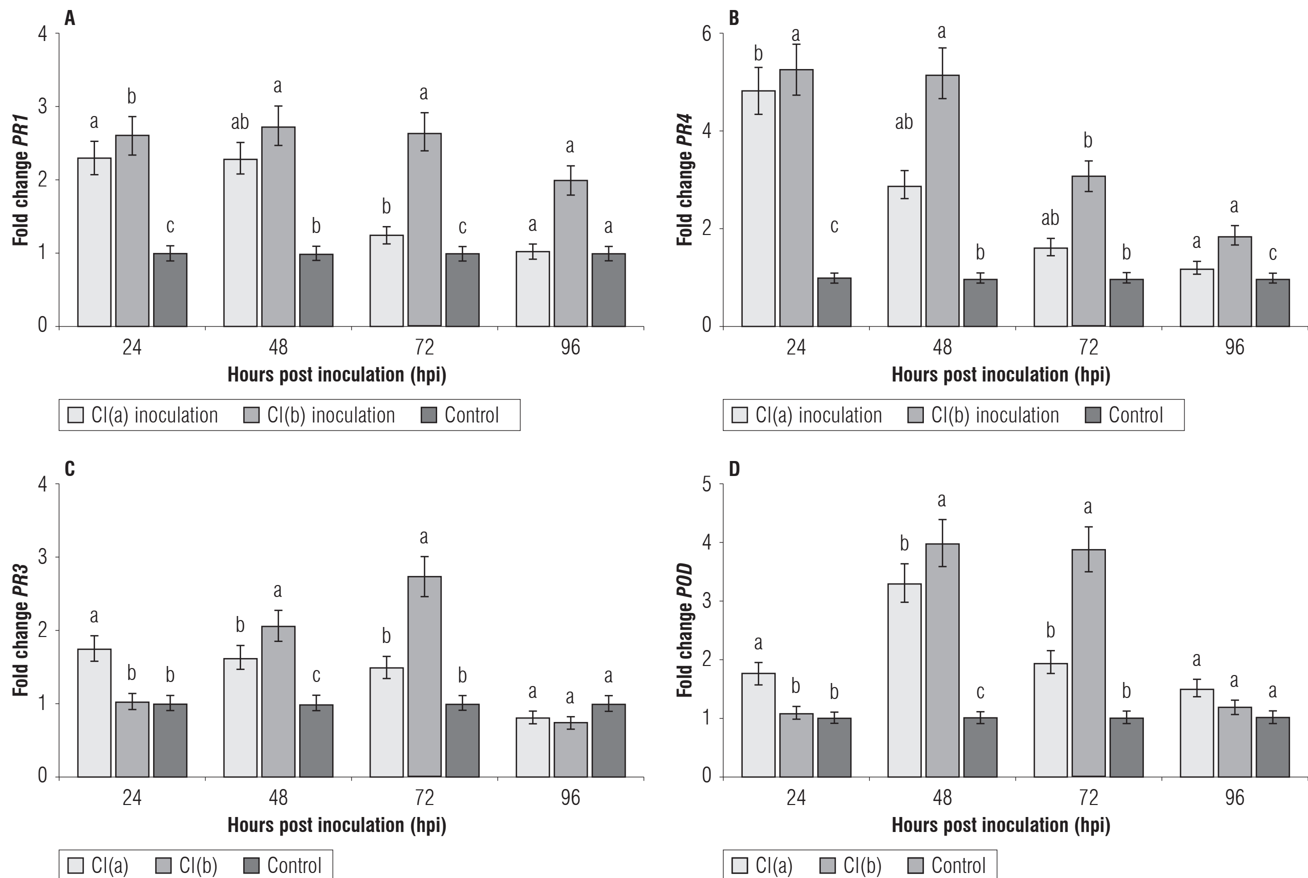

FIGURE 5. Differential expression of bean defense genes in Sutagao plants inoculated with Cl(a) and Cl(b) isolates of *C. lindemuthianum* at different time points. A) Change in *PR1* expression; B) Change in *PR4* expression; C) Change in *PR3* expression; D) Change in *POD* expression. Analysis of variance (ANOVA) of repeated measures was performed followed by the Tukey's test. Bars show the standard error of the mean calculated from 30 individual plants. Different letters represent values with significant differences between treatments ($P<0.05$).

the two isolates, which were maintained until the end of the evaluation period (96 hpi). An increase in *Cac1* expression was detected in isolate Cl(a) from 24 to 72 hpi, with a decrease at 96 hpi. In contrast, *Cac1* expression levels were low for isolate Cl(b) (Fig. 6).

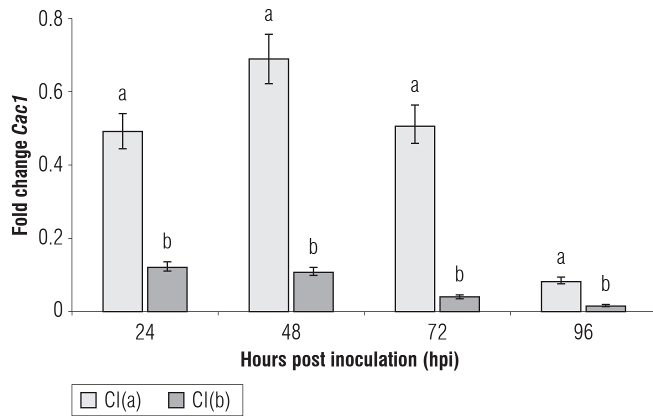

FIGURE 6. Differential expression of the *C. lindemuthianum Cac1* gene in Sutagao bean plants inoculated with Cl(a) and Cl(b) isolates at different time points. Analysis of variance (ANOVA) of repeated measures was performed followed by the Tukey's test. Bars show the standard error of the mean calculated from 30 individual plants. Different letters represent values with significant differences between treatments ($P<0.05$).

Discussion

In this work, we characterized two *C. lindemuthianum* strains morphologically and pathogenically, revealing contrasting results. Isolate Cl(a) acquired salmon coloration conferred by the production of acervuli and conidia on PDA medium and was more virulent than isolate Cl(b), which did not sporulate on PDA medium. Based on the described results the *C. lindemuthianum* isolate Cl(a) was categorized as virulent and Cl(b) was classified as hypovirulent, indicating a correlation between variations in morphological characteristics and virulence. Fu, Shin *et al*. (2022) also associated the morphological characteristics of *Colletotrichum scovillei* with its infective capacity on bell pepper fruits. In that case, the production of larger conidia, a lower germination rate, and the absence of appressoria formation at the tip of the germ tubes, influenced the generation of a successful infection, which was reflected in the absence of anthracnose symptoms in bell pepper fruits (Fu, Shin *et al*., 2022).

The alterations in the fungus morphology, particularly in the infective structures, could be related to the deletion of certain genes or epigenetic changes involved in the regulation of conidial germination and appressorium formation, which are associated with low pathogenicity (Fu, Park *et al*., 2022; Jiang *et al*., 2021; Romero *et al*., 2024; Yamauchi *et al*., 2004). In most plant pathogenic fungi, MAPKs regulate the formation of infection structures that are essential for penetration and colonization within the plant. Among MAPKs, MAP kinase 1 (PMK1) is essential for appressorium formation, plant penetration and infection process (Fu, Park *et al*., 2022). For example, deletion of this gene (*Cspmk1*) in *C. scovillei* resulted in morphologically abnormal conidia, delayed germination and loss of virulence in pepper fruits (Fu, Shin *et al*., 2022).

For molecular identification of *Colletotrichum* at the species level, different markers such as the internal transcribed spacer (ITS) region of ribosomal RNA, actin, β-tubulin, glyceraldehyde-3-phosphate dehydrogenase, and chitin synthase 1 have been recommended (Ruiz-Campos *et al*., 2022). However, in the phylogenetic analyses of *Colletotrichum* spp., the ITS region is an important molecular marker with discriminatory power because it can separate taxa at the species complex level very well, defining the clades of species of this pathogen (Guevara-Suarez *et al*., 2022). *Colletotrichum* species associated with cultivated plants in Colombia, Ecuador, Peru, and Venezuela have been distributed in five clades: Acutatum, Boninense, Gigasporum, Gloeosporioides, and Orbiculare. The last clade includes the species *C. lindemuthianum*, which is restricted to the hosts *Phaseolus vulgaris* and *P. coccineus* (Fabaceae) (Guevara-Suarez *et al*., 2022).

In this study, the ITS marker confirmed that both isolates belong to the species *C. lindemuthianum*, although they presented morphological and pathogenicity differences. At the molecular level, the Cl(a) isolate presented identity with *C. lindemuthianum* accession KR012909, which corresponds to an isolate reported as an endophyte obtained from germinated bean seeds of cultivar CN (Cabeza Negra) in Colombia (Parsa *et al*., 2016). In the phylogenetic analysis, this isolate was placed in the third group of *C. lindemuthianum* within the Orbiculare clade (Guevara-Suarez *et al*., 2022), where there were also sequences of isolates of this fungus from the bean cultivar Cargamanto from the Antioquia Department in Colombia (Fig. 1). In contrast, an unexpected result was found with the isolate Cl(b) which presented an identity with accession JX546805, belonging to group 2 of the Orbiculare clade, where there are ribosomal RNA sequences of bean isolates from the United States, Germany, France, and the Netherlands (Damm *et al*., 2013; Guevara-Suarez *et al*., 2022; Liu *et al*., 2013). Furthermore, the ITS sequences of Cl(a) and Cl(b) did not match other *Colletotrichum* species from the Orbiculare clade, specifically *C. orbiculare* and *C. trifolii*, which are crop pathogens,

or the other five weed-infecting species *C. malvarum*, *C. bidentis*, *C. sidae*, *C. spinosum*, and *C. tebeestii* (Damm *et al*., 2013). Several molecular markers and PCR-based typing strategies (Ansari *et al*., 2004; Mahuku & Riascos, 2004) have been used for the assessment of fungal species variability. However, in this case, an ITS-based characterization allowed relating macroscopic, microscopic and virulence traits, contributing to a comprehensive polyphasic analysis of *C. lindemuthianum* isolates.

The *Colletotrichum* species clustered in the Orbiculare clade also express the *Cac1* gene, which encodes adenylate cyclase, involved in the production of cyclic AMP (cAMP) from ATP. cAMP controls the phosphorylating activity of protein kinase A (PKA) and cooperates with the MAPK cascade, leading to conidial germination, appressorium penetration, and fungal growth (Fu, Park *et al*., 2022; Jiang *et al*., 2021; Romero *et al*., 2024; Yamauchi *et al*., 2004). In this study, the expression of the *Cac1* gene of *C. lindemuthianum* was higher in the virulent isolate Cl(a) with respect to the hypovirulent Cl(b). We hypothesize that the observed morphological changes could result from altered expression or deletions of this gene or other key genes. Homologs to the *Cac1* gene have been detected in several plant pathogenic fungi and are considered essential for a successful infection process, mainly in the early stages (Jiang *et al*., 2021). It has been detected in the colonization process of *Magnaporthe oryzae*, *Fusarium graminearum*, and in different *Colletotrichum* species such as *C. lagenarium*, *C. scovillei*, and *C. lindemuthianum* (Bormann *et al*., 2014; Fu, Park *et al*., 2022; Romero *et al*., 2024; Yamauchi *et al*., 2004; Yin *et al*., 2018; Zhou *et al*., 2012). Romero *et al*. (2024) showed an increase in the expression of *Cac1* in *C. lindemuthianum* race 7 at 24 hpi during the initial stages of infection, although under our conditions, the highest expression was at 48 hpi in Cl(a), demonstrating that the expression of *Cac1* at initial stages is critical for conferring virulence.

Regarding the defense response of Sutagao bean plants exposed to Cl(a) and Cl(b) isolates, we found that plants infected with the virulent isolate Cl(a) presented the lowest expression levels of the genes evaluated. In contrast, plants infected with the hypovirulent isolate Cl(b) exhibited higher expression. Similar results were reported by Alvarez-Diaz *et al*. (2022) in the bean cultivar BAT93, where a higher expression of the defense genes *PR1*, *PR10* and *PR5* was observed at 48 and 72 hpi when exposed to the low-virulence *C. lindemuthianum* strain C531. Contrastingly, a lower expression of these genes was observed when the plants were exposed to the high-virulence *C. lindemuthianum* strain 100 (Alvarez-Diaz *et al*., 2022). Similarly, in a differential expression evaluation of defense genes in different bean cultivars, the lowest expression of *PR1*, *PR3*, *PR4*, and *POD* genes was detected late in the susceptible Sutagao cultivar. However, an earlier expression of these genes was detected in the resistant G2333 bean genotype in the presence of race 7 of *C. lindemuthianum* (Pedroza *et al*., 2022). This behavior was similar to that found in our plants when infected with the hypovirulent isolate Cl(b).

Furthermore, in this work, plants infected with the hypovirulent Cl(b) isolate showed an early increase in *PR1* gene expression at 24 and 48 hpi. This is similar to what was reported by Shams *et al*. (2020) and Alvarez-Diaz *et al*. (2022), who highlighted the importance of this protein in the early defense response of bean against *C. lindemuthianum*. *PR1* is considered a marker of salicylic acid biosynthesis and is positively regulated in the defense process, showing higher expression during an incompatible interaction event in the absence of disease (Alvarez-Diaz *et al*., 2022). *PR1* expression may also be associated with a PTI- and/or ETI-type response, in which the signaling pathway associated with the production of salicylic acid (SA) is activated. SA participates in activating the defense response against biotrophic and hemibiotrophic pathogens such as *C. lindemuthianum* (Peng *et al*., 2021). Early PR1 expression detected in plants inoculated with the hypovirulent Cl(b) between 24 and 48 hpi could correspond to the time when the pathogen was in a biotrophic state in the host (Nabi *et al*., 2024; Romero *et al*., 2024). According to fluorescence microscopy assays reported for the infection process of this fungus, after 24 hpi the conidia fully germinate and penetrate directly to cause infection, and by 48 hpi, it forms a primary hypha inside the cell (Nabi *et al*., 2024; Romero *et al*., 2024). Altogether, these results demonstrate that PR1 is essential in early defense response to *C. lindemuthianum* infection.

Conclusion

P. vulgaris-*C. lindemuthianum* interaction is highly specific and depends on the genetic characteristics of both the host and the pathogen. When bean plants were exposed to a virulent isolate (Cl(a)), they developed the disease very rapidly, leading to plant death. In this scenario, the plants failed to counteract the pathogen infection, which was related to lower expression of the *PR1*, *PR4*, *PR3*, and *POD* genes at 24, 48, and 72 hpi. In this case, the pathogen developed a successful infection, which was also associated with the early expression of the virulence factor *Cac1* during infection (24–48 hpi). This gene regulates the cAMP-dependent kinase-type phosphorylation cascade that allows for the development of infective fungal structures and ensures

disease onset. In contrast, bean plants of the Sutagao cultivar were confronted with a hypovirulent isolate Cl(b) of *C. lindemuthianum*, the disease development was delayed, and the symptoms were less severe. Here, plants showed higher expression of *PR1*, *PR4*, *PR3*, and *POD* genes at 24, 48, and 72 hpi, which counteracted early infection. This was also associated with lower expression of the fungal virulence factor *Cac1* at 24 and 48 hpi, which prevented the early formation of infective structures in the plants.

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Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author's contributions

Conceptualization: CS and AG; Methodology: CS and AG; Experiments: CS; Data analysis: CS and AG; Writing – original draft preparation: CS; Writing – review and editing: CS and AG; Funding acquisition: AG. All authors reviewed the final version of the manuscript.

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