Investigating the role of a CFEM effector protein in fungal interactions relevant for biocontrol

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Vita brevis, ars longa, occasio praeceps, experimentum periculosum, iudicium difficile. — Hippocrates

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-Isaak Iliopoulos

List of Figures

Figure no.	Title	Page no.
Figure 1	Close-up dual culture plate measurements	22
Figure 2	Climate chamber experiment (experimental set-up)	25
Figure 3	Comparative gene expression analysis of *cfem12* of *C. rosea* during interaction with fungal hosts *F. graminearum*, *B. cinerea* and *R. solani*	27
Figure 4	Hypersensitivity response to avr4 in various treatments	28
Figure 5	Subcellular localization	28
Figure 6	Mutant validation of mutants for *cfem12*	29
Figure 7	Selected mutant lines for *cfem12*	30
Figure 8	Growth in normal and under stress conditions	31
Figure 9	Sporulation experiment	32
Figure 10	Dual culture and secretion assay	33
Figure 11	Self-interaction experiment	34
Figure 12	Root colonization experiment	35
Figure 13	Climate chamber experiment	36
Figure 14	In planta experiment	36
Figure 15	Climate chamber experiment (2)	44

List of tables

Table no.	Title	Page no.
Table 1	Antibiotics used in this study their stock solutions and their working solutions	15
Table 2	Primers for qPCR to see expression of *cfem12* in different interactions	15
Table 3	Designed primers used for transient expression and subcellular localization of CFEM12 protein	16
Table 4	Root colonization primers used in the RT-qPCR	23

List of equations

Equation no.	Title	Page no.
Equation 1	Haemocytometer formula used for spore count	19

Abstract

This study explored the role of CFEM-domain-containing proteins in *Clonostachys rosea* strain IK726, a well-characterized biocontrol fungus with known mycoparasitic activity. Genome analysis of *C. rosea* IK726 identified 22 genes encoding CFEM proteins, including 11 proteins with a transmembrane domain, eight GPI-anchored proteins, and three proteins with predicted signal peptides. The proteins with signal peptides were predicted to be secreted. One of these secreted CFEM-domain proteins, CRV2T0013563 (named CFEM12), which also harbours an antimicrobial peptide domain, was selected for functional characterization by generating gene deletion strains. Four individual deletion mutants of *cfem12* were generated through *Agrobacterium tumefaciens* mediated transformation and validated through molecular techniques.

A comprehensive phenotypic assessment was conducted under both standard and stress conditions. Mycoparasitic and antagonistic assays were performed using dual culture plate confrontation and culture filtrate secretion tests against the plant pathogens *Botrytis cinerea*, *Fusarium graminearum*, and *Rhizoctonia solani*. A climate chamber experiment evaluating biocontrol efficacy against *F. graminearum*; an in-planta experiment evaluating the biocontrol efficacy against *B. cinerea*; root colonization on wheat; subcellular localization studies; transient gene expression assays to assess differences between the wild type (WT) and mutants.

The phenotypic analysis showed no significant difference related to growth or stress tolerance (NaCl, caffeine, SDS, sorbitol) between the *C. rosea* WT and *cfem12* deletion strains. However, conidial production in *cfem12* deletion strains was significantly affected ($p < 0.001$). While mutants did not differ from the WT in their ability to overgrow pathogens in dual culture assays, they exhibited a significantly reduced capacity to inhibit *B. cinerea* in secretion assays ($p < 0.001$) compared to the WT. No differences were observed in self-interaction between *C. rosea* mutant and WT lines. Furthermore, the mutants showed no significant differences from the WT in their ability to colonize wheat roots, control *F. graminearum* under controlled climate chamber conditions and to control *B. cinerea* in an in planta experiment.

Lastly, the transient expression of *cfem12* gene did not supress hyper sensitive response (HR) induced by the gene *avr4* in *Nicotiana benthamiana* and the subcellular localization-confocal analysis did not provide any information about the localization of the gene.

In summary, although *cfem12* is not essential for growth, stress tolerance, root colonization, or mycoparasitic overgrowth, it plays a significant role in conidiation and the secretion of antifungal compounds in *C. rosea*.

Keywords: antagonisms, biocontrol efficacy, CFEM domain, *Clonostachys rosea*, fungal antagonism, gene deletion, mutant characterization, mycoparasitism.

Contents

1.Introduction 10
1.1 Background 10
1.2 Aim and specific objectives 10
1.3 Literature review 11
1.3.1 Common in fungal extracellular domain-containing proteins 11
1.3.2 Endophytic fungi 12
1.3.3 Biocontrol agents: *C. rosea* and modes of action 13
2. Material and Methods 15
2.1 Media and antibiotic preparation 15
2.2 Gene expression analysis of *cfem12* in *C. rosea* (cDNA synthesis and RT-qPCR) 15
2.3 Hypersensitivity response and subcellular localization 16
2.3.1 Amplification of targeted sequence 16
2.3.2 Gateway cloning to generate entry vector and destination-expression vector 17
2.3.3 *A. tumefaciens* transformation 17
2.3.4 Agroinfiltration to *N. benthamiana* 17
2.4 A. tumefaciens mediated genetic transformation 18
2.4.1 Spore harvesting and co-cultivation 19
2.4.2 Colony formation 19
2.4.3 Mutant validation 19
2.4.4 Polymerase chain reaction (PCR) and gel electrophoresis 20
2.5 Phenotyping analysis of gene deletion strains 20
2.5.1 Growth phenotyping under normal and abiotic stress conditions 20
2.5.2 Sporulation 21
2.6 Antagonism tests 21
2.6.1 Culture filtrate secretion assay and dual culture plate confrontation assay 22
2.6.2 Self Interaction-Dual culture plate confrontation assay 22
2.7 Root colonization 23
2.7.1 Ratio of *C. rosea* to wheat (RT-qPCR) 23
2.7.2 Observations on growth media 24
2.8 Biocontrol efficacy 24
2.8.1 Climate chamber experiment 24
2.8.2 In planta experiment 25

2.9 Statistical analysis 26
3. Results and discussion 27
3.1 Relative gene expression of *cfem12* 27
3.2 Hypersensitivity response 27
3.3 Subcellular localization 28
3.4 *A. tumefaciens* genetic transformation and mutant validation 29
3.5 Phenotyping differences 30
3.5.1 Growth under normal and different stress conditions 30
3.5.2 Sporulation 32
3.6 Antagonisms test 32
3.6.1 Dual culture assay and secretion assay 32
3.6.2 Dual culture assay- Self interactions of *C. rosea* WT and mutants 33
3.7 Root colonization 34
3.8. Biocontrol efficacy 35
3.8.1 Climate chamber experiment 35
3.8.2 In planta experiment 36
4.Discussion 37
5.Future aspects 38
6.References 40
Popular scientific summary 43
Appendix 1 44

1.Introduction

1.1 Background

Common in fungal extracellular membranes (CFEM) domains, defined by eight conserved cysteine residues, are unique to fungi (Kulkarni et al. 2003). Initially identified in proteins of the plant pathogen *Magnaporthe grisea*, CFEM domains have since been found in various plant pathogenic and non-pathogenic fungi (Zhang, Z.-N. et al. 2015). Functional studies of CFEM genes by generating gene deletion strains in fungal pathogens such as *Verticillium dahliae* (Wang et al. 2022), *Fusarium graminearum* (Chen et al. 2021, Zuo et al. 2022), and *Lasiodiplodia theobromae* (Peng et al. 2021) resulted in decreased virulence, emphasizing their crucial role in fungal pathogenicity. However, their role in biocontrol fungi remains unexplored. This study focused on *Clonostachys rosea* strain IK726, originally isolated from barley roots in Denmark, that exhibits robust mycoparasitic activity and is highly effective as a biological control agent (Funck Jensen et al. 2021). The strain has shown efficacy against a wide array of plant pathogens, including *Alternaria* spp., *Bipolaris sorokiniana*, *Botrytis cinerea*, and several *Fusarium* species, all of which are significant threats to agriculture (Funck Jensen et al. 2021). Genome analysis of *C. rosea* IK726 has identified 22 CFEM-encoding genes, comprising 11 transmembrane proteins, eight GPI-anchored proteins, and three secreted proteins (Piombo et al. 2023).

1.2 Aim and specific objectives

While the specific functions of CFEM-domain proteins in *C. rosea* are not yet fully understood, studies in other fungi suggest that these proteins play roles in host-pathogen interactions, stress tolerance, and fungal development (Zhu et al. 2017, Guzmán-Guzmán et al. 2017, Dai et al. 2021). Based on this, we hypothesized that a CFEM-domain containing gene in *C. rosea*, may contribute to key biological functions such as mycoparasitism, plant colonization and stress adaptation.

Aim

To investigate the functional role of the CFEM-domain-containing gene CRV2T0013563 (named CFEM12) in *C. rosea* strain IK726, particularly in relation to antagonistic activity against plant pathogens, and plant-associated traits such as root colonization and biocontrol efficacy.

Specific Objectives

1. To generate *cfem12* deletion mutants using *Agrobacterium tumefaciens (A. tumefaciens)* mediated transformation and validate them using molecular techniques.
2. To investigate the capability of *cfem12* to supress HR induced by avr4 in *Nicotiana benthamiana (N. benthamiana)* and to investigate its subcellular localization in *N. benthamiana.*
3. To evaluate phenotypic differences between wild type (WT) and *cfem12* deletion mutants focussing on growth and development, antagonism, stress tolerance, plant root colonization and biocontrol.

1.3 Literature review

1.3.1 Common in fungal extracellular domain-containing proteins

The CFEM (Cysteine-rich fungal extracellular membrane) domain is a distinctive protein motif found exclusively in fungi, characterized by eight conserved cysteine residues that are thought to form disulfide bonds contributing to protein stability (Kulkarni et al. 2003). Originally identified in the plant pathogen *Magnaporthe grisea*, CFEM domains are now known to occur widely among fungi, particularly within the Ascomycota and Basidiomycota phyla, while being completely absent in plants, animals, and prokaryotes (Zhou et al. 2010). Proteins containing CFEM domains are structurally diverse and may be transmembrane, GPI-anchored, or secreted, enabling them to participate in various extracellular or membrane-associated functions (Piombo et al. 2023). Their biological roles are equally diverse and often reflect the ecological niche of the host fungus. In plant pathogenic fungi, CFEM proteins play diverse and critical roles in virulence and host interaction, mediating processes such as host adhesion, penetration, immune suppression, nutrient acquisition, and biofilm formation, all of which are essential for successful infection. For example, a CFEM effector in *Fusarium verticillioides* suppresses plant immunity and maintains fungal cell wall integrity, underscoring its importance in pathogenicity (Li et al. 2025). Similarly, CFEM proteins contribute to pathogenicity and stress tolerance in *B. cinerea* (Zhu et al. 2017) and modulate host immunity and virulence in *Verticillium dahliae* (Wang et al. 2022). Moreover, in *Candida albicans*, CFEM proteins are crucial for acquiring iron from host heme sources, a key function in iron-limited environments (Zhou et al., 2010). These roles often reflect the ecological niche of the host fungus. Functional characterization of CFEM proteins is commonly performed using genetic approaches such as

gene knockout or silencing via *A. tumefaciens*-mediated transformation (Michielse et al. 2005; Utermark and Karlovsky, 2008), in planta expression assays to evaluate effects on host-pathogen interactions (Bos et al., 2006; Oh et al., 2009), and bioinformatic prediction of secretomes to identify potential effectors (Piombo et al. 2023). These methodologies have advanced our understanding of the molecular mechanisms underlying CFEM-mediated fungal pathogenicity and host responses. Conversely, in non-pathogenic or saprophytic fungi such as *Saccharomyces cerevisiae*, CFEM proteins like CCW14 contribute to cell wall biogenesis and structural integrity (Kulkarni et al. 2003). From an evolutionary perspective, CFEM domains are believed to have originated in the last common ancestor of higher fungi, with subsequent gene and domain duplications contributing to their expansion and functional diversification (Zhou et al., 2010; Piombo et al. 2023). Due to their fungal specificity and involvement in crucial interaction processes at the host-pathogen or host-symbiont interface, CFEM proteins are of increasing interest in fungal biology, with potential applications in antifungal therapy, biocontrol strategies, and the engineering of beneficial plant–fungus relationships.

1.3.2 Endophytic fungi

Endophytic fungi are a diverse group of microorganisms that colonize internal plant tissues without causing disease symptoms. These fungi are increasingly recognized as crucial contributors to plant health and resilience, engaging in mutualistic interactions that can enhance plant growth, stress tolerance, and defence mechanisms.

Endophytic colonization benefits plants in several ways. First, these fungi can enhance plant resistance to both biotic and abiotic stressors through the modulation of plant immune responses and the production of bioactive secondary metabolites (González-Teuber, 2019). For instance, endophytes often trigger induced systemic resistance (ISR), which primes plant defence pathways without eliciting a full defensive response until an actual threat is detected.

In agricultural contexts, endophytic fungi are gaining attention as eco-friendly alternatives to chemical fertilizers and pesticides. They can increase nutrient acquisition, promote hormone production (such as auxins and gibberellins), and help plants manage drought or salinity stress (González-Teuber, 2019; Bonfante & Anca, 2009). These traits make them promising candidates for sustainable agriculture and biotechnological applications.

The interaction between endophytic fungi and other microbes, particularly mycorrhizal fungi, and bacteria, forms a complex network of mutualism. According to Bonfante & Anca (2009),

these multipartite associations are orchestrated through intricate signaling pathways and may synergistically promote plant growth and health.

Furthermore, the challenge of applying endophytic fungi in field settings lies in understanding their specific compatibility with host species, their environmental adaptability, and methodological standardization for inoculation (Fungal Biology Reviews, 2022). Nevertheless, current research underscores their untapped potential as agents for biocontrol and plant growth promotion.

1.3.3 Biocontrol agents: *C. rosea* and modes of action

Biocontrol fungi are beneficial microorganisms that naturally suppress plant pathogens and enhance plant health through a variety of mechanisms, including direct antagonism, competition for resources, and stimulation of the plant's own defence systems. By leveraging these diverse modes of action, biocontrol fungi contribute to sustainable agriculture by reducing the need for chemical pesticides and promoting environmentally friendly crop protection (Fravel et al. 2005; Funck Jensen et al. 2021).

C. rosea formerly known as *Gliocladium roseum* is an ecologically adaptable ascomycete fungus with significant potential as a biocontrol agent (BCA) in sustainable agriculture. It has been widely isolated from various environments such as soil, rhizospheres, and decaying plant materials (Funck Jensen, D. et al. 2021) and is notable for its broad-spectrum antagonistic activity against plant pathogens including *B. cinerea*, *Fusarium spp.*, and *Alternaria spp.* (Karlsson et al. 2015). The biocontrol mechanisms of *C. rosea* include mycoparasitism, antibiosis, competition, and induced systemic resistance (ISR) (Funck Jensen, D. et al. 2021). Mycoparasitism involves direct attack on other fungi through the secretion of cell wall-degrading enzymes (CWDEs), including chitinases, β-1,3-glucanases, and proteases. These enzymes degrade the cell walls of phytopathogens, enabling *C. rosea* to colonize and outcompete them. In terms of antibiosis, the fungus produces bioactive secondary metabolites such as sorbicillinoids and peptaibols with antifungal activity (Derntl et al. 2016; Fatema et al. 2018). Through competition, *C. rosea* efficiently colonizes space and sequesters nutrients, thereby preventing pathogen establishment (Xue et al. 2003). Another important feature is its ability to act as an endophyte, promoting plant health and inducing plant defence responses (Sun et al. 2020; Gao et al. 2022). The induction of ISR has been documented in various crops and may involve signalling pathways mediated by jasmonic acid and ethylene (Zhang et al. 2021). On a genomic level, *C. rosea* possesses a genome ranging from 55.4 to 58.3 Mb, with

more than 14,000 annotated genes, including those encoding NRPSs, PKSs, ABC transporters, and CWDEs (Karlsson et al. 2015). These gene families support its capacity for secondary metabolism and environmental adaptation. In addition to its agricultural benefits, *C. rosea* has demonstrated utility in environmental sustainability. Certain strains can degrade synthetic polymers such as poly(ε-caprolactone), contributing to bioplastic degradation (Li et al. 2022). Furthermore, the fungus can produce volatile hydrocarbons, pointing to its potential in bioenergy production (Sun et al. 2023). The fungus is also viable for commercialization. Solid-state fermentation methods have enabled large-scale production of conidia, and *C. rosea* spores show a shelf life of over 180 days under appropriate conditions (Sun et al. 2023). Commercial products based on *C. rosea*, such as *Prestop*®, have already demonstrated success in controlling plant diseases in field settings (Marrone, 2019, Fravel et al. 2005). These characteristics make *C. rosea* an attractive candidate for use as a biocontrol agent in agricultural systems.

Despite its proven potential, the widespread adoption of biocontrol fungi like *C. rosea* is still limited due to challenges such as variable field efficacy, high production costs, and limited shelf life of formulations. However, advancements in formulation technology, strain improvement, and integrated pest management strategies are expected to increase the reliability and adoption of biocontrol agents in the future (Marrone, 2019).

2. Material and Methods

2.1 Media and antibiotic preparation

Lysogeny broth (LB) was used as the growth medium for the bacteria with solid LB media for 1 liter consisting of 10gm Bactotryptone (Bacto™, Tryptone, Becton, Dickson and Company, MD, U.S.A.), 5gm yeast extract (Sigma-Aldrich, Burlington, MA, U.S.A), 10gm NaCl (Fisher Scientific, Loughborough, U.K.), and 15gm Difco™ agar (Becton, Dickson and Company, MD, U.S.A.) while liquid LB did not include agar. Potato Dextrose Agar (PDA) was prepared using powder from VWR Chemicals (PDA, Sigma-Aldrich, St. Louis, MO) and Potato Dextrose Broth (PDB) was prepared from Difco™ Potato Dextrose Broth (Becton, Dickson and Company, MD, U.S.A.). Induction medium (IM) and GM7 medium for selection of transformants were prepared based on the protocol by Karlovsky (2008). Hansen's Agar (HA) was used in some cases and was prepared by dissolving 4gm of glucose (Sigma-Aldrich, Burlington, MA, U.S.A), 4gm of yeast extract, 10 gm of malt extract (Sigma-Aldrich, Burlington, MA, U.S.A), 15 gm of Difco™ agar agar in one liter of distilled water and the pH was assorted to 5.5 using either HCl or KOH.All the media were autoclaved before use. Antibiotics kanamycin and spectinomycin were prepared by filter sterilization of kanamycin and spectinomycin dihydrochloride pentahydrate powder dissolved in distilled water (Alfa Aesar, Karlsruhe, Germany). Zeocin, rifampicin, acetosyringone, hygromycin and cefotaxime were provided by supervisors. All the antibiotics used in this study will be provided in Table 1 including their stock and working solution.

Antibiotics	Stock solution(mg/ml)	Working solution(µg/ml)
Hygromycin	40	100,150,200
Cefotaxime	200	200,400...
Kanamycin	50	50
Spectinomycin	100	100
Zeocin	100	50
Rifampicin	50	25
Acetosyringone	200	200

Table 1 Antibiotics used in this study their stock solutions and their working solutions.

2.2 Gene expression analysis of *cfem12* in *C. rosea* (cDNA synthesis and RT-qPCR)

*cfem12*_For.	5'----------CATCCCTGAGTGTGCCCTCGG----------3'
*cfem12*_Rev.	5'----------CAGTTTCGGCACCACACTTCTTGA---------- 3'

Table 2 Primers for qPCR to see expression of cfem12 while different interaction.

The expression of *cfem12* gene was evaluated in *C. rosea* during self-interaction (Cr-Cr) and the interaction with the fungal hosts *B. cinerea* (Cr-Bc) and *R. solani* (Cr-Rs) at two interaction

stages: before contact (BC) and after contact (AC). Supervisors provided the total RNA extracts from *C. rosea* in the different interactions. 1 µg of total RNA was treated with DNase I (Fermentas, St-Leon-Rot, Germany), following the manufacturer´s protocol. DNase-treated total RNA were transcribed into cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, U.S.A.).

The gene expression analysis was performed using real-time quantitative polymerase chain reaction (RT-qPCR) (Bio-Rad) in four to five biological replicates (4 biological replicates for the first five interactions, which were Cr-Cr BC, Cr-Cr AC, Cr-Bc BC, Cr-Bc AC and Cr-Rs BC) while the last interaction (Cr – Rs AC) had five biological replicates). Each biological replicate had one to two technical replicates.

Each RT-qPCR well contained a total volume of 10 µl, consisting of five µl of 2x EVA Green (Bio-Rad), 10 pgmol/µl of both forward and reverse primer (0.1 µl of the forward and 0.1 µl of the reverse primer-Table 2), 3.8 µl of nuclease-free water and 1000ng/µl of cDNA (1 µl). The housekeeping gene actin was used to normalise the expression levels across the different interactions. The RT-qPCR ran for 40 cycles and with the following specifications:95.0 C for 3 min, 95.0°Cfor 10 s, 60.0°Cfor 30 s ,72°Cfor 30 s and 95.0 °C for 1 min and the relative gene expression was calculated from threshold cycle (Ct) using $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

2.3 Hypersensitivity response and subcellular localization

2.3.1 Amplification of targeted sequence

*cfem12*_For.	5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAAGTTCTCCCTCGTCGCT-3'
*cfem12*_Rev.	5'GGGGACCACTTTGTACAAGAAAGCTGGGTTGGCATGGTATGATCGCTTGGAG--3'

Table 3 Designed primers used for transient expression and subcellular localization of CFEM12 protein.

Gene specific primers (Table 3) were used to amplify CFEM coding sequences from *C. rosea* plasmid DNA for *cfem12* and by PCR using DreamTaq Green PCR Master Mix (Thermo Fisher Scinetific, Baltics, Vilnus, Lithuania). A reaction mixes of 10µl DreamTaq master mix, 0.4µl of forward and reverse primers each. The three steps PCR condition included 95 °C for 2 min, followed by 30-35 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 10 minutes. The PCR product was analyzed using agarose gel electrophoresis, followed by gel extraction using the Gel Extraction Kit (Thermofisher Scientific).

2.3.2 Gateway cloning to generate entry vector and destination-expression vector

The entry clone was generated using gateway-specific donor vector, pDONR^{TM}/Zeo, while the destination vector pGWB505 (with GFP) and pGWB602 without GFP was used to generate the expression vectors. The low salt LB with Zeocin^{TM} selective antibiotic was used for pDONR^{TM} Zeo entry vectors and spectinomycin was used for the expression vector pGWB505 and pGWB602. The constructs were verified by colony PCR using gene-specific primers.

2.3.3 *A. tumefaciens* transformation

The expression vectors were cloned into C58 *A. tumefaciens.* In detail, 50 µl of *A. tumefaciens* strain C58 was thawed on ice and 350ng/µl of the expression vector and placed immediately on liquid nitrogen until fully frozen. The mixture was then incubated for five min at 37°C. One ml of LB was added to the mixture and left for four hours at 28°C on a rotary shake at 200rpm to incubate. It was then centrifuged at 4000rpm for 1 min, and the pellet was resuspended in 100µl LB. It was plated and left to grow for two days at 28°C in LBA supplemented by rifampicin and spectinomycin and two more days at room temperature. Overnight culture of single colonies in LB supplemented by antibiotics were used to create glycerol stock (1:1 of *Agrobacterium* and 50% glycerol) stored at -80°C.

2.3.4 Agroinfiltration to *N. benthamiana*

The culture stored in glycerol was refreshed in 10ml LB supplemented with spectinomycin and rifampicin. They were grown at 200 rpm at 28°C for 2 days. Induction buffer with 10mM MES buffer, 150µM Acetosyringone, 10mM $MgCl_2$ and Milli-Q water to make total 250ml volume was prepared. The grown culture was centrifuged at 3500rpm for 10 minutes at room temperature, and the supernatant was discarded. The pellet was washed by resuspending it in 5ml induction buffer. The culture was incubated at 200 rpm at 28°C for 2 hours. After two hours, the optical density (OD) of the culture was adjusted to 0.6 - 0.8 for the construct without GFP *(cfem12*: pGWB602 and empty vector) and to 0.2 for the construct with GFP (*cfem12*: pGWB505 and free GFP). *N. benthamiana* was grown with 16-hours photoperiod for 3 weeks. The induced *Agrobacterium* culture was infiltrated into the leaves of *N. benthamiana* using a blunt-end syringe. All the infiltrated regions were marked.

2.3.4.1 Hypersensitivity response specifics

For the hypersensitivity response, overnight cultures of the transient expression vector *cfem12*, the empty vector pGWB602, avirulence gene (*avr4*), and a negative control were grown in 10ml of LB media at 28°C with shaking at 180 rpm. *Avr4*, a chitin-binding effector recognized

by Cf4 receptor in resistant tomato plant induces a strong hypersensitive response (Joosten et al., 1997), making it a reliable positive control. Following induction, the cultures were adjusted to an optical density OD of 0.5 for *cfem12*, the empty vector pGWB602, and the negative control, while the avr4 culture was diluted to an OD of 0.3. Avr4 was added to all the spots and infiltrated 24hrs after the other treatments to determine whether our gene of interest could suppress the hypersensitive response. These bacterial suspensions were infiltrated into 2-week-old *N. benthamiana* plants on the abaxial side of the leaves. A total of eleven plants were used, with two leaves per plant, and each leaf received four infiltrations: two on each side of the mid rib.

2.3.4.2 Subcellular localization specifics

Prior to constructing the expression vector, the subcellular localization of the effector *cfem12* was predicted using the EffectorP Fungi 3.0 web tool. The analysis suggested that *cfem12* is dual localized in the cytoplasm and the apoplast. Based on this prediction, the effector gene was cloned into the p505 vector, which contains a GFP fusion for visualization.

To improve fluorescence signal detection at both 24- and 48-hours post-infiltration, the p505-*cfem12* construct was co-infiltrated with the silencing suppressor P19 from Tomato bushy stunt virus (TBSV). P19 was used to suppress post-transcriptional gene silencing in *N. benthamiana*, enhancing transient gene expression by stabilizing mRNA transcripts and promoting protein accumulation (Voinnet et al. 2003). Agrobacterium cultures carrying p505-*cfem12* and P19 were co-infiltrated at a 1:1 ratio into *N. benthamiana* leaves. Confocal microscopy was performed to assess the subcellular localization of the GFP-tagged effector. Due to the unavailability of the fluorescence signal from GFP, both alone and while being co-infiltration with p19, the gene of interest, *cfem12*, was initially introduced using the same technique, gateway cloning, to an RFP vector, which was p660.The selection of p660 was made through the antibiotic spectinomycin.

2.4 A. tumefaciens mediated genetic transformation

The *A. tumefaciens* containing a CFEM gene deletion cassette was provided by supervisors. The complete transformation was guided through a protocol called Genetic transformation of filamentous fungi by *A. tumefaciens* (Utermark and Karlovsky 2008) and the mutagenesis is based on homologous recombination, which facilitates targeted gene insertion or deletion /replacement (Schenk, P. M. et al. 2008).In detail, overnight culture of *A.tumefaciens* grown in LB supplemented with the antibiotic's rifampicin and spectinomycin was centrifuged at 4000 rpm for 5 min at room temperature. The pellet was washed twice using liquid IM and was

resuspended in IM containing 200 μM acetosyringone. After the OD was measured, it was diluted to 0D=0.15 and was left to grow until the OD reached approximately 0.30.

2.4.1 Spore harvesting and co-cultivation

C. rosea culture was flooded with 5 ml sterile deionised water. Secondly, spores were loosened from the mycelia by rubbing the sterile spreader across the PDA surface. The resulting suspension was then filtered through sterile Mira cloth and funnel into a Falcon tube. The spore concentration of the filtrate was estimated to be about 1×10^7 spores/ml using a haemocytometer using the following formula (Equation 1).

$$spores/ml = \frac{number\ of\ spores\ in\ 4\ haemocytometer\ squares}{4} \times dilution\ factor \times 10000$$

Equation 1 Haemocytometer formula used for spore count.

1-1 mixture of *Agrobacterium* and *C. rosea* spores was prepared and left for 1 hour to mix together at room temperature at 100 rpm. 200 μl of this mixture was placed onto the surface of a cellophane sheet on IM agar plate supplemented by 200 μM acetosyringone and spread equally using a drigalski spatula. The plates were placed at 23^0C for 60 hours.

2.4.2 Colony formation

After the passing of the 60 hours a dense layer of mycelium was formed, the cellophane sheets were transferred to GM7 plates containing hygromycin and cefotaxime. Cefotaxime was used to eradicate *A. tumefaciens* and hygromycin was used, because the deletion cassette was there to replace our gene of interest with the hygromycin resistance gene making this an effective selection. The GM7 plate were placed at 23° C for 7-10 days and the colonies of the transformants were visible on the background of decaying mycelium. The colonies were transferred into new PDA plates containing again hygromycin and cefotaxime and let to grow at 20°C.

2.4.3 Mutant validation

For mutant validation, RNA was extracted using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) following the instructions of the manufacturer. In detail,100 mg of tissue was placed in 2ml tubes and 600 μl of lysis solution was added. The mixture was homogenized using a homogenizer at 5500 rpm for 2 cycles for 30 sec with 10 s in-between on ice to cool down. The tubes were incubated on 60°C for 5 minutes supplemented by 1% mercaptoethanol. The tubes were centrifuged at max speed for 3 minutes and the supernatant was transferred into a filtrate column. The column was centrifuged for 1 minute at max speed. The filtrate column was discarded, and same amount of binding solution was added

to the flow through lysate and mixed together by pipetting up and down 5 times. It was transferred into a binding collum and centrifuged for 1 min at max speed. This step was repeated twice if needed. The flow through was discarded every time. After the binding was complete 500 µl of washing solution was placed into the binding column. This step was repeated 2 more times with the other washing solution. The binding column was centrifuged alone for 2 min at max speed to remove residual of ethanol. Lastly 50 µl of elution buffer was placed into the centre of the column left for 2 minutes at room temperature and centrifuged at max speed for 2 minutes. To increase the concentration of the RNA this step was repeated. After the RNA isolation was completed, it was quantified using a Nanodrop (ND-1000 Nanodrop spectrophotometer, Seven Werner), to investigate if it was successful by observing the ratios of 260/280 and 260/230. If the ratio was found low, then in some cases re-isolation was conducted.

2.4.4 Polymerase chain reaction (PCR) and gel electrophoresis

PCR was conducted using DreamTaq Green PCR Master Mix or Phusion Master Mix from ThermoFisher Scientific (Baltics, Vilnus, Lithuania). In each PCR tube 1000 ng/µl of cDNA or 100 ng/µl DNA (1 µl) (10 µL) was added, followed by 10 pmol/µl of each primer (total 0.4µl) and 5 µl of 2X DreamTaq Green/Phusion and 3.6 µl water. The following protocol was used: an initial enzyme activation/cDNA-DNA denaturation step at 95 °C for 2 min, followed by 30-35 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 10 minutes. When DreamTaq Phusion was added the protocol changed to 98°C for 30s, followed by 35 cycles at 98°C for 30s,65°C for 30s and 72°C for 2min, with a final extension for 72°C for 10 minutes. The PCR products were runed into TBE gels containing 1% agarose and for 30 minutes at 120 V and the size marker that was used was Gene Ruler™ DNA Ladder Mix (100-10000 bp) (Thermo Fisher Scientific).

2.5 Phenotyping analysis of gene deletion strains

To assess whether the deletion of *cfem12* influenced the biology of *C. rosea*, a series of phenotypic assays including growth under normal and abiotic stress conditions and sporulation were conducted.

2.5.1 Growth phenotyping under normal and abiotic stress conditions

To investigate the response of fungal cells to various abiotic stresses, PDA medium supplemented with different agents were used. In detail, 1 agar plug from each mutant was placed to the middle of the petri dishes (3 replicates for each mutant for all 5 different

conditions) for and left to grown and measurements were made for growth after both 8 and 15 days at 20°C. PDA with 0.05 M sodium chloride (NaCl) was used to simulate salt-induced osmotic stress, which can lead to cellular dehydration and ionic imbalance, thereby affecting membrane stability and metabolic processes (Hohmann, 2002). A concentration of 1 M sorbitol was added to PDA to impose non-ionic osmotic stress, mimicking desiccating conditions by reducing water availability without affecting ionic composition, a condition known to influence cell turgor and trigger stress response pathways (Saito & Posas, 2012). Caffeine at 0.1% was incorporated as a chemical stressor known to interfere with cell cycle regulation and DNA repair mechanisms, particularly by targeting checkpoint kinases and MAPK signalling pathways (Lenardon et al., 2009). Lastly, PDA supplemented with 0.025% sodium dodecyl sulfate (SDS) introduced membrane and cell wall stress, as SDS is a detergent that disrupts lipid bilayers and denatures proteins, making it a valuable tool for assessing cell wall integrity and stress resilience (Levin, 2005). These stress conditions provide insight into fungal adaptive mechanisms and are commonly used in functional studies of stress signalling and tolerance. The original SDS was supposed to be 0.05% but only a few replicates were able to survive.

2.5.2 Sporulation

To quantify sporulation, a calibration model was established to correlate optical density (OD) measurements at 600 nm with spore counts obtained using a hemacytometer, achieving 99% accuracy. The model was generated using a *C. rosea* WT. The spores were harvested, serially diluted (10-fold), and OD readings were recorded. Spore counts were then determined using a hemacytometer to establish the correlation. For the assay, one agar plug was placed in the centre of a small Petri dish and incubated at 25°C for 10 days. Sporulation was assessed for three biological replicates of each mutant and the WT. Spores were harvested using Milli-Q water and a sterile spreader. The resulting spore suspension was filtered through Mira cloth to remove hyphal fragments. Spore concentration was measured using a spectrophotometer at 600 nm, with each biological replicate was plated in five technical replicates in a 96 well plate (Sarstedt AG&Co.KG, Germany).

2.6 Antagonism tests

To evaluate the antagonistic and mycoparasitic differences of *C. rosea* WT and the *cfem12* deletion strains, two complementary assays were employed: a culture filtrate secretion assay, which assessed the inhibitory effects of secreted metabolites on pathogen growth, and a dual culture plate confrontation assay, which evaluated direct fungal interactions, including

overgrowth of *C. rosea* as a proxy for mycoparasitism. The pathogens that were used in both of these studies are *F. graminearum*, *B. cinerea* and *R. solani*.

2.6.1 Culture filtrate secretion assay and dual culture plate confrontation assay

For the culture filtrate secretion assay, 4 agar plugs from each mutant were placed into 100 ml of PDB and let to grow on room temperature at 150 rpm. After 3 days, the PDB was filtrated using a pump and the mycelial biomass of mutants and the WT were dried and measured. The 100ml of PDB was further placed into 9 tubes (3 replicates for each pathogen) and 1 agar plug from each pathogen was placed and after 4 days the pathogens were freeze-dried and again measured, and the graph was created. The control condition only had pure PDB.

For the dual culture plate confrontation assay, 1 agar plug from the *C. rosea* WT and the *cfem12* deletion strains were placed at the edge of the petri dish (9 cm diameter) and after 11 days of incubation at 20°C the pathogens were inoculated on the same plate. After 3-5 days of the inoculation meet point the overgrowth of the mutants was measured (Figure 1) and the graph was created.

***Figure 1 Close up dual culture plate**. Example indicating the interaction between the mutant cfem12 34-2 and the pathogen B. cinerea. The mutant and the pathogen are placed in the left and the right side of the plate, respectively and the blue straight line indicates the meeting point and red dotted line indicate the overgrowth in 5 days. The measurement was conducted between the blue and the red line.*

2.6.2 Self Interaction-Dual culture plate confrontation assay

For the *C. rosea* self-interactions the same day both types were placed into the same petri dishes and the total distance covered in 14 days post inoculation was measured. Statistical analysis was only conducted between the self-interaction of WT, between the interaction of WT and the mutant and between the self-interaction of the mutant and the graph was created.

2.7 Root colonization

Triticum aestivum(wheat) seeds were sterilized using a mixture of both detergent and sodium hypochlorite (2%). After the seeds have been air dried, they were left to grown at 20ºC vertically until the root reached the approximately length of 2-3 cm. The roots have then been dipped into 10^6 spore concentration of both the mutants and the WT and left to petri dishes with enough water to grow for 5 days. For each mutant and the WT 6 biological replicates were prepared. After the passing of the 5th day, 2 experiments took place.

2.7.1 Ratio of *C. rosea* to wheat (RT-qPCR)

C.rosea Actin Rev.	5' --TCGGCAGTGGTGGAGAAGGTGT----- 3'
C.rosea Actin For.	5' --GTTCTGGATTCCGGTGATGGTGTC--- 3'
T. asvestivum tubulin Rev.	5'- -CGCCAGTGTACCAATGCAAGAAA--- 3'
T. asvestivum tubulin For.	5'- -GCTCACATCTCGTGGGTCACAGA ---3'

Table 4 Root colonization primers used in the RT-qPCR.

DNA was extracted using a CTAB DNA extraction protocol available inhouse (DNA isolation from *Neurospora sp*. in 1.5 ml tubes). In detail, roots from 3 plants (consisting of 1 biological replicate) were placed on 1.5 ml tubes and 600 µl of CTAB 3% was added to the tubes. The 3% of CTAB consisted of 3g CTAB in 32,6 ml of deionized water 15 ml of 1 M Tris-HCl pH 8, 52 ml of 5M NaCl and 0,4M ETDA pH 8. The mixture of CTAB and roots was homogenized using a homogenizer at 6000 rpm for 4 cycles of 30 s. The samples were vortexed and incubated at 65ºC for 30 min shaking in-between. The samples were centrifuged for 10 min at 10.000 rpm and the supernatant was transferred into a new 1.5 ml tube.1 volume of chloroform was added to the samples, vortexed and they were centrifuged for 10 min at 13. 000rpm.The supernatant was again transferred into a new 1.5ml tube and 1 volume of isopropanol was added to the tube. The samples were mixed gently and placed at -20ºC for 20min.The samples were then centrifuged for 20min at 13.000 rpm. The supernatant was discarded, and the left was left to dry briefly before being washed by 200 µl of 70% EtOH by centrifuge at 13.000rpm for 5 min. The supernatant was again discarded, and the left was dissolved in 50 µl of TE-buffer. The DNA was quantified using a Nanodrop and it was diluted to 20ng/µl.The qPCR was performed in a total volume of 10 µl containing 5 µl of 2x EVA Green (Bio-Rad), 10 pgmol/µl of both forward and reverse primer (0.1 µl of the forward and 0.1 µl of the reverse primer-Table 4), and 100ng/µl of cDNA (4.8 µl). The expression of the housekeeping gene actin (*C. rosea*) or tubulin(wheat) in WT was used to normalise the expression in mutants-WT type. The qRT-PCR ran for 40 cycles and with the following specifications:95.0 C for 3 min, 95.0°Cfor 10 s, 60.0°Cfor 30 s ,72°Cfor 30 s and 95.0 °C for 1 min and the relative gene expression was calculated from threshold cycle (Ct) using $2^{-\Delta\Delta CT}$method (Livak & Schmittgen, 2001). Both

$2^{-\Delta\Delta CT}$ methods were divided, and the ratio of *C. rosea* / wheat was calculated, and the graph was created.

2.7.2 Observations on growth media

One root of each biological replicate was cut into same sized pieces and placed into PDA plates to grow for 4 days, and if any differences were observed they were reported. The plates also indicated the direction of the root (0 to end), to also observe if *C. rosea* WT and mutants were able to grow with the root and on the other direction.

2.8 Biocontrol efficacy

2.8.1 Climate chamber experiment

A controlled bioassay was performed to assess the efficacy of *C. rosea* WT and deletion strains of *cfem12* as biological control agents (BCAs) against *F. graminearum* strain PH-1, the causal agent of foot and root rot in wheat (*Triticum aestivum*). Wheat seeds were surface sterilized by sequential washing with detergent and 2% sodium hypochlorite, followed by three rinses in sterile deionized water and air-drying under sterile conditions.

Spore suspensions of *C. rosea* WT and *cfem12* deletion strains were harvested from PDA cultures, filtered through sterile Mira cloth to remove hyphal fragments, and adjusted to a final concentration of 1×10^6 spores/ml using a haemocytometer. Seeds were divided into seven treatment groups: (A) non-inoculated control, (B) inoculated with *F. graminearum* only, and (C–G) inoculated with *F. graminearum* and treated with either the *C. rosea* WT or one of the gene *cfem12* mutants. Seed coating was achieved by shaking seeds in their respective spore suspensions for 30 minutes at 100 rpm.

Seeds were sown in sand-filled pots (3 seeds per pot-Fig 2A, B, C)) arranged in five trays, each representing a biological replicate. All trays were maintained in a climate chamber under controlled environmental conditions: a 12-hour photoperiod (100–150 µmol m^2 s^{-1} light intensity), 70 ± 5% relative humidity, and a temperature range of 15–20°C. In treatments B through G, inoculation with *F. graminearum* was carried out by placing a 3–5 mm PDA plug colonized with fungal mycelium adjacent to the seeds. Sterile PDA plugs were used in the non-inoculated control (A).

Following 3 weeks of incubation (Fig.2D), disease severity was evaluated using a standardized 0–4 scale (Fig.2E), based on root and coleoptile browning. Root and shoot lengths, fresh and dry biomass were also measured using a scale and a ruler to assess plant growth performance.

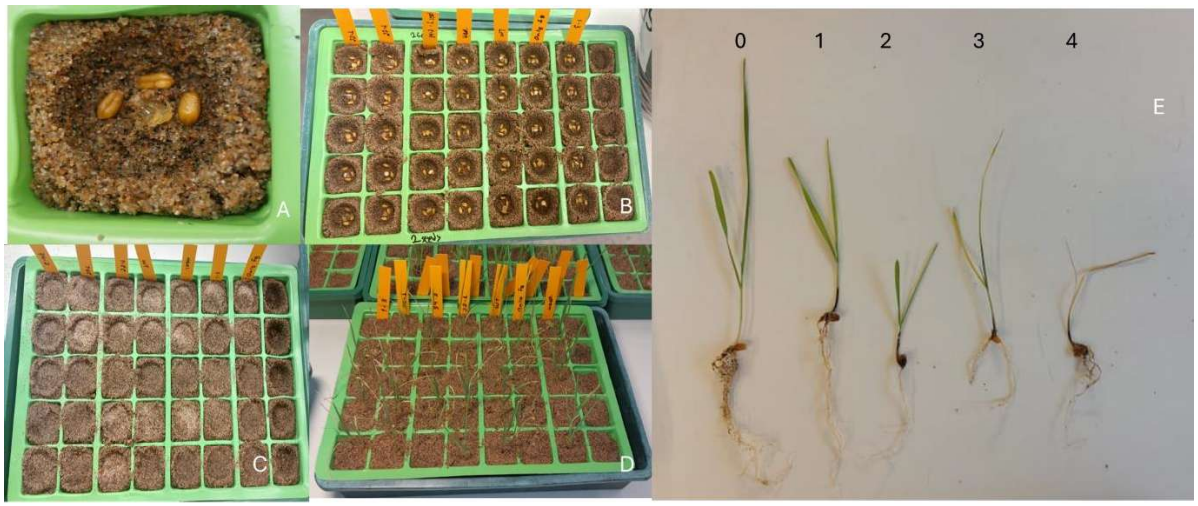

Figure 2 Climate chamber experiment (experimental set up). (A)*close up of each pot. Each pot consisted of 3 wheat seeds and F. graminearum agar plug in the middle(B) tray close up before and after sand sealing. (D) 3 weeks after being placed into a growth chamber, the trays were taken out and different traits were measured. An example is the sand disease score severity (E)*

2.8.2 In planta experiment

A controlled in planta experiment was conducted to evaluate the efficacy of *C. rosea* WT and deletion strains of the gene *cfem12* against *B. cinerea*, the causative agent of grey mold, using *N. benthamiana* as a model plant. *N. benthamiana* plants were grown for two weeks under a 16-hour photoperiod. *B. cinerea* was cultured on HA medium to enhance sporulation, while *C. rosea* WT and mutant strains were cultured on PDA medium.

Spore suspensions of both *C. rosea* (WT and deletion mutants) and *B. cinerea* were prepared and counted using a hemacytometer based on a previously described method (Equation 1). Final concentrations were adjusted to 10^6 spores/mL for *C. rosea* and 10^5 spores/mL for *B. cinerea*. *C. rosea* spore suspensions were mixed with Tween 20 and applied to *N. benthamiana* leaves twice. Plants were then enclosed in boxes covered with plastic film to maintain humidity. Two days later, *B. cinerea* spores were mixed together with half of PDB and also glycerol, left for 1 hour at 100rpm at room temperature and were initially inoculated onto the same leaf areas. Tween 20 was also added to the *B. cinerea* spores before inoculation and due to presence of HR after the inoculation of leaves with *C. rosea* spores one spot on each leaf was left intact for experimental purposes.

Disease progression was assessed 3 days post-inoculation by measuring the necrotic lesion area. Graphical representation of lesion size was generated. Each treatment included three

replicate pots. As a positive control, plants were inoculated with *B. cinerea* alone (10^5 spores/mL), while the initial application consisted of water with Tween 20 to evaluate any potential phytotoxic effects of the surfactant.

2.9 Statistical analysis

The statistical analysis was done through the program SPSS (IBM statistical analysis). Multivariate analysis of variance (MANOVA) or One-way ANOVA Tukey HSD/Games-Howell was performed, and all the results listed below are based on that. In brief, the critical p-value was assorted to 0.05. The null hypothesis (Ho) addressed was that no differences will be observed in the different conditions. Depending on the significance they were marked as Ns (no significance > 0.05), * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ or the significance is marked by letters to indicate significant differences between groups.

3. Results and discussion

3.1 Relative gene expression of *cfem12*

The expression of *cfem12* was evaluated in *C. rosea* during self-interaction and the interaction with the fungal hosts *B. cinerea* and *R. solani* at two interaction stages: before contact and after contact (Fig.3). The gene *cfem12* showed significant upregulation at after contact stage of self-interaction ($p<0.05$) compared to the contact stage. However, its expression was significantly downregulated in both the interaction stages with *B. cinerea* ($p<0.05$) compared to the respective interaction stages of self-interaction control, and significant upregulation after the contact stage of interaction with *R. solani* ($p<0.01$).

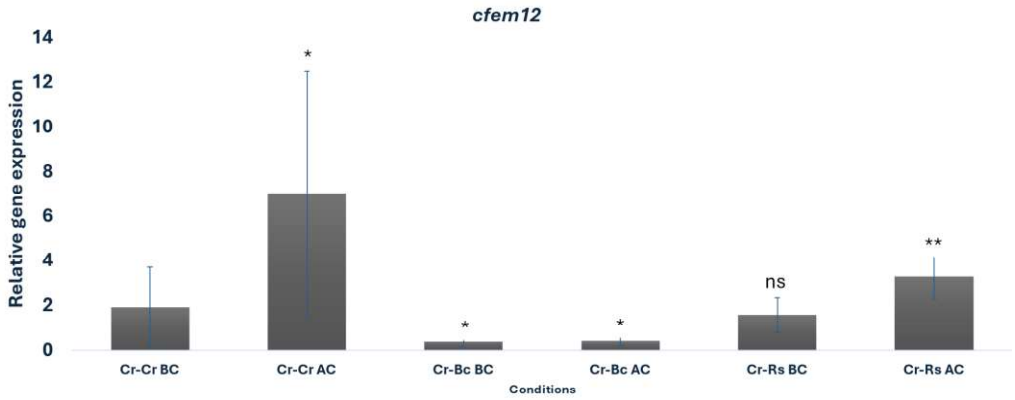

Figure 3 Comparative gene expression analysis of cfem12 *gene in C. rosea during interaction with fungal hosts F. graminearum, B. cinerea and R. solani at two time points. Each bar in the x-axis presents a different interaction while the y-axis presents the mean 2^-ΔΔCT (Relative transcription level). The symbols indicate: ns (no significance > 0.05), * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001. Cr stands for Clonostachys rosea, Bc for Botrytis cinerea Rs for Rhizoctonia solani BC stands for before contact and AC for after contact. All data have been normalized by Cr-Cr BC.*

3.2 Hypersensitivity response

Since CFEM proteins are shown to suppress HR response in plant and thereby contribute to fungal pathogenesis (Wang et al. 2022), an experiment was performed using *N. benthamiana* leaves to evaluate whether *cfem12* can suppress HR when co-infiltrated with the *avr4* (Fig.4). The *N. benthamiana* line used in this experiment carries an R gene that specifically recognizes Avr4, triggering the HR was used in this experiment. Leaves were infiltrated with various treatments: buffer (IM), empty vector (EV), and two replicates of *cfem12*. Figure 4 shows that *cfem12* did not suppress the HR. All treatments, including those with *cfem12*, displayed HR

symptoms similar to those seen in the buffer and EV control. This indicates that *cfem12* does not interfere with or suppress the *avr4*-induced HR in *N. benthamiana*.

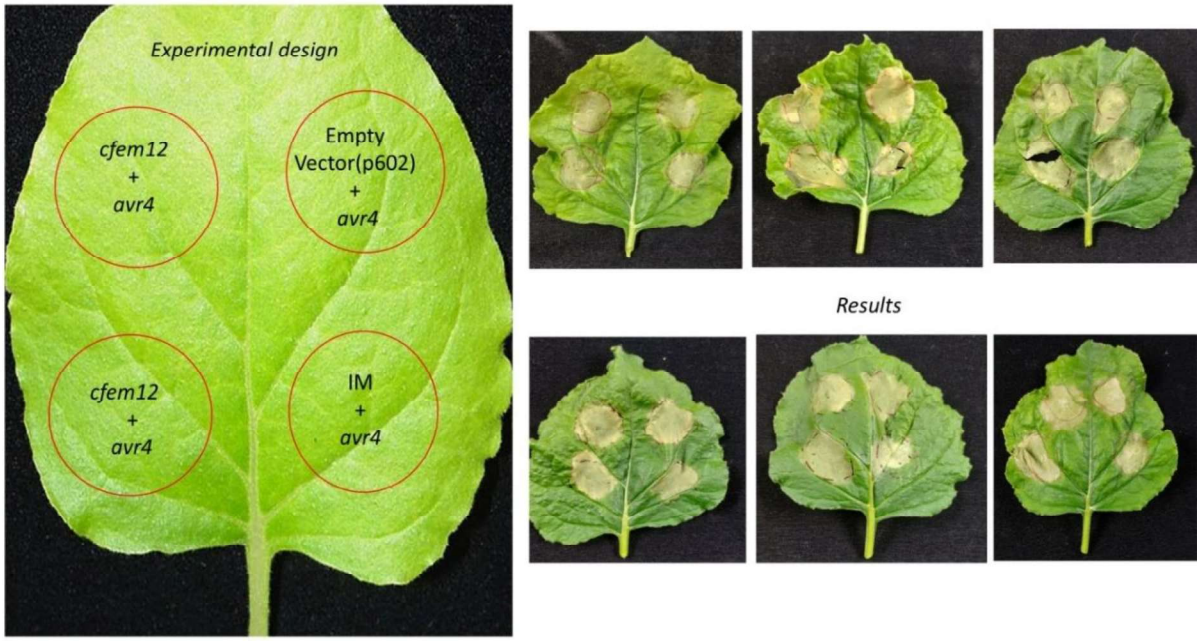

Figure 4 Hypersensitivity response to avr4*. The gene of interest (cfem12), the empty vector (EV-p602) and IM (induction media), which was used as a negative control were agroinfiltrated at day 1 and after 24 hours avr4 was also agroinfiltrated to the same spots in the leaves. 6 leaves were photographed after 4 days post the inoculation of avr4.*

3.3 Subcellular localization

Leaves infiltrated with the free GFP-p505 construct showed clear fluorescence around the plasma membrane (Figure 5A and 5B). In contrast, leaves infiltrated with GFP tagged *cfem12*, either alone or co-infiltrated with the silencing suppressor p19, did not exhibit any detectable GFP fluorescence, making it impossible to determine the subcellular localization of the construct (Figure 5C).

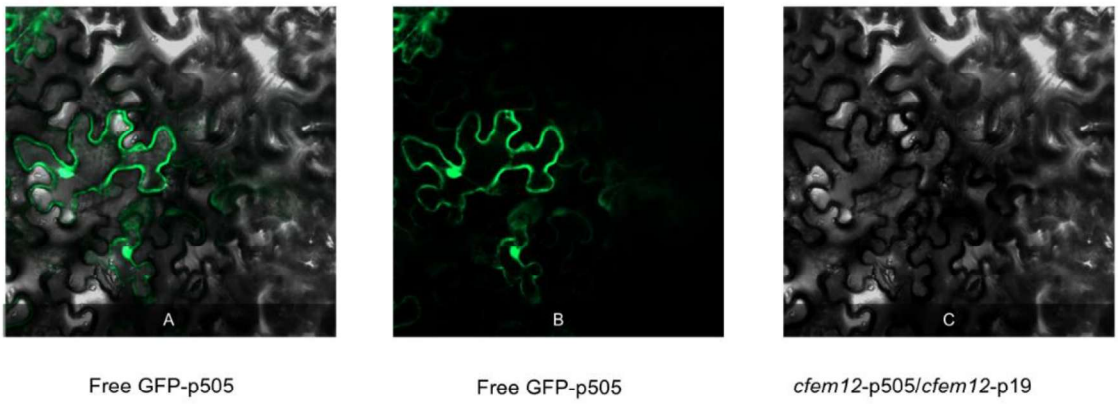

Figure 5 Subcellular localization of GFP constructs in N. benthamiana epidermal cells. *Confocal microscopy images showing localization patterns of GFP fluorescence. (A) and (B) represent cells expressing free GFP-p505, (C) shows cells expressing the cfem12-p505 and cfem12(p505) when co-infiltrated with p19 construct.*

3.4 *A. tumefaciens* genetic transformation and mutant validation

During previous work, eight independents' colonies of putative mutants for *cfem12* were created. Total RNAs from these mutant strains were isolated for the validation through RNA extraction. This RNA was then converted into cDNA and used in PCR reactions with primers specific for actin and *cfem12*.It was expected that all mutants would produce PCR products of the same size as the WT, but with no bands, indicating that *cfem12* was successfully knocked out. Gel electrophoresis results (Fig.6) showed that mutants 1 (1-3), 4 (22-1), 7 (27-1), and 8 (34-2) exhibited this type of projection compared to both the genomic DNA (gDNA) and complementary DNA (cDNA) from the WT. Consequently, these mutants were classified as mutants of the target gene (Fig.7).

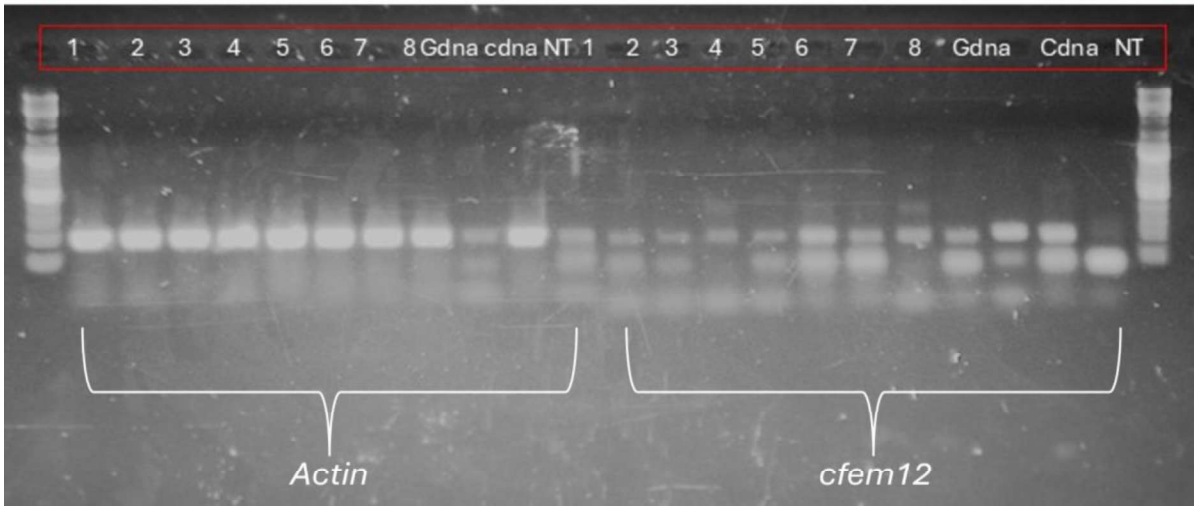

Figure 6 RNA validation of mutants for cfem12 via RT-PCR products visualized on 1% agarose gel. *Mutants (1 to 8) have had their RNA isolated, and their cDNA has been used for PCR with both actin primers and with primers for cfem12 and NT for both stands for non-template control for both primer pairs. Gdna stands for genomic DNA and Cdna for complementary DNA. Both Gdna and Cdna derived from C. rosea WT.*

Figure 7 RNA validation of mutants for cfem12 via RT-PCR and visualized on 1% agarose gel. *From in total eight mutants (Figure 6) for cfem12 mutant lines 1,4 7 8 have been selected due to no expression levels of the gene. as it can be seen from the intensity/difference of the band between the mutants and the gDNA and the cDNA of WT. NT stands for non-template control, Gdna for genomic DNA and Cdna for complementary DNA. Both Gdna and Cdna derived from C. rosea WT.*

3.5 Phenotyping differences

3.5.1 Growth under normal and different stress conditions

Growth assays on PDA and under various stress conditions indicated that the deletion of *cfem12* did not affect the vegetative growth or stress tolerance (Fig.8A, Fig 8B), as the mutant strains exhibited growth comparable to the WT across all tested conditions. While the deletion itself had no discernible effect, the different stress treatments varied in their impact on growth. Specifically, growth inhibition increased in the following order: caffeine (0.1%) < SDS (0.025%) < sorbitol (1 M) < NaCl (0.5%) < PDA (Fig 8C).

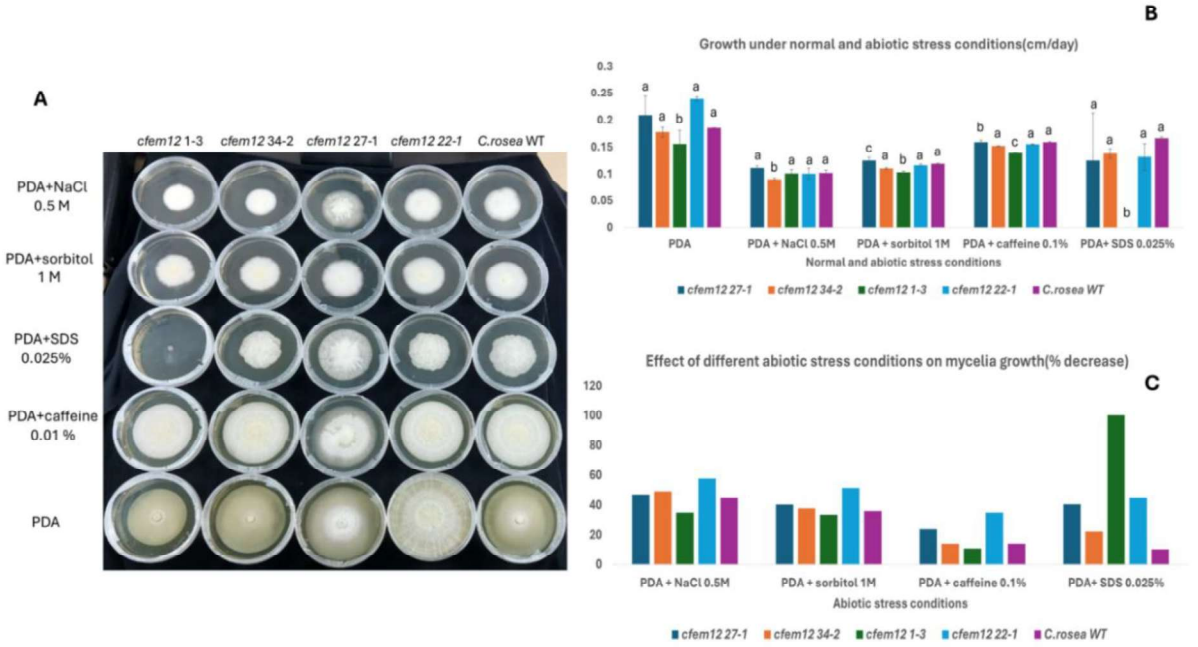

Figure 8 Growth and effect of different stress conditions on the growth of C. rosea and C. rosea cfem12 mutants*. A) Visual representation of C.rosea WT and deletion strains on PDA and PDA supplemented by agents 15 days post inoculation(dpi) B) Bar chart presenting the average growth of two measurements made after both 8 and 15 days in different stress conditions (PDA + NaCl 0.5M, PDA+sorbitol 1M, PDA + caffeine 0.1% and PDA +SDS 0.025%) of C. rosea and C. rosea mutants for gene cfem12. 3 replicates for both C. rosea WT and the mutants (cfem12 27-1 cfem12 34-2, cfem12 1-3 and cfem12 22-1). Statistics are based on observations on day 8 and the letters represent same groups or different groups. C) Bar charts presenting the average effect of different stress conditions (PDA + NaCl 0.5M, PDA+sorbitol 1M, PDA + caffeine 0.1% and PDA +SDS 0.025%) in reduction of growth measured after both 8 and 16days for C. rosea WT and C. rosea mutants for gene cfem12. 3 replicates for both C. rosea WT and the mutants (cfem.2 27-1 cfem12 34-2, cfem112 1-3 and cfem12 22-1).*

3.5.2 Sporulation

The OD measurements revealed that deletion of *cfem12* significantly impaired *C. rosea* sporulation on PDA. After 10 days, the mutant strain produced notably fewer spores compared to the WT ($p < 0.001$), indicating that *cfem12* plays a role in *C. rosea* sporulation (Fig.9C).

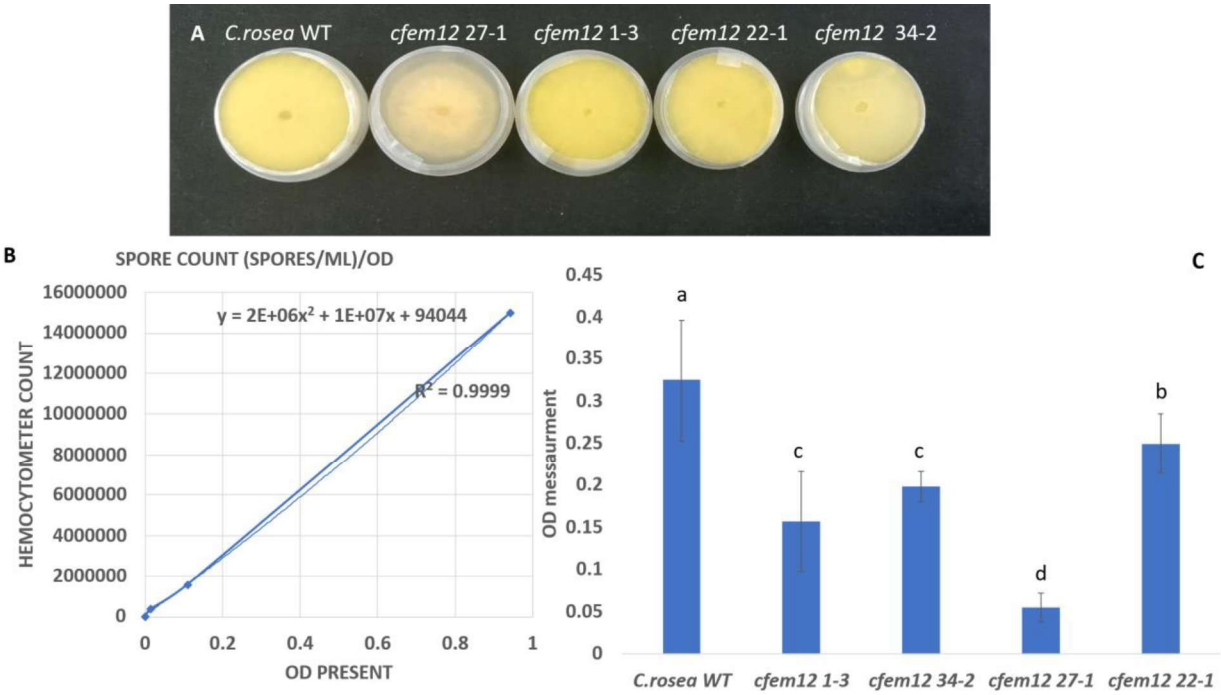

Figure 9 Sporulation experiment*. Visual representation presenting one plate of each the WT and the mutants that were used for spores counting. B) Chart indicating the correlation graph between OD and spore count. The model was created based on serial dilutions 1:10 and calculating both the OD and the spore count with the hemacytometer. The equation of all the measured spore count is based on $y = 2E+06x^2 + 1E+07x + 94044$ with an accuracy of 99,9%. C)Chart indicating either the spore count or the OD measurements on 600nm conducted on 10days old C. rosea WT and C. rosea mutants for cfem12. Statistics are based on observations on day 10 and the letters represent groups who share statistical differences or not. Each group had 3 biological replicates and 5 technical replicates.*

3.6 Antagonisms test

3.6.1 Dual culture assay and secretion assay

In the dual culture assay, no significant differences were observed between the deletion strains for *cfem12* and WT in their ability to overgrow the plant pathogens *B. cinerea*, *F. graminearum*, and *R. solani* (Figure 10A, B). However, in the secretion assay (Figure 10C, D), where in some cases equal amounts of fungal biomass were present (WT and the mutants *cfem12* 34-2 and *cfem12* 27-1), significant differences ($p < 0.001$) were detected in the inhibition of *B. cinerea* growth. Specifically, the mutants exhibited reduced antagonistic activity compared to the WT. In contrast, no significant differences were observed between mutant and WT in their effects on *F. graminearum* and *R. solani* in the secretion-based assay.

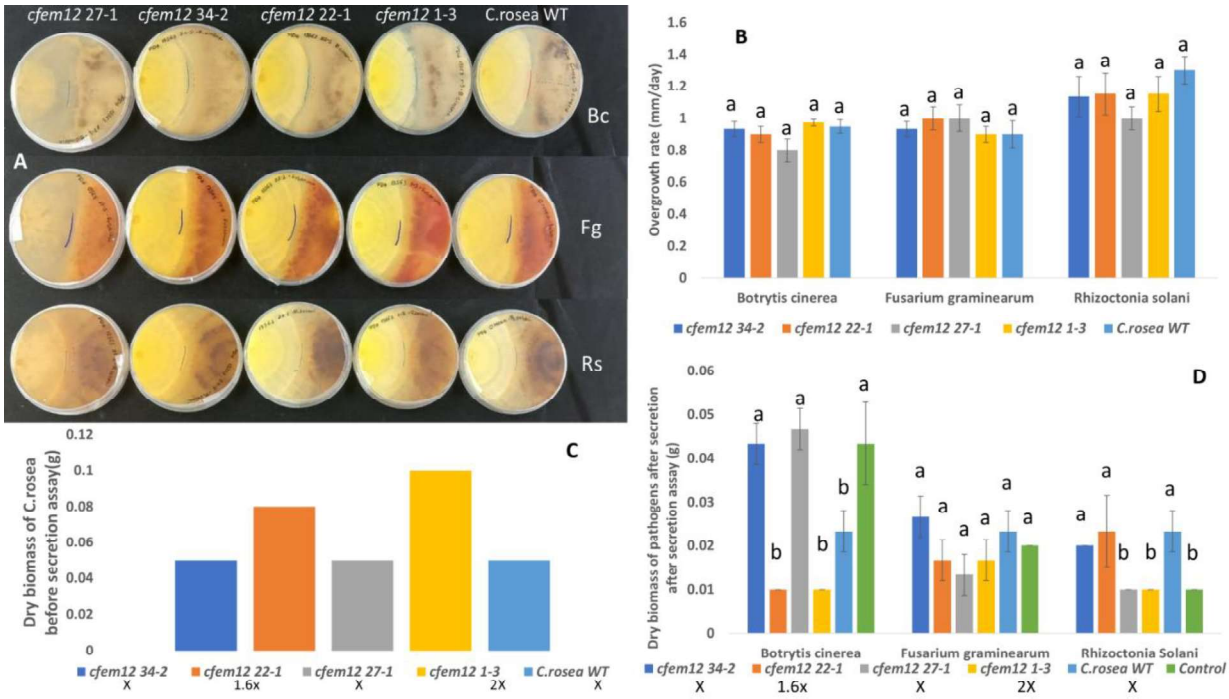

Figure 10 Dual culture and secretion assay experiments. *A) Visual representation of the plates 5 days after contact. The acronyms indicate Bc-Botrytis cinerea, Fg Fusarium graminearum and Rs stands for Rhizoctonia solani. B) Bar chart presenting the overgrowth of C. rosea and C. rosea cfem12 mutants in dual culture with the pathogens B. cinerea, F. graminearum and R. solani. 4 replicates for both C. rosea WT and the mutants (cfem12 27-1 cfem12 34-2, cfem12 1-3 and cfem12 22-1). Measurements were conducted 5 days after contact and the letters represent statistical different groups C, D) Secretion assay results where C indicate the dry biomass of C. rosea after grown in PDB for 3 days and D represents the growth of the pathogens in the media containing PDB and C. rosea metabolites after 4 days.*

3.6.2 Dual culture assay- Self interactions of *C. rosea* WT and mutants

Since the expression of *cfem12* was upregulated at after contact stage of self-interaction, a dual culture self-interaction experiment was performed to investigate whether CFEM12 is involved in self-recognition. No differences were observed either between the self-interaction of WT, the interaction of WT with the mutant and between the self-interaction of mutants in the total area covered (Figure 11). All of them had the ability to recognize and interact with each other (Fig.11A, B and C) and the only differences that can be seen ($p<0.001$) in the mutants *cfem12* 1-3 and *cfem12* 34-2 (Fig.11D) is due to the growth rather than their ability to recognize.

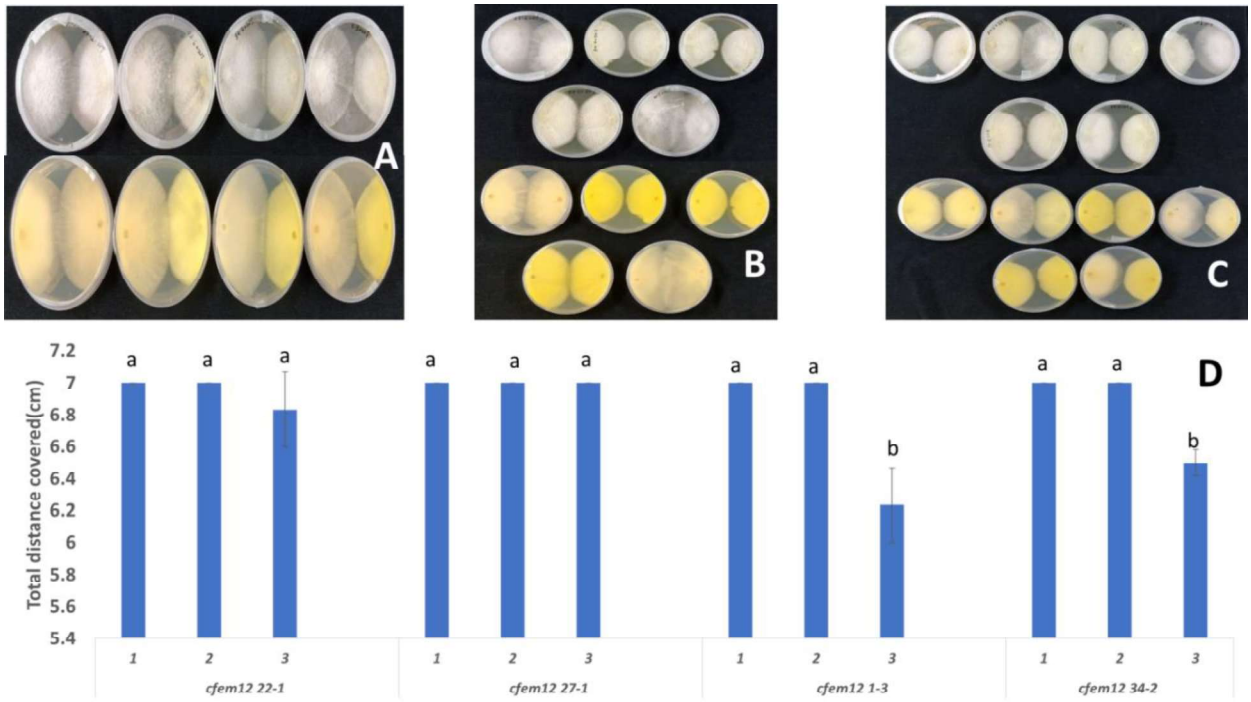

Figure 11 Self to self-interaction experiment. *Bar chart presenting the growth of C. rosea WT and C. rosea mutants for cfem12 during the self-self-interactions 14 days post inoculation. The acronyms cfem12 1-3, cfem12 27-1, cfem12 34-2 and cfem12 22-1 indicate the mutants, All the possible combinations were tested (A, B, C) but statistical analysis was only made between the self-interaction of WT (B, Dbar1), between the interaction of WT and the mutant (A, Dbar2) and between the self-interaction of the mutant. (B, Dbar3). Statistics are based on observations on day 14 and the letters represent groups who share statistical differences or not.*

3.7 Root colonization

Root colonization was assessed through both biomass quantification (Figure 12A) and visual observations on PDA plates (Figure 12B). Although the deletion strain of *cfem12* exhibited approximately a two-fold increase in *C. rosea* biomass compared to the WT, the difference was not statistically significant (Fig.12A). Therefore, it was concluded that the deletion mutants did not possess an enhanced ability to colonize wheat roots. The same results can also be observed by the visual assessments on PDA plates (Fig.12B), which showed no observable differences in colonization patterns between the mutants and the WT.

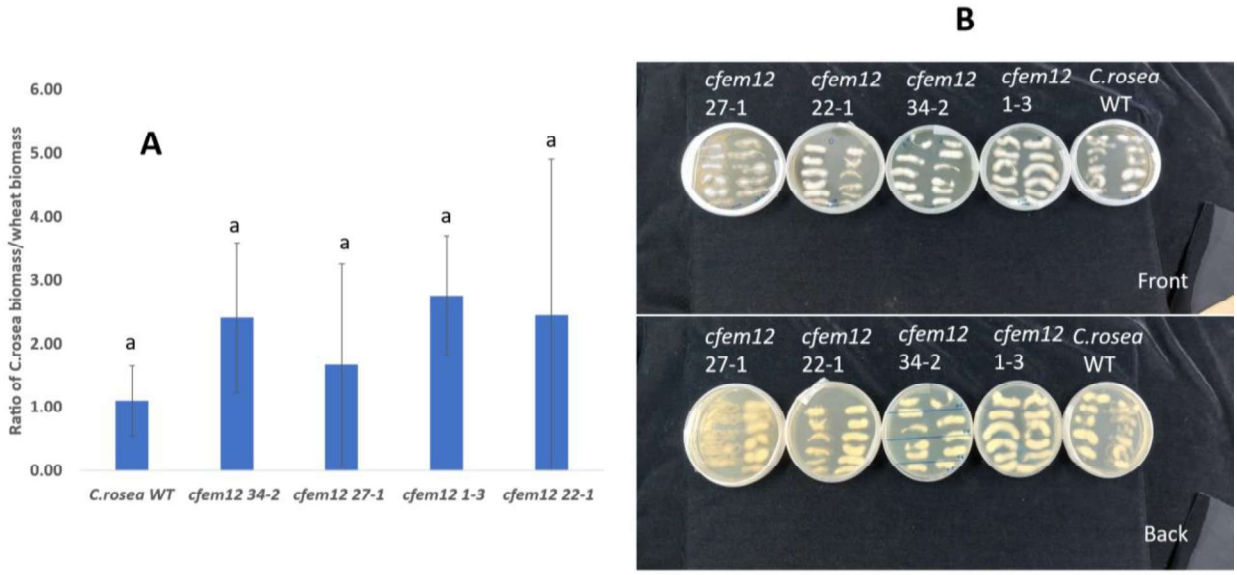

Figure 12 Root colonization experiment. *(A) Bar chart presenting the ratio of C. rosea WT and deletion strain for the gene cfem12 and wheat to wheat using specific primers for either tubulin in wheat or actin for C. rosea. Statistics are based on observations after the DNA extraction and RT-qPCR and the letters represent groups who share statistical differences or not. (B) Equally cut roots on PDA plates after 4 days at 20°C. 2 plates for each mutant and WT were prepared.*

3.8. Biocontrol efficacy

3.8.1 Climate chamber experiment

The biocontrol efficacy of *C. rosea* WT and deletion strains of *cfem12* against fusarium foot rot caused by *F. graminearum* was evaluated through a climate chamber experiment. Based on the results presented in the Figure 12, it can be concluded that the deletion of *cfem12* did not alter the biocontrol ability of *C. rosea* against *F. graminearum*. No significant differences were observed between the mutant and wild-type strains across multiple parameters, including shoot length (Fig.13A), root length (Fig.13B), disease severity score (Fig.13C), and fresh biomass (Fig.13D). Dry biomass was also measured, showing a 100% correlation with fresh biomass as shown in Figure 15 (Appendix 1).

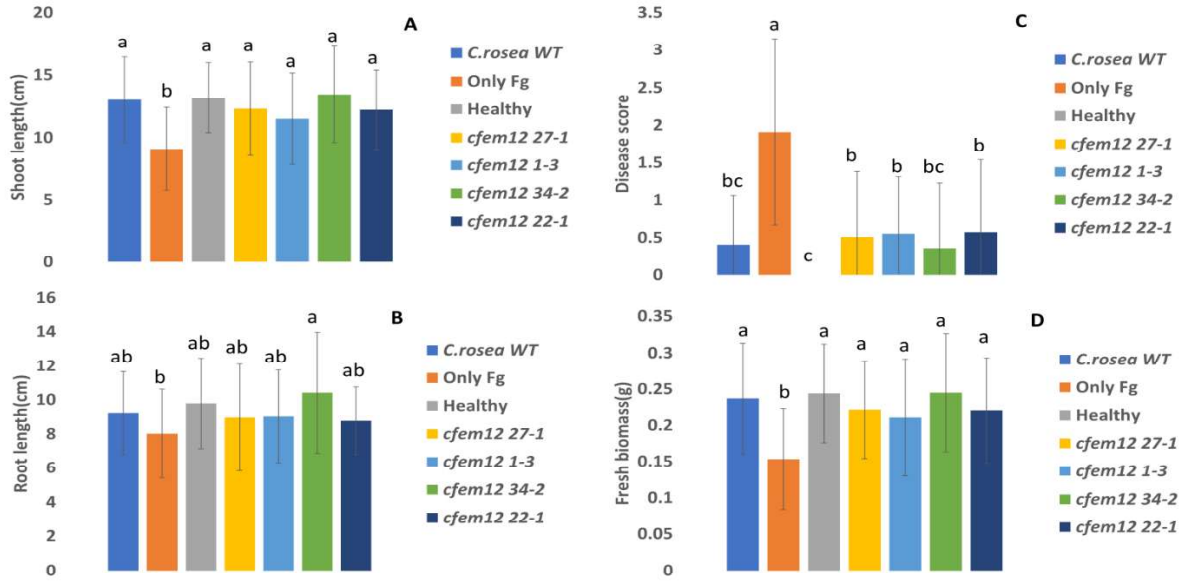

Figure 13 Climate chamber experiment. *Bar charts presenting the measurements conducted after 3 weeks regarding of shoot length(A) root length(B), disease severity score(C) and fresh biomass(D). Statistics are based on observations after 3 weeks and the letters represent groups who share statistical differences or not.*

3.8.2 In planta experiment

The biocontrol efficacy of *C. rosea* WT and deletion strains of *cfem12* against *B. cinerea* was further assessed using an in-planta experiment on *N. benthamiana* leaves (Figure 14A). Based on the results presented in Figure 14, deletion of *cfem12* did not affect the biocontrol capability of *C. rosea* against *B. cinerea*. No significant differences were observed between the mutant and wild-type strains in their ability to reduce the necrotic lesion area caused by *B. cinerea* (Figure 14B).

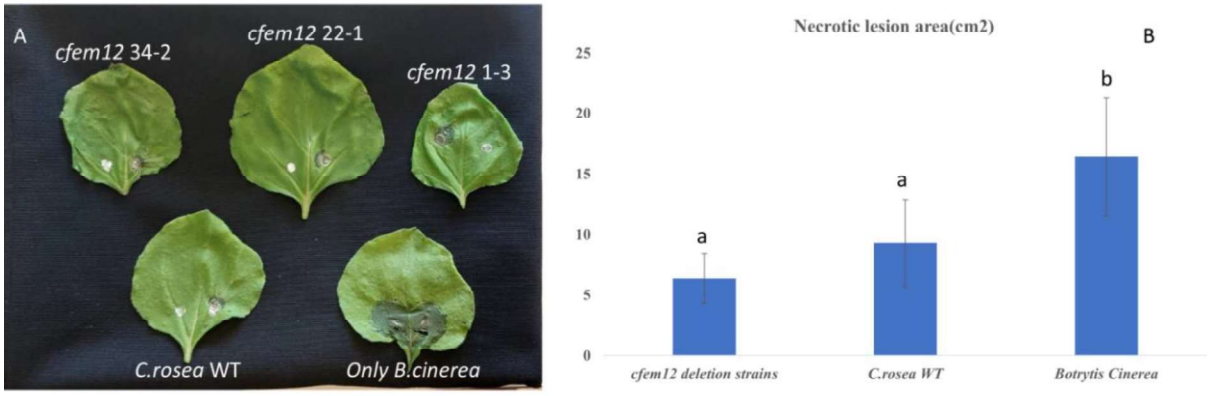

Figure 14 In planta experiment*. (A)Visual representation of one leaf for the mutants (cfem12 34-2, cfem12 22-1 cfem12, 1-3), the WT and one leaf that was inoculated only with B. cinerea. B) Bar chart presenting the measurements regarding the necrotic lesion area conducted after 3 days post inoculation with B. cinerea and the letters represent groups who share statistical differences or not.*

4.Discussion

In this study, our primary aim was to investigate the role of CFEM12, a secreted protein from the *C. rosea* IK726 strain that contains a CFEM domain motif, using a series of molecular and phenotypic assays.

To explore whether *cfem12* functions similarly to effector-like CFEM proteins described in pathogenic fungi, HR suppression and subcellular localization assays were performed. In contrast to CFEM genes in *Fusarium verticillioides* and *B. cinerea*, which have been shown to suppress plant immune responses and localize to the host interface (Li et al. 2025; Zhu et al. 2017), *cfem12* did not exhibit HR suppression in *N. benthamiana*. This finding suggests that *cfem12* do not act as a classical effector on immune suppression. The failed GFP localization attempt leaves its subcellular distribution unresolved, though the lack of suppression already argues against a host-directed effector role.

Additionally, CFEM proteins have also been linked to fungal development and stress responses. In *B. cinerea*, CFEM deletion impacts sporulation and stress tolerance (Zhu et al. 2017). In our study, *cfem12* mutants in *C. rosea* showed significantly reduced sporulation but retained normal growth and stress resistance. This partial overlap suggests *cfem12* is important for sporulation but may have diverged functionally from its counterparts in pathogenic fungi, possibly reflecting different ecological pressures in biocontrol species.

Interestingly, *cfem12* expression was differentially regulated during fungal interactions, aligning with prior observations in *Trichoderma* where CFEM genes like *tacfem1* respond to microbial competition (Guzmán-Guzmán et al. 2017). However, despite this transcriptional response, biocontrol performance of *cfem12* mutants was inconsistent across pathogens. Secreted metabolite activity against *B. cinerea* was reduced, indicating *cfem12* may influence secondary metabolite production. Yet, this reduction was not reflected in dual culture or in planta antagonism assays and thereby should be more investigated. For *Fusarium graminearum*, no impact of *cfem12* deletion was observed in any assay, suggesting pathogen-specific effects or functional redundancy.

The lack of a strong phenotype in root colonization also contrasts with previous findings in endophytic fungi such as *Falciphora oryzae*, where CFEM gene deletion impaired host interaction (Dai et al. 2021). In *C. rosea*, *cfem12* mutants maintained normal colonization of

wheat roots, implying that *cfem12* may not play a central role in root symbiosis—or that functional compensation occurs via other CFEM family members.

Collectively, these findings indicate that *cfem12* contributes to fungal sporulation and secreted antifungal activity but does not act as a key effector or colonization factor. Its function appears to diverge from that of CFEM proteins in pathogenic fungi, supporting the idea that gene families involved in host interactions can undergo functional specialization depending on ecological context.

5.Future aspects

While the primary focus of our study was on the gene *cfem12*, several promising avenues remain for future investigation. Our analysis suggests that this gene may play a significant role in the interaction between *C. rosea* and *B. cinerea*, but further experimentation is necessary to validate its function. Two key directions worth pursuing are:

First and foremost, a detailed in-silico analysis to identify potential interactions between CFEM12 and key proteins from *B. cinerea* could provide crucial insights into the molecular basis of this interaction. Such interactions could be confirmed using RNA-seq data from dual culture assays, followed by functional assays such as Co-immunoprecipitation (Co-IP) and Yeast Two-Hybrid (Y2H) systems. These experiments could reveal direct physical interactions between CFEM12 and *B. cinerea* proteins, further elucidating its role in biocontrol.

Secondly, conducting a metabolomics analysis of both the WT and mutant strains would help uncover how the deletion of *cfem12* affects secondary metabolite production, particularly regarding the mutants' diminished ability to inhibit *B. cinerea* in secretion assays. Understanding the biochemical shifts in the mutant strains could provide valuable insight into the antifungal mechanisms impaired by the loss of this gene.

In addition to *cfem12*, two other CFEM-domain-containing genes in *C. rosea* are worth exploring. These genes may have similar or distinct roles in pathogen interaction and antifungal activity, warranting further functional characterization to gain a comprehensive understanding of the CFEM protein family in *C. rosea*.

For other pathogens, like *F. graminearum* and *R. solani*, results from the dual culture assays and climate chamber experiments suggest that *cfem12* does not significantly contribute to

antagonism against these pathogens. This reinforces the specificity of the gene's role in biocontrol, which warrants further exploration in pathogen-specific contexts.

Regarding the hypersensitive response (HR), our findings indicated that *cfem12* did not suppress HR triggered by Avr4. However, numerous other genes are known to trigger HR. For example, in *Phytophthora infestans*, effectors like AVR3a, AVR2, and AVRblb2 are recognized by corresponding potato R genes (R3a, R2, and Rpi-blb2), triggering HR (Bos et al., 2006; Oh et al., 2009). Similarly, in *Magnaporthe oryzae*, effectors like AVR-Pita, AVR-Pii, and AVR-Pik activate HR through recognition by their respective rice R genes (Kanzaki et al., 2012). In *Fusarium oxysporum*, the effector SIX3 (AVR2) induces HR in tomato lines carrying the I-2 resistance gene (Rep et al., 2004), while *Verticillium dahliae's* Ave1 effector is recognized by the tomato Ve1 receptor, triggering HR and conferring resistance (de Jonge et al., 2012). These examples highlight the diverse strategies pathogens use to manipulate or trigger plant immunity through HR-inducing effectors.

Finally, although we observed no significant differences between wild-type and mutant strains of *C. rosea* in terms of growth or stress tolerance—except for the observed variation in sporulation—additional experiments could provide a more complete picture of the gene's broader biological role. Future studies could test more environmental stress conditions, analyze spore and mycelial size, and measure spore germination rates, which were not specifically assessed in this study. Despite observing growth from plate cultures, spore germination rates remain an unexplored area that could offer more biological insight into the role of *cfem12* in fungal development and pathogen inhibition.

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Popular scientific summary

This study aimed to investigate the role of CFEM proteins in *Clonostachys rosea*, a fungus with significant potential as a biological control agent. By aligning with the United Nations Sustainable Development Goals (SDGs), Zero Hunger (Goal 2), Responsible Consumption and Production (Goal 12), and Life on Land (Goal 15) these findings would help at reducing dependence on synthetic pesticides. This can be achieved by the usage of *C. rosea* to support sustainable agriculture, environmental conservation, biodiversity protection, and soil health. Insights into *C. rosea's* genetic mechanisms and advanced fungal genetics can establish a foundation for safer, more effective pest management strategies. Apart from that, the implementation of biological control agents, more specifically *C. rosea* can also help to enhance crop yields, improve disease resilience, and address global food security challenges while meeting EU regulatory standards. By investigating in detail one CFEM protein, CFEM12, particularly in relation to its biological fitness, antagonistic activity against plant pathogens, and plant-associated traits such as root colonization and biocontrol efficacy we can evaluate the possible potential of those proteins in specificity towards pathogens. Future investigation of the other CFEM proteins in *C. rosea*, CRV2T0016446 and CRV2T0010487, could also provide further evidence of the importance of these proteins in specific interaction with pathogens, and help design tailored pest management solutions for sustainable farming systems. By uncovering the mechanisms behind *C. rosea*'s effectiveness, this research underscores the value of science-driven solutions to foster collaboration among researchers, farmers, and policymakers, paving the way for a future where agricultural productivity and environmental stewardship go hand in hand.

Appendix 1

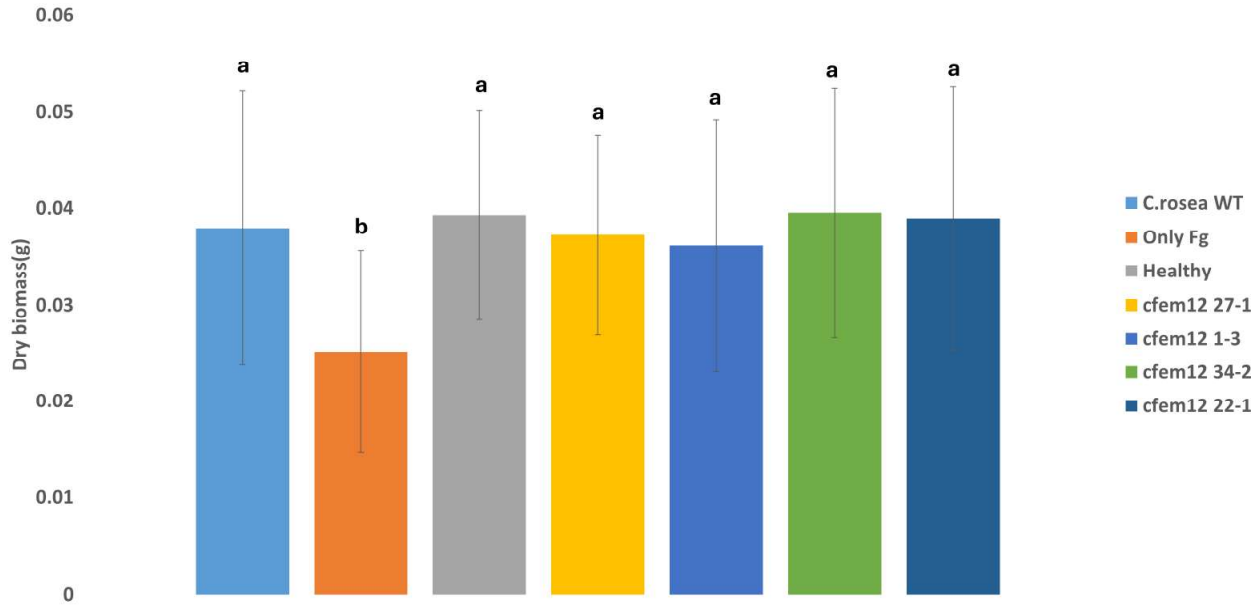

Figure 15 Climate chamber experiment (2). *Bar charts presenting the measurements conducted after 3 weeks regarding of dry biomass. Statistics are based on observations after 3 weeks and the letters represent groups who share statistical differences or not.*

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