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Relative Expression of Genes Elicited by *Clonostachys rosea* in *Pinus radiata* Induces Systemic Resistance

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Abstract: Radiata pine is one of the most commonly planted tree species in Chile due to its fast growth and desirable wood and pulp properties. However, its productivity is hampered by several diseases. Pitch canker disease (PCC) caused by *Fusarium circinatum*, is considered the most damaging disease to the pine forest industry. Several control measures have been established, with biological control emerging as an environmentally friendly and effective way for *F. circinatum* control. Previous studies support the value of *Clonostachys rosea* in reducing PCC damage, with evidence suggesting a potential induced systemic resistance (ISR) triggered in radiata pines by this agent. Ten-month-old radiata pine plants were pre-treated with *C. rosea* on a substrate at 8 and 1 days before inoculation with *F. circinatum* on the stem tip, and expression levels were determined for *DXS1*, *LOX*, *PAL*, and *PR3* genes 24 h later. Lesion length was 45% lower on plants pre-treated with *C. rosea* and infected with *F. circinatum* compared to non-pre-treated and infected plants. Additionally, *LOX* and *PR3* were induced 23 and 62 times more, respectively, in comparison to untreated plants. Our results indicate that *C. rosea* causes an ISR response in pre-treated plants, significantly increasing the expression of resistance genes and reducing lesion length.

Keywords: Monterrey pine; biological control agent; ISR

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1. Introduction

One of the most economically important pine species in the world is *Pinus radiata* D. Don. It is also the most planted commercial tree species in the Chilean forest industry, thanks to its exceptionally fast growth rate and wood properties [1,2]. However, the productivity of this species can be diminished by fungal and pest diseases, competition from weeds, and abiotic stress [3–7].

Fusarium circinatum is the causal agent of pitch canker, a disease that affects over 60 *Pinus* species [8] and also *Pseudotsuga menziesii* [9]. This disease is considered the most significant issue for conifers worldwide [10]. The major symptom of the disease is the formation of cankers, necrotic lesions present in the stem and branches that release large quantities of resin. Canker development can lead to branch and tree death [11,12]. This pathogen also affects seeds and seedlings, but identification can be challenging due to similarities with damping off and root rot caused by other fungal diseases [11,13]. The pathogen can be spread by insect vectors, water splashes, wind, and soil [3,11,14,15].

Pinus radiata is considered one of the most susceptible species to this pathogen worldwide [11]. In Chile, *F. circinatum* was detected in 2002, affecting *P. radiata* nursery seedlings and clonal hedge plants in the central–south region of the country [16]. Currently, it is possible to find isolated trees killed by the pathogen in young plantations up to four years

of age; however, secondary spread has not been observed. For this reason, it is believed that the diseased trees were probably infected in the nursery [10,17]. This disease behavior is similar to the situation described in South Africa with *P. patula*, where the pathogen attacked seedlings in nurseries and was not associated with the typical symptom of resinous cankers in plantations. However, outbreaks of pitch canker lesions in South African pine plantations were reported later [18]. Additionally, Wikler et al. (2003) [3] demonstrated that the disease is more severe and progresses faster on managed lands than in native populations in California, indicating a potential risk of future contamination of plantations. Therefore, the implementation of control methods to prevent or delay the occurrence of pitch canker outbreaks in plantations is of the utmost importance.

Currently, there are some measures being developed or utilized in nurseries, including the cultural and chemical control [10,19] development of genetic control through the detection of quantitative trait loci (QTLs) associated with resistant genotypes [2] and the selection of candidate genes that could be employed for the early identification of individuals potentially resistant to the pathogen [20]. Finally, there are some examples of biological control through the selection of antagonist microorganisms against *F. circinatum* under in vitro and greenhouse conditions [13,21].

One of the antagonists that performed best against the pathogen was *Clonostachys rosea* (teleomorph *Bionectria ochroleuca*), a non-pathogenic and cosmopolitan fungus widely distributed worldwide. This fungus exhibited a wide trophic plasticity, acting as a saprophyte in a variety of soils, as an endophyte or epiphyte on live plants, and also as a mycoparasite [22–24]. Thus, the antagonistic activity of *C. rosea* demonstrates a broad spectrum, with reports in the tomato, soybean, lettuce, and rose, and it is currently acknowledged as a strong biological control agent (BCA) against pathogenic fungi such as *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Botrytis cinerea* which affect various crops of agronomic and forest importance [23,25–29]. In the forestry field in Chile, studies aimed at evaluating this BCA against important diseases such as gray mold caused by *B. cinerea* on seedlings of *Eucalyptus globulus* [30,31], and damping off caused by *Fusarium circinatum* in *Pinus radiata* [13], demonstrated a reduction in the diseases through various mechanisms, including substrate competence and parasitism. Sutton et al. (1997) [32] suggested that induced resistance could be one mechanism used by *C. rosea* to control diseases caused by some pathogens. Induced systemic resistance (ISR) is defined as the increased resistance exhibited by plants when appropriately stimulated by an inducer agent, leading to physical or chemical responses that enable plant protection when challenged with a pathogen [33,34]. This priming phenomenon involves the jasmonate/ethylene (JA/ET) signaling pathway and is induced, but not restricted, by non-pathogenic organisms such as endophytic or rhizogenic fungi and bacteria [35]. It is possible that inducing agents trigger some pathways involving multiple polygenic responses. In this case, when the ISR has been activated, prolonged resistance against multiple pathogens can be achieved [36,37].

Main Genes Involved in Systemic Resistance

The JA/ET signaling pathway leads to the induction of pathogenesis-related (PR) proteins, including PR3, a basic chitinase; PR4, a chitin-binding protein, in conjunction with β-1,3-glucanase, have been implicated in the resistance to several fungal pathogens. Chitinases are enzymes that hydrolyze β-1,4 glycosidic bonds linking N-acetylglucosamine residues of chitin. They play a direct role in plant defense by inhibiting the growth of hyphae which invade the intercellular space [38] Considered as useful markers for host defense responses, they confer disease resistance based on antimicrobial activity both in vitro [39] and in planta [40]. The enzyme phenylalanine ammonia-lyase (PAL) catalyzes the reaction from L-phenylalanine to ammonia and trans-cinnamic acid. This enzyme serves as an entry point into the phenylpropanoid metabolism, regulating the production of monolignols. It has been associated with defense responses, playing a key role in lignification and wound protection [41,42]. In cell cultures of *Pinus banksiana* and *Picea abies*, an increase in *PAL* transcript levels precedes an increase in PAL activity and lignification

triggered by a fungal elicitor [43,44]. On the other hand, lipoxygenase enzymes (LOX) catalyze the dioxygenation of polyunsaturated fatty acids, adding molecular oxygen at either the C-9 or C-13 residues of linoleic or linolenic acid. This process leads to the formation of 9- or 13-hydroperoxylinoleic or -linolenic acid, respectively [45,46]. The hydroperoxidation of polyunsaturated fatty acids by LOX produces the precursor for the synthesis of jasmonic acid. Jasmonic acid, in turn, triggers the expression of various genes that play a role in defense responses in plants [47] (Rosahl 1996). Additionally, 1-deoxy-D-xylulose-5-phosphate synthase (DXS) is an enzyme that catalyzes the first step of the methylerythritol phosphate (MEP) pathway [48]. In plants, the MEP pathway is involved in terpenoid biosynthesis, providing the main components for oleoresin synthesis, with defensive roles against herbivores and pathogens in conifers [49,50].

Induced resistance mechanisms have been shown to be effective measures to provide protection and are implemented in the agricultural sector. However, very little is known about the mechanisms present in forest trees, specifically in widely planted conifer species such as *P. radiata* [36,51]. For this reason, our goal was to evaluate the behavior of the Cr7 strain of *C. rosea* as an elicitor of ISR on a *P. radiata*-resistant genotype and evaluate four candidate genes for the priming phenomenon associated with ISR in this forest species.

In a previous study conducted by this research group, resistant and susceptible genotypes of *P. radiata* were tested against *F. circinatum*. Various strains of *C. rosea* were applied to the pine plants' substrate to induce resistance against the pathogen. It was found that only the resistant genotype of *P. radiata* showed evidence of ISR, with two *C. rosea* strains decreasing the lesion length by almost 50% compared to the uninduced control. This demonstrates the feasibility of using selected strains of this biocontrol agent to restrict pitch canker disease [52].

2. Materials and Methods

2.1. Plant Material

A previously characterized *P. radiata*-resistant genotype (R) was included because of its previously reported priming effect against *F. circinatum* in response to *C. rosea* elicitation [52]. The plant material was ten months old and provided by BioForest S.A., Concepción, Chile. All clones were maintained under controlled conditions of 80% relative humidity, 25 °C, and a 12/12 photoperiod, starting two weeks before the first application of the *C. rosea* strain and continuing until the completion of the experiments in the greenhouses at BioForest S.A.

2.2. Clonostachys rosea and Fusarium circinatum Strain Culture Conditions

C. rosea strain Cr7, isolated from healthy radiata pine roots and belonging to the Forest Pathology Lab collection at the University of Concepción, was included in this study. This strain was previously tested for its biological control activity (BCA), providing over 80% protection against the damping off disease caused by *F. circinatum* under greenhouse conditions [21]. Furthermore, this strain triggered an elicitation of ISR, reducing the damage caused by the pathogen by almost 50% [52]. An aggressive strain of *F. circinatum*, isolated from symptomatic *P. radiata* hedges, Pr44-4641 [13], was used to induce damage to plants. The antagonistic fungi and the pathogen were stored in tubes containing potato dextrose agar (PDA) as a culture medium at 4 °C prior to the assays. The fungal strains were replicated in Petri dishes containing PDA and incubated at 25 °C for seven days to obtain fresh inoculum before each assay. Images of both fungi are provided in Supplementary Figure S1.

2.3. Induced Systemic Resistance Assay

The assay consisted of five treatments, which included an absolute control treatment with plants not subjected to an apex cut or strain inoculations (AT), a negative control for wound effects with apex cut but no strains applied (W), and three treatments including an apex cut plus *C. rosea* application (Cr), *F. circinatum* inoculation (Fc), or *C. rosea* application plus *F. circinatum* inoculation (Cr + Fc). Before each assay, PDA dishes with each strain were

prepared as described previously. Conidia were collected from the surface of the mycelium using a spatula, then suspended in sterile deionized water, and filtered through six sheets of sterile gauze under aseptic conditions. To elicit the systemic response, *C. rosea* Cr7 was applied to the corresponding plant substrate in a volume of 15 mL (1×10^7 conidia/mL). This treatment was applied twice as the following: eight days before inoculation and the day before inoculating the clones with *F. circinatum*. The pathogen was inoculated at the apex of each plant by making a blunt cut and applying a 5 microliter drop at a final concentration of 1×10^5 conidia/mL. Each treatment consisted of three replicates for molecular analysis and ten replicates for lesion length evaluation. The disease severity caused by the pathogen was evaluated 120 days post-inoculation by measuring lesion length in millimeters using a digital caliper (0.1 mm resolution, ±0.02 mm precision, generic brand). The removal of the external tissue allowed for better visualization of the xylem necrosis and was used to evaluate the lesion length more precisely.

2.4. RNA Extraction and Quantitative Real-Time PCR Analysis

Total RNA was extracted from 5 cm fragments of each shoot (stem or cut apex), measured from the point of inoculation. All samples were collected 24 h after inoculation with *F. circinatum*, and the uninoculated controls were sampled simultaneously. The extraction followed the CTAB method of Chang et al. (1993) [53]. The samples were dissolved in 20 µL of RNase-free water, and the concentration was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The purity of the samples was assessed based on a 260/280 ratio between 1.9 and 2.1 and a 260/230 ratio greater than 2.0. Sample integrity was visually checked by electrophoresis in 2% agarose gels. Before cDNA synthesis, each sample of 1 µg of RNA was treated with DNase I (Fermentas, Waltham, MA, USA) to remove genomic DNA contamination. First-strand copy DNA was synthesized by reverse transcription using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA), following the manufacturer's instructions and stored at −80 °C before use.

Gene expression was determined using quantitative real-time PCR (qPCR) with the fluorophore SYBR Green for detection using the StepOnePlus system (Applied Biosystems, Waltham, MA, USA). The primers used for the four target genes, namely 1-D-deoxyxylulose 5-phosphate synthase (DXS), lipoxygenase (LOX), phenylalanine ammonia-lyase (PAL), and pathogenesis-related protein (PR3), along with two endogenous reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ubiquitin (UBQ), as previously reported by Donoso et al. (2015) [20], are detailed in Table 1. Primer pairs that were not previously reported were designed using the AlleleID 6.0 software. The qPCR reaction used 10 ng of cDNA as a template, 10 µL Power SYBR® Green PCR Master Mix (Applied Biosystems), and the corresponding primers at a concentration of 450 nM, in a total volume of 20 µL. Amplification was carried out under the following conditions: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C in 96-well optical reaction plates (Applied Biosystems). The specificity of the amplified products was verified using the melting curve method. The temperature was increased by 0.3 °C from 60 to 95 °C for 118 cycles to detect any non-specific amplification patterns. The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method [54]. For qPCR analysis, three biological replicates (ramets) were considered for each treatment, and three technical replicates were performed for each ramet. The calibrator sample corresponded to a sample of AT treatment. PCR efficiency was determined for each gene by measuring the cycle threshold (CT) to a specific threshold for a serial dilution of cDNA samples. The primers used for qPCR are listed in Table 1.

Table 1. Primers used in quantitative real-time PCR analysis for four targets and two endogenous reference genes (*hkp*).

Gene	Forward (5′-3′)	Reverse (5′-3′)	GenBank ID Gene	Amplicon	Reference
DXS	TGGGAAGGCGGGTTGGT AAAG	TTGATTGTGTCCAGGAGAGG TGTC	EU439293	97	This work
LOX	AGGCAGTGGAAATGGAAA GTTTGG	CAAGCGTGAGTGAGTTGAG GAAG	JQ262756	191	This work
PAL	GGAGCCACTTCTCACAGGAG	CCGGGTAGTATCTTCGGACA	U39792	123	[55]
PR3	AAACCTGGATTGCAACAACC	TTATGGCAAACGGGTACACA	AF457093	150	[20]
GAPDH (*hkp*)	GCTCCCAGCAAGGATGCCCC	AGCCAAAGGGGCCAAGCAGT	L07501	117	[20]
UBQ (*hkp*)	TGGCCGGGCAGGATCAAACG	TCCCCTCGTAAACGCCTCCC	BM133596	122	[20]

2.5. Statistical Analysis

The statistical software Prism 5.0 (GraphPad Software, MO, USA) was used to perform analysis of variance (ANOVA). Gene expression data were subjected to one-way ANOVA to test the effect of treatments. Before conducting ANOVA, the assumptions of normality and homogeneity of variance were verified. Tukey tests were used to determine statistically significant differences between treatments ($p < 0.05$).

3. Results

3.1. Effect of the Application of C. rosea Strain Cr7 on the Damage Caused by F. circinatum in a Resistant Genotype of P. radiata

Two different external symptoms were observed when we evaluated the disease development due to *F. circinatum* infection: dark brown color development on the shoot and dehydration of the affected zone. Plants with no treatment or cut at the top (AT) showed no damage. In the case of wound treatment W, a slight resin exudation and local tissue oxidation were observed. This damage was confined to the cut zone and did not increase over time, maintaining the same size of just a few millimeters at 120 days after the start of the experiment. Both the AT and W control plants were not statistically different. The application of *C. rosea* (Cr) to the substrate did not result in statistically significant differences with the W control either. The damage is attributed to the apex cut. Extensive damage was evident in the case of *F. circinatum* treatment (Fc), with the pathogen developing from the apical zone, causing xylem necrosis at the stem level and needle death. Nevertheless, this damage was notably reduced on plants with the additional application of *C. rosea* (Cr + Fc), as shown in Figure 1.

After the needles were removed, a detailed assessment of the stem damage was conducted. AT plants showed no damage while W and Cr plants showed minimal damage due to the apical cut, which was restricted to less than a millimeter from the top of the plants (Figure 2). Cr + Fc treatment resulted in a nearly 30-fold increase in lesion length compared to the W control (6.6 cm). However, treatment with the *F. circinatum* pathogen alone caused approximately 60 times more damage than the same control, reaching 14.6 cm, as illustrated in Figure 2. The data clearly show a reduction in the damage caused by the presence of *C. rosea* in the soil of the Cr + Fc treatment.

3.2. Relative Expression of Genes Involved in the Defensive Response of P. radiata Plants

No significant differences in the relative expression of the *DXS1* gene were observed between the absolute and apex cut controls AT and W. Furthermore, the expression levels of this gene were not significantly altered by the Cr treatment, suggesting that the mechanical wound induced on W and the application of Cr to the substrate did not affect the gene expression of *DXS1*. Nevertheless, its relative expression was notably reduced by Cr + Fc treatment compared to the controls and Cr treatment. A significant increase in *DXS1* expression was observed in plants inoculated with the pathogen (Fc), with expression levels doubling those of the controls and being nearly 20 times higher compared to the Cr + Fc treatment (Figure 3A).

Figure 1. Damage observed on *P. radiata*-resistant (R) plants treated with *C. rosea* C7 strain and inoculated with *F. circinatum* Pr44-4641 strain at 120 days post-treatment. W, apex cut without any strain applied. Cr, only *C. rosea* was applied to the substrate. Cr + Fc, *C. rosea* was applied at the substrate level and *F. circinatum* was inoculated on the cut apex. Fc, only *F. circinatum* was inoculated on the cut apex. The vertical scale bar on the right represents 10 cm. The W plant was not in the original picture and was added here for comparison. However, all pictures were taken on the same day under the same conditions, with no further editing.

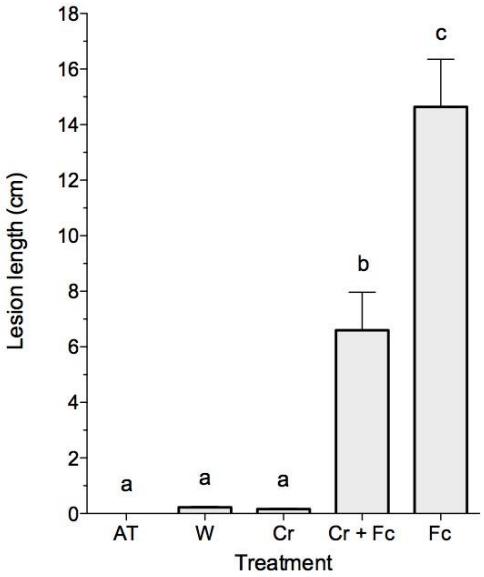

Figure 2. Lesion lengths observed 120 days after infection with *F. circinatum* on a resistant (R) genotype of *P. radiata*. AT, absolute control without the apex cut or strains applied. W, apex cut control. Cr, *C. rosea* strain applied to the substrate. Cr + Fc, *C. rosea* was applied to the substrate 8 and 1 days before *F. circinatum* inoculation on the top cut. Fc, only *F. circinatum* was inoculated at the top cut. Bars show the mean ± SE, $n = 6$. Different letters indicate statistically significant differences between treatments (Tukey test, $p < 0.05$).

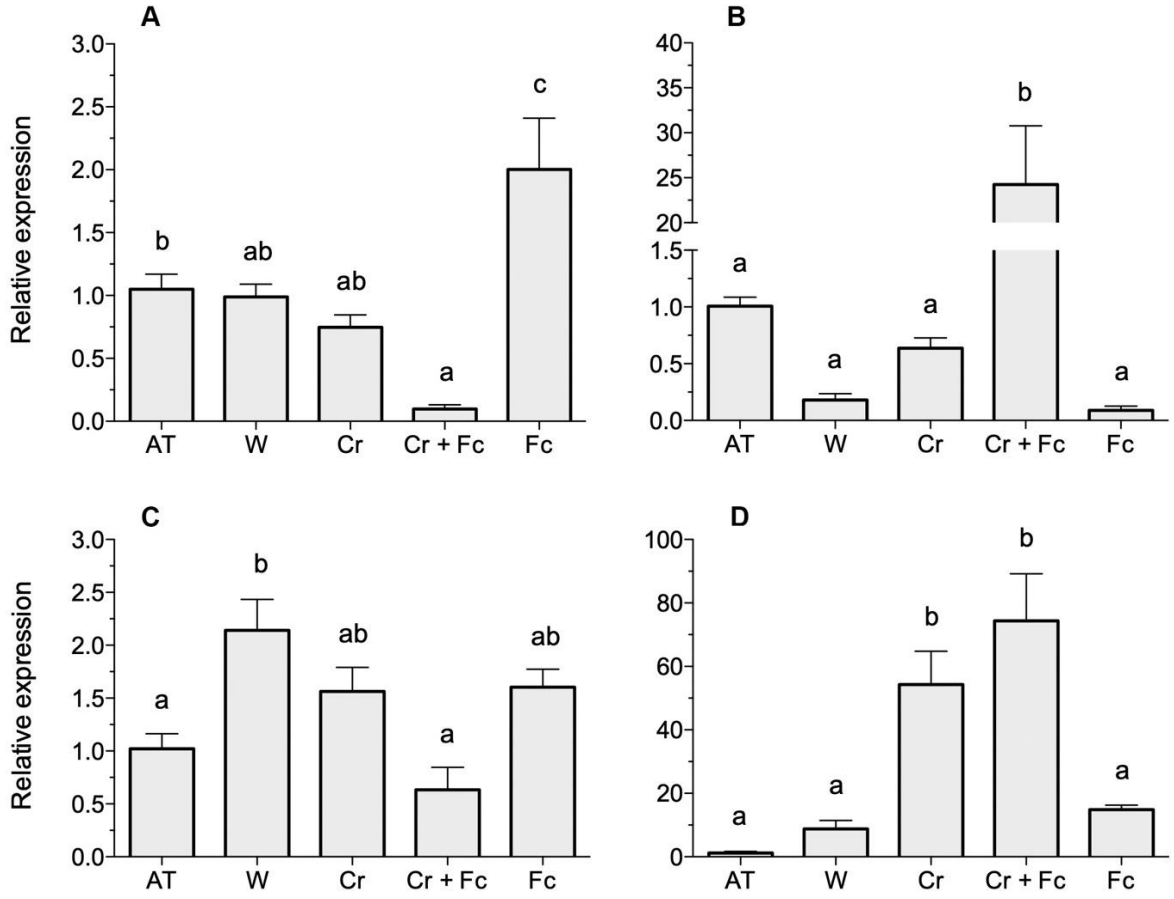

Figure 3. Relative expression of selected genes related to pathogenic attack on *P. radiata* plants 24 h after *F. circinatum* inoculation. (**A**) *DXS1*. (**B**) *LOX*. (**C**) *PAL* and (**D**) *PR3*. AT, absolute control without the apex cut or strains applied. W, apex cut control. Cr, *C. rosea* strain applied to the substrate. Cr + Fc, *C. rosea* was applied to the substrate 8 and 1 days before *F. circinatum* inoculation on the top cut. Fc, only *F. circinatum* was inoculated at the top cut. Bars show the mean ± SE, $n = 3$. Different letters indicate statistically significant differences between treatments (Tukey test, $p < 0.05$).

The relative expression of the *LOX* gene was lower, but not statistically significant, for all treatments except for Cr + Fc when compared to the AT control plants. The pre-treatment with the Cr7 strain of *C. rosea* after infection with *F. circinatum* increased the expression of this gene by 23 times compared to the control (At). However, as mentioned earlier, neither the Cr nor Fc treatments alone caused significant effects on the expression of this gene (Figure 3B).

Transcript accumulation for the *PAL* gene was low and was not significantly induced by treatments, including the *C. rosea* strain (Cr and Cr + Fc), when compared to the AT control. Only the cut control W showed statistically significant results, but with low induction levels compared to AT (Figure 3C).

On the other hand, in the case of the *PR3* gene, a significant increase in transcript accumulation was observed in plants pre-treated with *C. rosea* Cr and Cr + Fc. The expression levels were 45 and 62 times higher compared to the absolute control, respectively. Both were significantly different from the W and Fc treatments. Although not statistically significant, it is noteworthy that there was an increase in *PR3* activity in W and Fc compared to intact AT plants (Figure 3D).

4. Discussion

The objective of this research was to evaluate the potential induction of ISR by the *C. rosea* Cr7 strain on a preselected pitch canker disease-resistant (R) genotype of *P. radiata*.

The relative expression of four genes, which have exhibited the "priming" effect associated with induced systemic resistance (ISR) in previous publications, was evaluated.

The preventive application of the Cr7 strain of *C. rosea* 8 and 1 days prior to inoculating the pathogenic agent *F. circinatum* effectively reduced the lesion length by 45% compared to the length of plants infected with the pathogen only in assays conducted over 120 days (Cr + Fc treatment 6.6 cm versus Fc treatment 14.6 cm, see Figure 2). This percentage aligns with the findings of Moraga-Suazo et al. (2016) [52], who reported a 48% reduction using the same biological material but over a shorter period of 60 days post-inoculation. The efficacy of *C. rosea* as a biological control agent against various pathogenic fungi has been demonstrated in different pathosystems. For example, it has been effective in controlling *Botrytis cinerea* on the strawberry [56] and tomato [57], *Fusarium culmorum* in wheat [58] and *Plasmodiophora brassicae* in rapeseed [59]. In this last report, the authors suggested that *C. rosea* (as the commercial product Prestop®, Massó agro department, Barcelona, España) induces systemic resistance (ISR) in rapeseed by activating the phenylpropanoid and ET/JA pathways [59].

Defense signaling in plants can be induced by the following tree resistance systems: (i) systemic acquired resistance (SAR), which is activated by pathogenic agents that cause necrosis in tissues, triggering the hypersensitive response (HR; [60]); (ii) systemic induced resistance (SIR), induced by biotic or abiotic elicitors that do not cause an HR [61]; and (iii) induced systemic resistance (ISR), which is triggered by the exposure of roots to specific strains of rhizobacteria and non-pathogenic, growth-inducing fungi in plants [33,37]. SAR is a salicylic acid (SA)-dependent response and is associated with the accumulation of PR proteins. On the other hand, ISR depends on the synthesis of ethylene (ET) and jasmonate (JA) and is independent of SA, not being related to the accumulation of PR proteins [62]. SIR is closely related to ISR and also requires the synthesis of JA, ET, and the activation of systemic protease inhibitors and proteins related to wound response [63]. The role of *C. rosea* in ISR has been previously established in tobacco [26] and in the cucumber [64]. The protective effect noted here also aligns with ISR, as it is induced by pre-treatment with a specific strain of *C. rosea* on the roots of *P. radiata*. Additionally, it is only effective for a resistant genotype of the plant and not for a susceptible genotype of the same species [52] (Moraga-Suazo et al., 2016). We further investigated the expression levels of some genes related to ISR that could play a role in the induced resistance of the R genotype of *P. radiata*.

One of the primary responses to pathogenic infection in conifers, caused by fungi or insects, is the accumulation of oleoresin. This compound is a complex of terpenes, mainly mono- and diterpenes [65]. Monoterpenes contribute to antimicrobial activity and play an important role in restricting pathogenic attacks in conifers [65,66]. One of the metabolic pathways involved in monoterpene biosynthesis is the methylerythritol phosphate (MEP) pathway, which starts with the 1-deoxy-D-xylulose 5-phosphate synthase enzyme (DXS) [48]. Most plants have multiple copies of *DXS*, which can be divided into two categories. Type I (*DXS1*) is expressed constitutively in photosynthetic tissues and is likely involved in substrate supply for primary isoprenoid biosynthesis, while type II (*DXS2*) appears to be involved in substrate supply for terpenoids, particularly implicated in ecological interactions [67]. In this work, we analyzed the relative expression of *DXS1* in *P. radiata* plants. There were no differences between the AT and W controls or Cr treatment. However, a decrease in the Cr + Fc treatment was observed, although it was not statistically different from the W and Cr treatments. The greatest difference in expression levels was observed when comparing Cr + Fc with Fc, with levels 20 times higher for Fc expression. Additionally, Fc treatment doubled the basal expression level compared to the AT or W controls. This suggests that the *DXS1* gene is induced only by the presence of the pathogen, and not by pre-treatment with *C. rosea*. This aligns with studies on *Picea abies*, which show a constitutive expression level for the *PaDXS1* gene, without induction by wounds, fungal elicitors, or defense signaling [68]. It was suggested that type 1 genes participate in primary metabolism, associated with photosynthetic tissues for the biosynthesis of chlorophyll and carotenoids [67]. In contrast, the two *DXS* type II genes in *P. abies* are responsive to several

stimuli previously associated with defense activation and resin production in conifers, including fungal infection, mechanical wounds, chitosan, and treatment with methyl jasmonate (MeJA) [68–70]. In *Pinus densiflora*, there was a reported differential expression of *DXS* genes, with higher transcript accumulation for the *PdDXS2* gene compared to the *PdDXS1* gene in response to wounds and MeJA [71]. Nevertheless, it is well known that *F. circinatum* has the ability to grow on resin ducts of *P. radiata* and use them to colonize and spread throughout the host [1]. Moreover, recent research indicated that *P. radiata* exhibits an inefficient defense activation when confronted with this pathogen, with a low number of perception and defense response genes induced by its presence, in comparison to the much more resistant *P. pinea* [72]. We found a low but significant induction of *DXS1* only with the exposure to *F. circinatum*, indicating a response to the pathogen but at a modest level.

Lipoxygenases (LOXs) are a functionally diverse class of dioxygenases that catalyze the addition of oxygen to polyunsaturated fatty acids to produce hydroperoxides. The hydroperoxidation of linoleic and linolenic acids by *LOX* creates the precursor for jasmonic acid synthesis [47,73], which is a key phytohormone related to multiple physiological processes, abiotic and biotic stress responses, and secondary metabolite accumulation [74,75]. We observed a significant increase in *LOX* induction 24 h after *F. circinatum* inoculation following *C. rosea* pre-treatment. This indicates that this pre-treatment with the specific strain Cr7 induces higher lipoxygenase activity in *P. radiata* plants. This activity was only present when both fungi were present, as each strain alone did not show any significant induction. Similar results have been observed in lettuce plants pre-treated with *Paenibacillus alvei* K165 for the biological control of *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. The combination of K165 with each pathogen induced higher expression levels of *LOX* [76]. On the cucumber, the application of *Trichoderma asperellum* strain T203 reduced necrotic lesion size when confronted with *Pseudomonas syringae*. This upregulated *LOX1* and other genes, modulating an ISR jasmonate/ethylene defensive response [73]. On the peanut, Sailaja et al. (1998) [77] reported a significant *LOX* enzymatic activity induced by the treatment of *Bacillus subtilis* AF1 against *Aspergillus niger* pathogenic attack. *LOX* activity increased the production of two hydroperoxides: 13-hydroperoxy-octadecadienoic acid and 13-hydroperoxy-octadecatrienoic acid. Both have demonstrated in vitro growth activity against *A. niger*.

Studies on olive trees using *Trichoderma harzianum* strain Th97 for the biological control of *Fusarium solani* showed a significant increase in transcript accumulation for *LOX*. However, the expression was lower when Th97 was applied preventively (without the pathogen present) or after a pathogenic attack [78]. The results, along with the upregulation of genes encoding ethylene biosynthesis, provide evidence that *T. harzianum* strain Th97 induces an ISR response in olive trees when exposed to *F. solani*. The previous research suggests an important role for *LOX* as an ISR marker, activating the defense pathway mediated by JA [79,80].

The responses associated with PAL gene expression are related to phenylpropanoid pathway activation. This gene codes for the phenylalanine ammonia-lyase enzyme, which catalyzes the first step in the pathway, thus regulating the synthesis of flavonoids, isoflavonoids, and phenolic compounds, as well as monolignols needed for lignin production [41,81,82]. These products enable anatomical defense activation, such as cell wall lignification, and chemical defenses related to flavonoid and phytoalexin production. Phenolic accumulation is related to both passive and systemic defenses. The intensity of these defenses is associated with the accumulation of phenolic acid precursors [83].

Studies that have evaluated the expression of this gene as a resistance promoter in plants when exposed to elicitors of fungi and pathogens have demonstrated a significant induction in several pathosystems. The application of chitosan induced *PAL* transcription in *P. patula* plants, enhancing the resistance response to *F. circinatum* [80]. On the potato, a fungal inducer derived from the incompatible pathogen *Trichothecium roseum*, called elicitor$_{80}$, enhanced the resistance against *Fusarium sulphureum* by increasing *PAL* transcription and systemically inducing the phenylpropanoid pathway. This effect is only achieved when

both elicitor$_{80}$ and the pathogen *F. sulphureum* are simultaneously applied, not when the elicitor or the pathogen is applied alone [84].

Likewise, Amira et al. (2017) [78] reported a significant induction of *PAL* expression when olive trees were treated with *T. harzianum* strain Th97, with a notable increase when this biocontroller was applied preventively before infection with the pathogen *F. solani*. In cotton plants, the PevD1 protein secreted by the pathogenic fungus *Verticillium dahliae* was utilized as an elicitor to trigger a defense response in the plants, leading to increases in *PAL* transcription and enzymatic activity [85].

In the cucumber, the biocontroller *Trichoderma asperellum* also increased PAL expression when confronted with *P. syringae*. This induction was mediated by the JA/ET pathway during the plant's defense response [73,79].

These responses are not restricted to whole plants; they also occur in cellular vine cultures exposed to filtrates of *Eutypa lata* and *Trichoderma atroviride*, a pathogen and a biological control agent, respectively. Both filtrates acted as elicitors and induced *PAL* expression, with higher transcript levels and, consequently, higher total phenolic content observed for *T. atroviride* [86].

Noteworthy, despite the fundamental role of PAL in plant defense, the gene was not induced in *P. radiata* in the presence of *F. circinatum*, which hindered the reinforcement of cell walls through lignification [72]. In our experiments, only the wound treatment showed a modest increase in transcript levels compared to the absolute control. This could be explained by the fact that PAL has been associated with lignin biosynthesis and wound protection. The low transcript levels of the *PAL* gene found in this study suggest that this particular defensive pathway is not efficiently activated by the *F. circinatum* pathogen in this genotype of the radiata pine, nor is it induced by *C. rosea*.

Pathogenesis-related proteins (PR proteins) are a structurally diverse group of proteins that are strongly induced by pathogenic attack or stress in plants [87]. Among this diverse group, PR3 proteins consist of chitinases, which play a direct role in plant defense by hydrolyzing chitin, thereby inhibiting the growth of hyphae which invade intracellular space. Chitinases are particularly related to ISR responses, stimulating JA/ET pathways [73,79]. Several investigations have studied the expression of *PR3* with various fungal elicitors. For instance, on cellular cultures of vines, elicitors derived from *T. atroviride* (a biocontrol agent) and *E. lata* (a pathogenic fungus) increased *PR3* transcripts. However, the highest transcript levels and chitinase activity were reported for treatment with *T. atroviride* elicitors [86]. Cucumber plants treated with the *T. asperellum* T203 strain and inoculated with the *P. syringae* pathogen doubled their transcript levels for the *Chit1* gene (a class III chitinase) on leaves compared to plants infected with the pathogen alone, demonstrating an increase in chitinase levels [73]. In the case of carrot plants, treatment with chitosan and alexin as elicitor compounds enhanced plant resistance to *Alternaria radicina*, increasing transcript levels and chitinase activity [88]. Class IV chitinases are also encoded by *PR3* genes, which are localized in the apoplast [89,90] and are believed to be part of the early defense response in plants, acting directly on the hyphae of invading fungi [91]. The action of these chitinases on the fungi releases elicitors that diffuse to plant cells and induce the expression of other defense genes [92]. On olive trees, *T. harzianum* strain Th97 has been used as a biocontrol agent for *F. solani*, resulting in a significant increase in transcripts of the *CHI* gene (a class I chitinase, *PR3*) in plants that were pre-treated with the *T. harzianum* strain. Furthermore, there was an even higher expression level observed when both the biocontroller and the *F. solani* pathogen were present [78]. Other fungi and bacteria reported to have activity as ISR stimulating agents include *Azospirillum* sp. B510 on rice [93], actinobacteria on *Arabidopsis* [35], *Burkholderia phytofirmans* PsJN on grapevine [94], *F. oxysporum* S162 on the tomato [95], and *Trichoderma harzianum* on *Pinus radiata* [96]. The expression patterns of chitinases and peroxidases have been studied in wheat seeds treated with *C. rosea* as a preventive measure before inoculation with *Fusarium culmorum*. One study demonstrated both the induction of resistance against the pathogen and a growth promotion effect in the plants, providing an additional benefit [58].

We observed a significant increase in *PR3* transcript levels when *C. rosea* strain Cr7 is present, a phenomenon that is consistent with an ISR response. The biological controller primes the plant to react more effectively when attacked by a pathogenic agent. When both the biocontroller and *F. circinatum* pathogen are present (Cr + Fc), the increase in *PR3* transcripts is even higher, which aligns with a more efficient plant response to the pathogen's aggression. Zamora-Ballesteros (2021) [72] noted that several *PR3* genes were upregulated in *P. radiata* and *P. pinea* when exposed to *F. circinatum* infection, emphasizing the importance of these genes for defense. The same phenomenon has been observed in the plant *Trichoderma* spp.'s pathosystem, where *Trichoderma* acts as a priming agent on the plant, enabling a quicker and more robust response when a pathogen attacks, resulting in increased resistance [73,79].

Our observations reveal that the *C. rosea* Cr7 strain reduced the symptoms of the disease caused by *F. circinatum* on *P. radiata*. Additionally, the application of this *C. rosea* strain significantly increased the expression of *LOX* and *PR3* genes, indicating an induced systemic resistance response in the plants pre-treated with this biocontrol agent.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f15050854/s1, Figure S1: *Clonostachys rosea* (left) and *Fusarium circinatum* (right) growing on potato-dextrose agar (PDA) petri dishes.

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