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Induction of PR proteins and resistance by the biocontrol agent *Clonostachys rosea* in wheat plants infected with *Fusarium culmorum*

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ABSTRACT

Clonostachys rosea (CR) is a common worldwide saprophyte with destructive effect against several plant pathogenic fungi showing antagonistic features against a wide variety of pathogens. We recently isolated a strain of *C. rosea*, named CR47, from wheat crown infected with *Fusarium culmorum* (FC); this strain proved to be effective against *Fusarium* seed borne diseases of cereals under field condition. In this paper the function of *C. rosea* applied as seed treatment on wheat seedling growth was investigated. In addition, we investigated the expression pattern of peroxidases and chitinases as well as PR4 proteins following both CR treatments of seeds and FC infection and also in the three-component system pathogen–antagonist–wheat. Several chitinase isoforms were induced by CR-treatment both in coleoptiles and roots, whereas some peroxidase isoforms were induced only in the presence of both antagonist and pathogen. In the latter case, it seems that CR-treatment by itself promotes plant growth and reduces the peroxidase expression, while enhances some chitinase isoforms probably involved in cell wall disruption. Moreover, both the antagonist and the pathogen studied induced PR4 protein expression, which probably exerts its role on the invading microorganisms by a translation-inhibitory process that could be ascribed to their ribonuclease activity.

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1. Introduction

Plant diseases play a direct role in the destruction of natural resources in agriculture. Not only growing crops but also stored fruits are prey to fungal infections, soil borne pathogens being the most aggressive [1]. The use of microorganisms that antagonize plant pathogens is risk-free when it results in enhancement of resident antagonists. Moreover, the combination of such biological control agents (BCAs) with reduced levels of fungicide (integrated control) promotes a degree of disease suppression similar to that achieved with full fungicide treatment [2].

In the past few years, *Trichoderma* spp., the most common saprophytic fungi in the rhizosphere, has received considerable attention as potential biocontrol agent for a number of soil borne pathogens [3]. The mechanisms by which *Trichoderma* isolates control pathogenic populations in the rhizosphere have been extensively studied. In most cases, the antagonistic process relies on the production of antibiotics [4] and/or hydrolytic enzymes [5] associated with possible competition for nutrients in the rhizosphere [6].

In the last years, several *Clonostachys rosea* (syn *Gliocladium roseum*; teleomorph *Bionectria ochroleuca*) strains proved to be effective bioagents against either soil borne or seed borne pathogens [7]. The fungus *C. rosea* (CR) is a common worldwide saprophyte with destructive effect against several plant pathogenic fungi [8]. In fact, a growing number of reports demonstrate that CR strains have antagonistic features against a wide variety of phytopathogenic fungi [8–10].

The modes of action of *C. rosea* as BCA are less understood than that of *Trichoderma* spp.; nevertheless mycoparasitism, substrate competition, enzymatic activity and induced resistance are thought to play a significant role [8,11,12]. Moreover, production of secondary metabolites may also exert an antibiotic effect as known from other biocontrol fungi [10,13].

C. rosea has been found in association with many different parts of living organisms as well as dead plant material indicating a high versatile ecological fitness [8]. A study conducted by Lubeck et al.

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Abbreviations: BCA, biocontrol agent; CR, *Clonostachys rosea*; DAT, days after treatment; FC, *Fusarium culmorum*; PR, pathogenesis related.

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[14] demonstrated that *C. rosea* IK726 can be transformed with the GUS and GFP marker genes and used as a tool for monitoring the strain in ecological studies. A formulation of this strain has also been developed [15]; therefore, its commercial potential seems very promising.

Our team has isolated a strain of *C. rosea*, named CR47, from wheat crown infected with *Fusarium culmorum* and it has been evaluated in an *in vivo* screening program [16]. The strain has proven effectiveness against *Fusarium* seed borne diseases of cereals under field condition [9] and showed to be a possible partner for pesticides in integrated disease management of wheat. In fact, CR47 was compatible with the fungicides carboxin, thiram, triticonazole and guazatine that are active ingredients of commercial products used for wheat seed treatments to control foot rot disease [17]. It was also compatible with several herbicides, such as flufenacet, chlorotoluron, chlorosulfuron and pendimethalin, which are commonly applied in Italian wheat cultivation at the pre-emergence stage [17]. Further investigations indicated that this strain was rhizosphere-competent being able to colonize wheat roots [13] and it was able to parasitize the mycelium of different species of *Fusarium* responsible of wheat foot rot disease [11] by producing cell wall lytic enzymes [12].

Moreover, it has been shown that *C. rosea* can control disease caused by *F. culmorum* as effectively as seed treatment with a recommended fungicide [15]. This latter concept, along with the recent demonstration that infection with beneficial fungi causes host plants to respond more rapidly and efficiently to pathogen attack [18], raises the question whether nonpathogenic fungi, such as *C. rosea*, stimulate the plant defense response, leading to the activation of genes and ultimately to the accumulation of defense molecules.

Among these are pathogenesis related (PR) proteins coded by the host plant that accumulate in response to pathogen infection or other signals related to plant defense responses [19]. Several PR proteins have been characterised at molecular level and shown to have antifungal activity *in vitro* [19]. Several PR proteins show enzymatic activity such as β-1,3-glucanase and chitinase (PR2 and PR3, respectively), both involved in the degradation of microbial cell wall structural polysaccharides [20,21] and PR4 and PR9, characterised by ribonuclease and peroxidase activity, respectively [22,23]. No enzymatic activity has been found up to now for proteins belonging to PR1 and PR5 families, nevertheless several genes belonging to these classes have been over expressed in transgenic plants strengthening the defensive role proposed for the corresponding proteins [24].

The aim of this research was to study the role of the biocontrol agent *C. rosea* applied as seed treatment on wheat seedling growth and on resistance induction against *F. culmorum* inoculation. The present study was also aimed at understanding the defense related response by comparing the expression pattern of selected defense related mRNAs and the induction of the corresponding proteins after infection with *F. culmorum*.

2. Materials and methods

2.1. Microbial cultures

The antagonist *C. rosea* strain 47 was isolated from wheat crown and the pathogen *F. culmorum* strain 17 from durum wheat seed. They were identified by CBS (Baarn, The Netherlands) and the code numbers refer to the position in the collection of the Dipartimento Protezione Valorizzazione Agroalimentare, University of Bologna, Italy.

The stock cultures obtained were stored at −80 °C in 2% potato dextrose broth (PDB, Difco, Sparks, MD, USA) amended with 15% glycerol (Sigma–Aldrich Co., St. Louis, MO, USA). The fungi were subcultured on 45 g L^{-1} of potato dextrose agar (PDA, Difco) and grown for 15 days at 25 °C for CR47 and 7 days at 21 °C for FC17 in the dark, then for further 4 days under 12 h cycles of fluorescent lamps and darkness to allow conidia production.

2.2. Fungal treatments and infection

2.2.1. C. rosea treatment

Wheat seeds (*Triticum durum* cv. Kronos) were surface-disinfected with 1% sodium hypochlorite solution for 5 min, rinsed in distilled sterile water and dried on sterile filter paper in a hood. Half sterilised seeds were treated with CR conidia suspended in water (1,000,000 spores ml^{-1}) for 30 min, whereas seeds soaked in distilled water for the same period of time were used as control. Both CR-treated and untreated seeds were sown in sterile sand in a tray and allowed to grow for different period of time at 25 °C. CR-treated and control seedlings were harvested 7, 8, 9 and 10 days after treatment (DAT) and ground to fine powder in a prechilled mortar in liquid nitrogen for protein extraction. Several seedlings from each tray were sampled and the length of leaves and roots, the thickness of seedling basal portions and the plant dry weight were measured. The experiment was carried out three times independently in the same conditions.

2.2.2. F. culmorum inoculation

F. culmorum inoculations were carried out on both CR-treated and control seeds. Before infection the samples were separately sown on wet sterilised paper and grown in the dark at 24 ± 1 °C. Roots of 6-day-old seedlings were covered with sterile sand and both CR-treated and untreated young plants were inoculated with FC. Inoculations were carried out by placing PDA blocks (3–4 mm) containing FC mycelium and conidia on each coleoptile, 1–2 cm above the sand surface. Sterile PDA blocks were also placed on coleoptiles germinated from untreated seedlings and used as control of pathogen infection. Inoculated, uninoculated and control seedlings were harvested at 7, 8, 9 and 10 days after inoculation and coleoptiles and roots were excised, separately weighted, rapidly frozen in liquid nitrogen and stored at −80 °C until further use. The experiment was carried out three times independently in the same conditions.

2.2.3. Assessment of disease

The disease caused by *F. culmorum* was evaluated on seedlings by examining the necrosis in the area of the inoculation 1, 2, 3 and 4 days after infection. The area below the PDA plug without the pathogen was also evaluated in order to check any browning of the healthy tissues. Moreover, samples of 10-day-old seedling either from CR-treated or FC infected as well as from FC infected after CR-treatment of seeds and control tissues were plated on PDA medium supplemented with 0.3 mg ml^{-1} of streptomycin sulphate (Sigma–Aldrich Co.) and incubated for 7 days at 21 °C in the dark, then for further 4 days under 12 h cycles of fluorescent lamps and darkness to allow conidia production. Samples were examined for the presence or absence of *F. culmorum* raised from wheat tissues by a light microscope (Carl Zeiss mod. ZM, Germany) at 500 magnification.

2.3. Protein extraction

Coleoptiles and roots harvested at different time intervals from CR-treated (T), FC-infected (I), CR-treated and FC-infected (T + I) and control (Co) tissues were frozen in liquid nitrogen and ground to fine powder using prechilled mortars. Two different extraction buffers were used depending on the following analysis. For peroxidase and chitinase activity as well as antifungal activity detection chilled 20 mM sodium acetate buffer (pH 5.5) containing

1% (w/v) polyvinylpolypyrrolidone (Sigma–Aldrich Co.) (Buffer 1) was added to the powder (1 ml g^{-1} fresh weight). Extractions were carried out at 4 °C for 60 min with continuous gentle stirring. Crude extracts were then centrifuged twice at 12,000 rpm for 20 min at 4 °C and the supernatant was filtered with GV Millex® Syringe Filter Unit (Millipore Corporation, USA) to remove particles, concentrated and desalted with Ultrafree®-4 Centrifugal Filter Unit (Millipore Corporation, USA).

For western blotting analysis chilled 50 mM Tris buffer (pH 7.4), containing 1% polyvinylpolypyrrolidone (w/v) (Buffer 2) was added to the powder (1 ml g^{-1} fresh tissue). Crude extracts were then centrifuged twice at 18,000 rpm for 30 min at 4 °C.

Protein concentration was determined by the protein–dye binding method of Bradford, using BSA as standard [25].

2.4. Antifungal activity

FC conidia were gently scraped from an 11-day-old culture grown on PDA, filtered through cheesecloth to remove hyphae and suspended in 2% malt (v/v) to give a final concentration of 10,000 conidia ml^{-1}. Antifungal activity was performed using assay mixtures containing 20 μl of conidia suspension and 20 μl of protein solution. Protein samples (3.5 mg ml^{-1}) from CR-treated, FC-infected, CR-treated and FC-infected as well as untreated tissues harvested at different time intervals and dissolved in Buffer 1 were used. The same volume of FC conidia diluted in 20 μl of Buffer 1 represented the control. Each assay mixture was placed onto sterilised glass microscope slides in a Petri dish and incubated at room temperature at 100% relative humidity for 20 h to allow germination of conidia. Germinated conidia were counted with a haemocytometer and observed under a light microscope (Leitz mod. Diaplan, Germany) at 125 magnification. The hyphal length of FC germinated conidia was determined using the grid-line intersect method [26]. The experiment has been performed twice with five replicates of 20 conidia each.

2.5. Detection of peroxidases

Peroxidase activity was assayed by measuring at 30 °C the absorbance increase at 470 nm due to the formation of tetraguaiacol from 0.46% (v/v) guaiacol and 13 mM H_2O_2 in 50 mM Tris buffer (pH 7.4). Crude protein extracts (2 mg total protein) from the samples harvested at different times from each treatment were added to 1.5 ml (final volume) of the reaction mixture. Peroxidase activity was calculated using an absorption coefficient for the tetraguaiacol of 26.6 $mM^{-1} cm^{-1}$ at 470 nm.

Peroxidase isoforms were detected in crude protein extracts from samples harvested at different times from each treatment using IEF on precasting gel T 5%/C 3% (CleanGel IEF, Amersham Biosciences) in the pH range 4–8 using a Multiphor II electrophoresis unit (Amersham Biosciences, Europe GmbH). Isoelectric focusing was carried out following the recommended running condition for CleanGel IEF. 5 μg of total protein were loaded in 10 μl final volume. After IEF the gels were soaked for 10 min in 0.1 M sodium phosphate buffer (pH 5.4) then immersed in 40 mM guaiacol (Sigma–Aldrich Co.) in the above buffer containing 5 mM H_2O_2 at room temperature until red bands appeared and subsequently fixed in water/methanol/acetic acid (6.5/2.5/1, v/v). The p*I* markers (Sigma–Aldrich Co.), ranging from p*I* 3.6 to 9.3, were used to estimate the p*I*s of the isoforms.

2.6. Detection of chitinases

Chitinase activity was performed on the same protein extracts as above. Chitinase assay was based on colorimetric determination of *p*-nitrophenyl cleaved from the chitin-analogous substrate, *p*-nitrophenyl-β-D-*N*,*N*-diacetylchitobiose (PNP). 10 μl of the substrate stock solution (2 mg ml^{-1}) were dissolved in 50 mM acetate buffer (pH 5.0) and added to the crude protein extracts (50 μg total protein) from each sample in a total volume of 0.5 ml. After an incubation for 2 h in a water bath at 37 °C, the reaction was stopped by adding 0.5 ml of 0.2 M Na_2Ca_3. Chitinase activity was calculated using an absorption coefficient for the *p*-nitrophenyl of 7000 $mM^{-1} cm^{-1}$ at 410 nm.

Chitinase isoforms were detected in overlaying gel containing glycol chitin [27]. IEF was performed as above using the same protein samples. After isoelectric focusing, gels were equilibrated in 0.1 M sodium acetate buffer (pH 5.2) in reciprocal shaker for 10 min, then covered with 7.5% polyacrylamide–chitin overlay gels containing 0.04 glycol chitin in 0.1 M sodium acetate buffer (pH 5.2); the sandwich gels were incubated at 40 °C for 2 h in a glass container under moist conditions. After incubation, overlay gels were separated from the sandwich gels and treated with 0.01% Calcofluor White M2R (Sigma–Aldrich Co.) in 0.5 M Tris (pH 8.8) at room temperature for 10 min. Chitinase isoforms were detected as dark bands against the fluorescent background excited by UV exposition (365 nm) of intact glycol chitin [26].

2.7. Statistical analysis

Symptoms data (referred to Table 1) and morphological characteristics (referred to Fig. 1) were analysed by *t*-test. Antifungal activity (see Table 2) as well as chitinase and peroxidase activity (Figs. 2 and 3) were statistically analysed by multifactor analysis of variance, software Statgraphics Plus 2.1. In the case of significant interactions, the means were compared by the standard error of the means (S.E.M.) at $P < 0.05$.

2.8. DNA isolation and Southern analysis

Genomic DNA was isolated from wheat leaves following the procedure described by Dellaporta et al. [28]. Five aliquots of 30 μg

Table 1
Effect of *F. culmorum* infection and CR-treatment either alone or in combination on coleoptile symptoms

	Days from *F. culmorum* inoculation							
	1		2		3		4	
	NA (mm)	DP (%)	NA (mm)	DP (%)	NA (mm)	DP (%)	NA (mm)	DP (%)
I	1.1 ± 0.20	75.2 ± 1,04	3.3 ± 0,41	80.5 ± 1.54	8.2 ± 0.51	81.6 ± 0.83	13.4 ± 1.24	80.5 ± 1.06
T + I	1.0 ± 0.44	10.4 ± 0.60***	1.4 ± 1.03**	60.7 ± 1.46***	1.5 ± 0.94***	62.3 ± 1.74***	1.1 ± 2.16***	70.2 ± 2.54***

I: *F. culmorum* inoculated coleoptile; T + I: CR-treated seeds and FC inoculated coleoptile; 1, 2, 3 and 4 days of inoculation correspond to 7, 8, 9 and 10 days after CR-treatment; NA: means of necrotic area below PDA plug with *F. culmorum*; DP: percentage of diseased plants, i.e. plants showing the necrotic area with respect to the total of examined plants; data are means ± standard deviation value (*N* = 5).

** $P < 0.01$.

*** $P < 0.001$ versus I according to Student's *t*-test.

Table 2
Hyphal length of *F. culmorum* in the presence of wheat protein extracts

Treatments	Coleoptile extracts (μm conidia^{-1})	Root extracts (μm conidia^{-1})
Co	2826.0	2826.0
nI/nT	1340.8	631.1
I	910.6	455.3
T	650.0	395.6
T + I	474.1	128.7

Conidia were germinated in malt (1%) in the absence of proteic extracts (Co), in protein extracts of coleoptiles and roots germinated from untreated (nI/nT), *F. culmorum* inoculated (I), *C. rosea* treated (T) and FC inoculated after CR-treatment (T + I) wheat seeds. Multifactor analysis of variance: significant interaction ($P < 0.05$) between treatments × coleoptile extracts and root extracts. S.E.M. (standard error of the interaction means): 90.54.

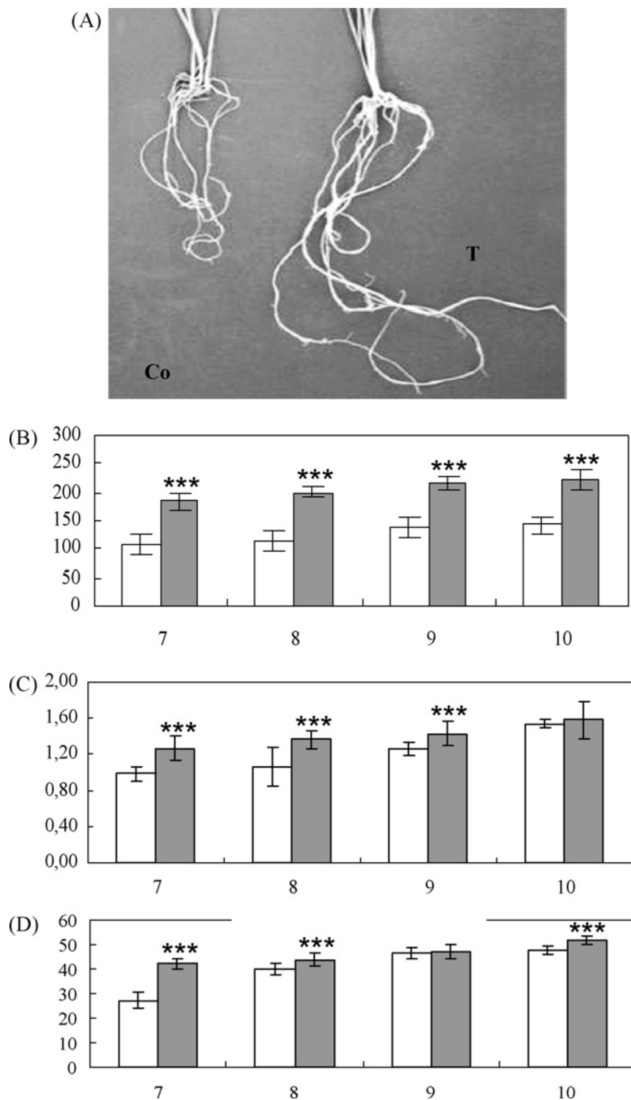

Fig. 1. Effects of *Clonostachys rosea* seed treatment on morphological characteristics of wheat seedlings. (A) *In vivo* effect of CR-treatment on wheat seeds: non-treated control (Co) and CR-treated sample (T); (B) root length; (C) thickness of seedling basal portions; (D) plant dry weight. In B–D white columns represent control tissues whereas grey columns are relative to CR-treated samples. Each bar represents the standard deviation. Data are means ± standard deviation value ($N = 5$). ***$P < 0.001$ versus control according to Student's *t*-test.

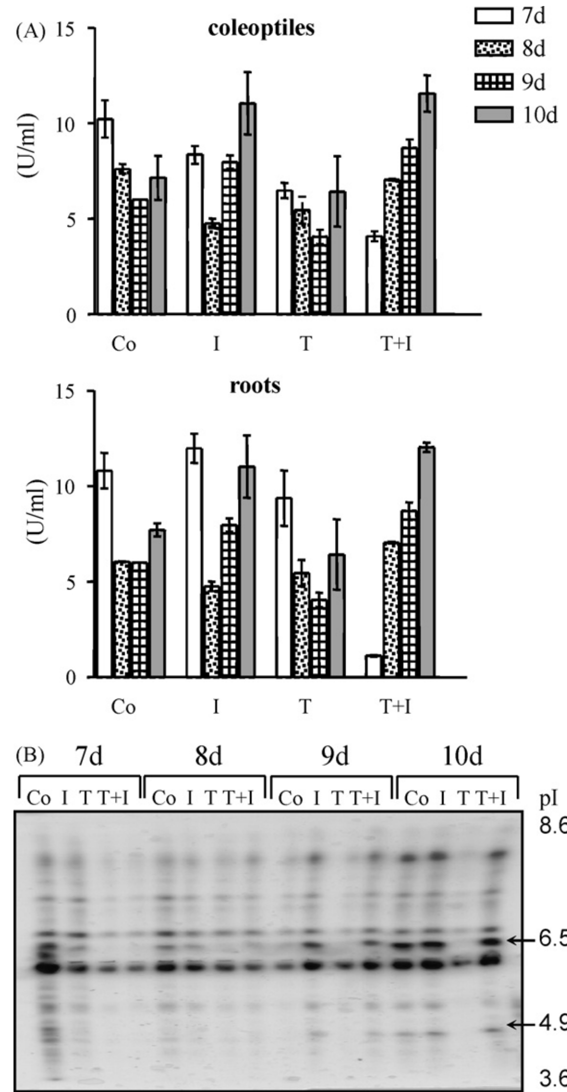

Fig. 2. Time course expression of wheat peroxidase in wheat coleoptiles and roots. Panel A: peroxidase activity of wheat coleoptiles (upper panel) and roots (bottom panel) in response to *F. culmorum* infection (I), CR-treatment (T), and *F. culmorum* infection on CR-treated seeds (T + I) detected 7, 8, 9 and 10 days after each treatment. Co: control seeds. Bars represent the standard deviation. Multifactor analysis of variance: significant interaction ($P < 0.05$) treatments × sampling time (S.E.M.: 0.82 for coleoptiles, 0.86 for roots). Panel B: peroxidase isoforms detection by IEF analysis of wheat coleoptiles as above after staining with guaiacol within the pH range 4–8. IEF markers (p*I*) are indicated.

genomic DNA were digested with restriction nucleases *Eco*RI, *Hind*III, *Bam*HI, *Nde*I and *Xho*I at 37 °C for 8 h in a final volume of 150 µl. DNA fragments were size separated on a 0.8% agarose gel and blotted on a nylon Hybond^{TM}-N membrane (Amersham Pharmacia Biotech, Uppsala, Sweden) as described by Sambrook et al. [29]. The UV cross-linked membrane was prehybridized (for 2 h) and hybridized (18 h) at 65 °C in 6× SSC, containing 5× Denhardt's reagent, 0.1 mg ml^{-1} salmon sperm and 0.5% (w/v) SDS. The *wPR4b* cDNA (EMBL accession no. AJ006099), encoding mature wheatwin2 [30], was labeled with ^{32}P-dCTP by random priming (Prime-it II kit, Stratagene) and used as a probe. After hybridization, membranes were washed twice in 2× SSC containing 0.5% (w/v) SDS for 20 min at 65 °C and twice in 0.5× SSC containing 0.1% (w/v) SDS at 65 °C for 20 min and then exposed to Kodak BioMax MS film with intensifying screens (Sigma–Aldrich Co.) at −80 °C.

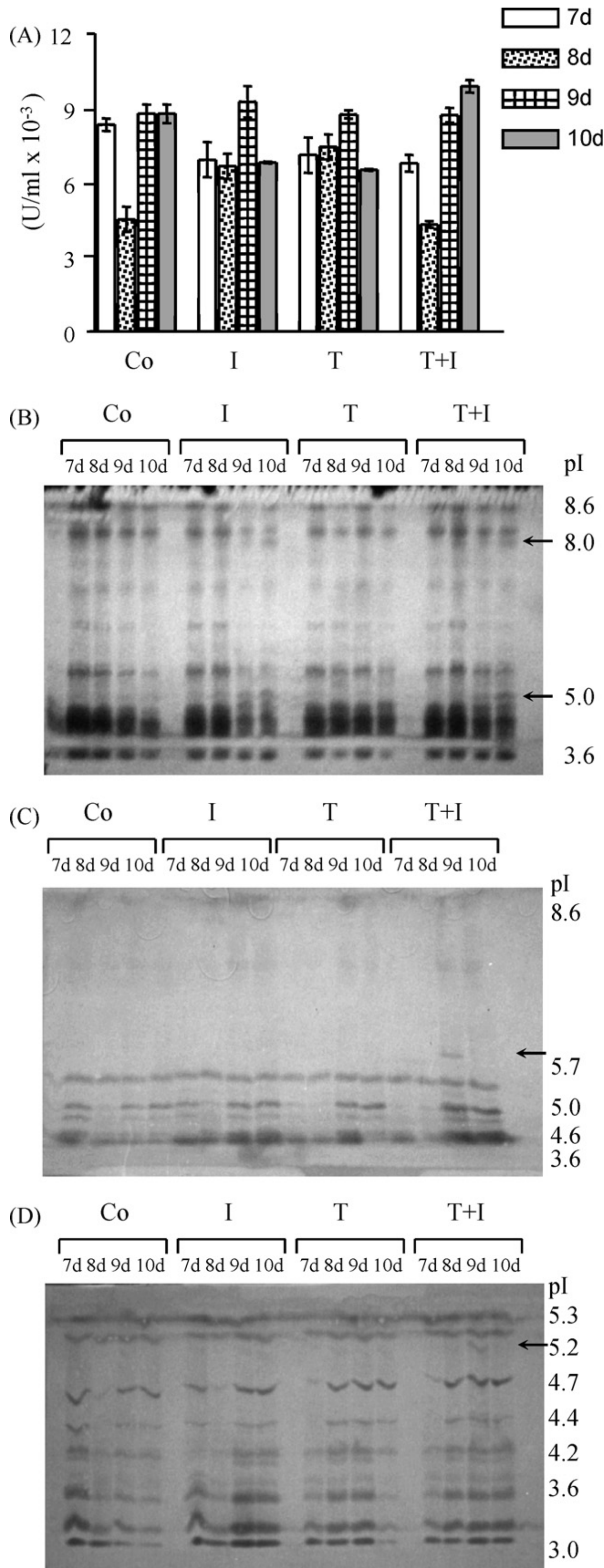

Fig. 3. Time course expression of wheat chitinases in wheat coleoptiles (B) and roots (A, C and D). Panel A: chitinase activity in response to *F. culmorum* infection (I), CR-treatment (T), and *F. culmorum* infection on CR-treated seeds (T + I) detected 7, 8, 9 and 10 days after each treatment. Co: control seeds. Bars represent the standard deviation. Multifactor analysis of variance: significant interaction ($P < 0.05$) treatments × sampling time (S.E.M.: 4.21×10^{-4}). Panels B–D: chitinase isoforms detection by IEF analysis of coleoptiles (B) and roots (C and D) harvested from the same samples after staining with guaiacol within the pH range 4–8 (B and C) and 3–5 (D). IEF markers (p*I*) are indicated.

2.9. RNA extraction and RT-PCR analyses

Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the recommended extraction conditions. RT-PCR was carried out in a final volume of 50 μl using the ready to go RT-PCR beads (Amersham Pharmacia Biotech, Uppsala, Sweden) mixed with 1 μg of total RNA and appropriate amounts of primers for PR genes amplification, following manufacturer's instructions. Specific primers for PR4 gene amplification were designed on the basis of the known sequences of wheatwin1 [31]. Forward PR4 primer was 5′-GCGACGTACCACTACTACCGGCCGGCG-CAGAAC-3′; reverse PR4 primer was 5′-GTGAAGACGGTGTCC-CAGTC-3′. PR1 specific primers were designed on the basis of the known sequence of the basic wheat PR1.1 [32]. Forward PR1 primer was 5′-GGACTACGACTACGGGTCCA-3′ whereas reverse PR1 primer was 5′-TGGTTTCTGTCCAACGACAT-3′. Heterologous primers for wheat PR5 gene amplification were designed on the coding region of a barley PR5 protein (EMBL, accession no. AJ001268). Forward PR5 primer was 5′-ATCACCAAGGATTGCCT-CAA-3′ and 5′-GTGAAGGTGCTGGTCTGGTC-3′ was the reverse PR5 primer. As an internal control of RNA amount, each RT-PCR experiment was also performed using specific oligonucleotides of a barley actin gene (EMBL accession no. AJ234400). Forward primer was 5′-CTCGCATATGTGGCTCTTGA-3′ and reverse primer was 5′-AGACCCAGACAACTCGCAAC-3′. All primers have been synthesised by Genenco Life Science (Italia) and used at 0.5 μM final concentration. RT-PCR was carried out with the following program: incubation at 42 °C for 30 min to allow the synthesis of cDNA, denaturation at 95 °C for 5 min, amplification for 30 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for PR4 and actin gene amplification and 58 °C for PR1.1 and PR5 gene amplification for 1 min, extension at 72 °C for 1 min. The amplified DNA fragments were separated in 1.5% agarose gels including ethidium bromide (0.4 mg ml^{-1}).

2.10. Western blotting

Clear supernatants from protein extracts prepared as above described (Section 2.3) were used for SDS-PAGE and immunoblotting following the procedure of Laemmli [33] and Towbin et al. [34], respectively. Polyclonal antibody was raised against wheatwin1 and goat anti rabbit-horse radish peroxidase (Cappel, Germany) was used as secondary antibody. 4-Chloro-1-naphthol (Sigma–Aldrich Co.) was used to detect positive bands.

3. Results and discussion

3.1. Beneficial effect of C. rosea

To detect the beneficial effect of CR on wheat seedlings the length of leaves and roots, the thickness of seedlings basal portion and the plant dry weight were measured both in CR-treated and untreated seedlings, 7, 8, 9 and 10 DAT. As shown in Fig. 1A and B, CR-treatment caused a strong root growth stimulation at all times whereas a less pronounced increase of the thickness of seedling basal portions was observed especially in the early stages of coleoptile development (Fig. 1C). Moreover, an increase of seedling dry weight was evidenced particularly at early stages of growth (Fig. 1D), but no effect on young leaves was evidenced (data not shown).

The strong stimulation of root growth, associated with the previous observation of the rhizosphere competence of CR [13], can produce positive effects on plants, as asserted by Harman et al. [35] which correlated a better root development, caused by beneficial microorganisms like *Trichoderma harzianum* strain T-22, with improved productivity of several plants and increased drought tolerance of maize [37]. In many cases, although in different experimental conditions, these responses are the result of direct effects on plants as decreased activity of deleterious root micro flora and inactivation of toxic compounds in the root zone. Furthermore, the beneficial fungi can increase nutrient uptake and the efficiency of nitrogen use and can solubilize nutrients in the soil.

3.2. Assessment of disease

F. culmorum, a widespread soil inhabiting species, is one of the most frequently isolated fungi from wheat kernels and the inoculum source is consequently seed transmitted. Infection of wheat seeds with *F. culmorum* spores results in a reduction of the germination capability of inoculated germlings respect to the control. Moreover, fungal colonization and symptom appearance are restricted to hypocotyl stems leaving unchanged root development. In fact, the infection of young seedlings produced an increase of necrotic lesions together with an increase of necrotic extension in infected plants (I), as shown in Table 1. As can be noted, the necrotic area (NA) of infected plants increases substantially during sampling interval, starting from about 1 mm at day 1 reaches about 14 mm at day 4. In parallel, the percentage of diseased plants (DP) was almost unchanged during the same sampling time. In order to verify the protective role of the biocontrol agent *C. rosea*, wheat seeds were treated with the antagonistic fungus before *F. culmorum* infection (T + I). In this case, on day 1 the necrotic areas have the same size in I and T + I plants but they are considerably lower in T + I plants with respect to I plants in the following days. In addition, there are much less diseased T + I plants (DP) according to the protective role of the antagonist. These results are in agreement with the previously reported protection of wheat plantlet by *C. rosea* [13] and are related to the plant defence reaction triggered upon CR-treatment. As expected, no necrotic lesions were present in CR-treated samples (T) as well as in control samples (nI/nT) (data not shown).

3.3. Antifungal activity

The antifungal activity of crude protein extracts either from wheat coleoptiles or roots were determined on *F. culmorum* growth. Protein extracts from both coleoptiles and roots from FC inoculated seeds (I), *C. rosea* treated seeds (T) and FC inoculated seeds after CR-treatment (T + I) were tested for their antifungal activity against *F. culmorum* conidia germination. Protein extracts from untreated healthy plants (nT/nI) were also used as a control. In the latter case, protein extracts from control plants proved to be ineffective towards the percentage of conidia germination (data not shown). On the contrary, conidia germination was significantly reduced with respect to the controls when using protein extracts from either coleoptiles or roots after all treatments (data not shown). As can be observed in Table 2 all protein extracts from control (nInT) plants and from treated plants (I, T and T + I) are effective in inhibiting hyphal growth of the pathogen. In fact, hyphal length of *F. culmorum* is significantly inhibited as compared to the control (Co) represented by *F. culmorum* grown in the absence of any protein extract. Moreover, protein extracts from both coleoptiles and roots from FC inoculated seeds (I), *C. rosea* treated seeds (T) and FC inoculated seeds after CR-treatment (T + I) affected the morphology of conidia, the germ tube elongation, the hyphal branching and caused, in some cases, the hyphal growth arrest (data not shown). Furthermore, all root protein extracts from treated and untreated seeds were significantly more effective ($P < 0.05$) than the corresponding coleoptile extracts. As can be noted in Table 2, protein extracts both from coleoptile and root in T + I were the more effective in hyphal length inhibition. However, it is worthwhile to notice that all plant extract are antifungal, regardless to prior treatment with BCA.

Microscopic observations showed that germ tubes and hyphae arose both from apical and central cells, while they emerged from tip cells in the control (data not shown). Moreover, root extracts from T and T + I produced hyphae with shorter and thicker cells and a clear branching with respect to the corresponding coleoptile extracts and they caused the growth arrest of hyphae, as determined after further 20 h of incubation (data not shown).

3.4. Peroxidase activity

It is well known from the literature that peroxidases are commonly expressed during plant–fungus interaction. In this paper we determined the total peroxidase activity of wheat coleoptiles and roots germinated from FC-infected (I) and CR-treated (T) seeds as well as from FC-infected after CR-treatment of seeds (T + I) and control tissues (Co). The enzymatic activities were performed on either coleoptiles (Fig. 2, upper panel A) or roots (Fig. 2, bottom panel A) on tissues harvested 7, 8, 9 and 10 days after each treatment. Peroxidase isoforms were also determined by isoelectric focusing on protein extracted from the same treated and untreated wheat coleoptiles (Fig. 2, panel B).

General decrease of peroxidase activity during sampling interval followed by an increase was evidenced in either coleoptiles or roots. Nevertheless, the combination of FC infection following CR-treatment (T + I) resulted in a strong significantly inhibition ($P < 0.05$) in the first sampling time. A large increase of activity in all sampling interval in both tissues in T + I was observed, thus revealing a positive interaction between the pathogen and the antagonist in plant tissues ($P < 0.05$). These experiments were performed four times independently obtaining similar results.

The different interaction of the two microorganisms (FC or CR) with plants suggests the involvement of different molecular recognition between both partners. *F. culmorum* is a severe pathogen of either seedlings or heads and it establishes the disease through several metabolic changes involving the synthesis and accumulation of PR proteins. *C. rosea* is a beneficial microorganism and its positive interaction with plant may involve a molecular recognition system like the well-reported interaction of *Trichoderma* species with plant (promoting plant growth) or the symbiotic mycorrhizal association. Peroxidase proteins may be rapidly involved in the peroxidation of substrate molecules, leading to the accumulation of highly toxic compounds (i.e. phenolic compounds). These compounds may contribute to plant resistance via their antifungal potential, but they can be toxic to the plant itself and it is assumed that plants activate a mechanism to repress peroxidase expression during the resistance process in order to maintain phenolic compounds below phytotoxic levels. *C. rosea* treatment, differently from *Trichoderma* and symbiotic mycorrhiza, represses the expression of peroxidases during seedling development, probably because it activates faster pathways to enhance root growth rather than to synthesize peroxidases that are not useful in the absence of a pathogen attack. In fact, Vallad and Goodman [36], reviewing several article on induced resistance, reported that plants meet physiological costs when deploying these defences which are often associated with reduced plant productivity when chemical inducers, like BTH, are

employed. This suggests that when wheat seedlings are stimulated by CR-treatment, they use their energy resources to promote root growth rather than other physiological process. On the contrary, upon FC inoculation following CR-treatment, plant metabolic changes result in the increase of PR proteins expression that leads to useful toxic compounds that can exert an active role against the invading pathogen. As with immunization, CR-treated plants may be sensitized to respond faster and to a greater extent to the pathogen attack.

As shown in Fig. 2B, several peroxidase isoforms can be evidenced from p*I* 3.6 and 9.3 in each coleoptile sample tested. A number of basic and acidic isoforms were constitutively expressed in protein extracts from untreated (Co), CR-treated (T), FC-inoculated (I) and FC-infected after CR-treatment (T + I) coleoptiles. As highlighted for the total peroxidase activity above described, CR-treatment brings about a repression of the expression of several isoforms; in particular, two isoforms with p*I* 4.9 and 6.5 (indicated by arrows in Fig. 2B) were strongly repressed by CR-treatment at all time. No significative differences were detected among isoforms from root protein extracts analysed 7, 8, 9 and 10 days from treatment (data not shown).

3.5. Chitinase activity

Exochitinase activity was detected in protein extracts from wheat roots analysed 7, 8, 9 and 10 days from the same treatments as above (Fig. 3, panel A). Chitinase activity in control tissues (Co) was mainly constant during sampling interval apart from the activity detected in 8-day-old wheat roots. Following *F. culmorum* infection (I) chitinase activity was lower at the first sampling interval with respect to the control but increased in the next two days and decreased in samples tested 10 DAT. Similar results were obtained in sample treated with the antagonist. Although the variations of chitinase activity were statistically significant in both experiments it is hard to relate this behaviour with respect to the treatment. However, what is more interesting is that the chitinase activity of the combined effect (T + I) is very similar to the one of the control. The latter results suggest that the presence of the antagonist helps the plants to balance the infection effects.

These experiments were performed four times independently showing similar results. In all experiments statistical analysis showed a *P*-value less than 0.05. Chitinase activity carried out on coleoptile protein extracts showed no differences between control and treated tissues (data not shown).

In order to further characterise chitinase activity, total proteins from either coleoptiles or roots of treated and untreated seedlings were submitted to IEF and then stained for endochitinase activity. As shown in Fig. 3 (panels B–D), several isoforms were present in protein extracts from both tissues. Chitinase profile of coleoptile protein extracts resolved in a pH range 4–8 (Fig. 3, panel B) shows one basic and one acidic isoform (indicated by arrows) specifically induced upon FC infection (I), more expressed in T + I samples and not detected either in Co or in CR-treated samples. This result might suggest that these isoforms could exert a role in the degradation of the pathogen cell wall.

Chitinase isoforms were also detected in root samples resolved by IEF either in the pH range 4–8 (Fig. 3, panel C) or 3–5 (Fig. 3, panel D). One chitinase isoform with p*I* 5.7 was detected after FC inoculation of CR-treated seeds in 9 DAT samples only (Fig. 3, panel C). Moreover, since several isoforms were unresolved at acidic pH we performed an isoelectric focusing in the acidic range of pH. As can be evidenced in Fig. 3, panel D, the isoform induced in 9 DAT samples harvested after FC inoculation of CR-treated seeds shows a p*I* value of 5.2. Since the acidic zone of the IEF gel in Fig. 3, panel B showed several unresolved bands the same samples were further analysed by using a 3–5 pH range, but no differences among samples were detected (data not shown). Taken together these results show that the interaction between pathogen and antagonist induces the expression of two different isoforms in wheat coleoptiles, whereas only one acidic isoform in root samples. Further experiments are in progress to identify and characterise these inducible chitinases.

3.6. Genomic organisation of PR4 genes

In order to study the genomic organisation of wheat PR4 gene family, Southern blot analysis was carried out with total DNA extracted from leaves of tetraploid wheat *T. durum* (cv. Kronos). Wheat genomic DNA was digested with *Eco*RI, *Hind*III, *Bam*HI, *Nde*I and *Xho*I which do not possess recognition sites in the known cDNA sequence coding wheatwin2, a PR4 protein previously characterised in *T. aestivum* [38]. The resulting fragments were hybridised with the *wPR4b* cDNA encoding wheatwin2 (data not shown). Several DNA fragments of different size were detected in all digests, showing a relatively low complexity in view of the allopolyploid nature of the *T. durum* genome that include two closely related genomes. Thus, a small multigene family in the genome of cv. Kronos presumably represents PR4 genes since some DNA fragments may represent different orthologs.

3.7. Induction of PR4 proteins

In order to investigate the expression pattern of PR4 proteins in wheat tissues treated with *C. rosea* either alone or in combination with *F. culmorum*, western blot analyses were performed utilising the anti-wheatwin antisera (Fig. 4). Time course expression of PR4 proteins was evaluated on both coleoptiles (panels A) and roots (panels B) from CR-treated (T), FC-infected (I), FC-infected following CR-treatment (T + I) and control tissues (Co) harvested 7, 8 and 10 days after each treatment. As shown in Fig. 4, panel A, PR4 proteins are not expressed in the early stages of control coleoptiles development but accumulated beginning from the eighth day suggesting a developmental regulation during wheat seed germination. On the contrary, seed treatment with the BCA *C. rosea* resulted in PR4 protein expression in the early stages of coleoptile growth suggesting the activation of a CR-mediated induction pathway. Moreover, the induction pattern of PR4 proteins in root tissues harvested from CR-treated wheat seeds (Fig. 4, panel B) is even more clear as they are only slightly detectable in the corresponding control tissues. On the basis of these results, we can assume that CR-treatment may have an advantageous effect on wheat seedling protection promoting the expression of defence proteins known to exert a crucial role in plant resistance. It is well documented that the role of PR proteins in plant defence mechanisms is notable as demonstrated by their induction following biotic and abiotic stresses [19 and references therein]. In particular, it has been reported that wheat PR4 proteins are induced in soft wheat (*T. aestivum*) coleoptiles in response to fungal infection [39] as well as after treatment with chemical agents that induce systemic acquired resistance and wounding [40] and therefore they can be considered useful markers of resistance. In this paper we report that *F. culmorum* inoculation (I) trigger a strong induction of PR4 proteins both in *T. durum* coleoptiles (Fig. 4, panel A) and roots (Fig. 4, panel B), highlighting a similar expression pattern as in *T. aestivum*. Interestingly, *F. culmorum* inoculation carried out on CR-treated (T + I) seeds trigger a stronger PR4 expression with respect to seed treatment with *C. rosea* alone both in coleoptiles (Fig. 4, panel A) and roots (Fig. 4, panel B). On the other hand, the expression levels of PR4 proteins either in FC inoculated seeds or in FC inoculated seeds

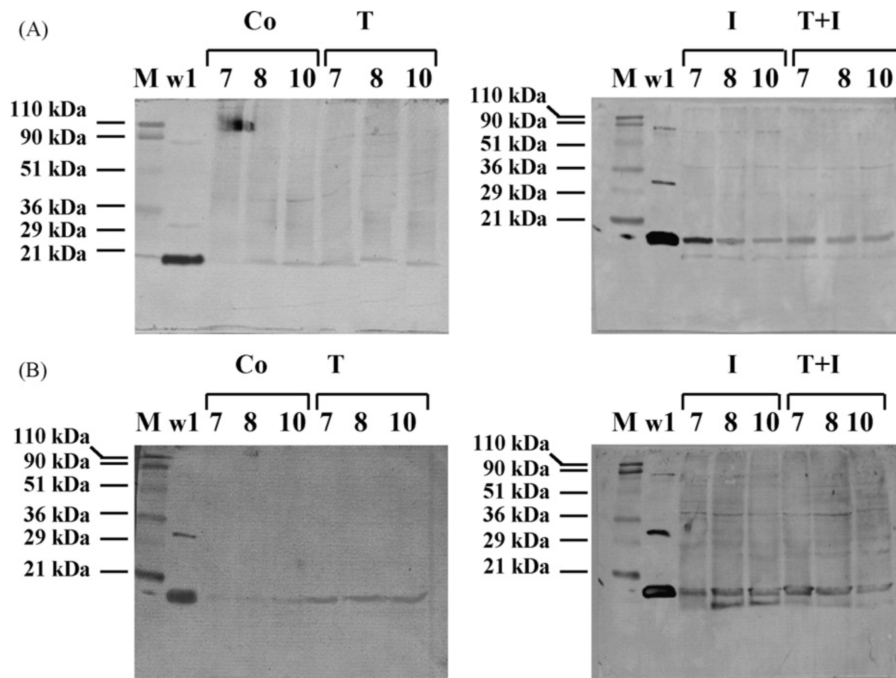

Fig. 4. Time course expression of wheat PR4 proteins in response to *F. culmorum* infection (I), CR-treatment (T) and *F. culmorum* infection on CR-treated seeds (T + I). Coleoptiles (A) and roots (B) were harvested 7, 8 and 10 days after each treatment as well as control seeds (Co). Western blot analysis was carried out using anti-wheatwin1 antisera. Molecular markers (M) and native wheatwin1 (w1) are indicated. The spot present in panel A, Co, around 100 kDa is an artefact.

following CR-treatment are similar suggesting that they do not exert a synergistic behaviour.

3.8. Induction of PR genes

In order to shed some light on transcriptional regulation of PR4 genes their expression pattern following CR-treatment and FC inoculation on CR-treated and untreated wheat seeds were analysed by using a RT-PCR approach. Time course induction patterns were evaluated in wheat coleoptiles and roots harvested from seedlings at different developmental stages. It is worthwhile to highlight that the primers used for PR4 gene amplification could detect more than one member of this family in analogy with that reported for *T. aestivum* PR4 family [41]. Moreover, to determine whether other PR genes are activated co-ordinately with those belonging to the group 4, we extended the analysis to the PR1.1 and PR5 genes, whose expression is used as an indicator of the activation of plant defence responses. Amplification of the housekeeping actin gene was used as internal control in the RT-PCR reactions. Fig. 5 shows the results of time course RT-PCR expression studies of the selected PR genes in wheat coleoptiles (panel A) and roots (panel B) in response to all treatments together with the relative controls. PR4 genes are not constitutively expressed in *T. durum* coleoptiles whereas are inducible either after CR-treatment (T) or upon FC inoculation (I) although with different temporal induction time. On the contrary, PR4 genes are developmentally regulated in control roots as shown in Fig. 5, panel B and they are also induced either after CR-treatment (T) or FC inoculation (I). Seed treatments with both antagonist and pathogen (T + I) have not synergistic effect on PR4 gene expression neither in coleoptiles or roots. As above described for PR4 proteins, these results on PR4 gene expression indicate similar transcriptional and translational regulation in both wheat tissues.

As shown in Fig. 5 transcripts corresponding to PR1.1 and PR5 genes were highly expressed in both coleoptiles (panel A) and roots (panel B) either in treated samples or in control tissues. These results indicate that these genes are constitutively expressed in both tissues and their synthesis is not influenced neither by the antagonist nor the pathogen. Several RT-PCR experiments using different dilutions of the cDNA samples used as template as well as low numbers of PCR cycles for all PR gene expression were performed. The results have been confirmed repeating the amplification experiments three times using the housekeeping actin gene as control. The time course transcription analysis experiments clearly indicate that, under our experimental condi-

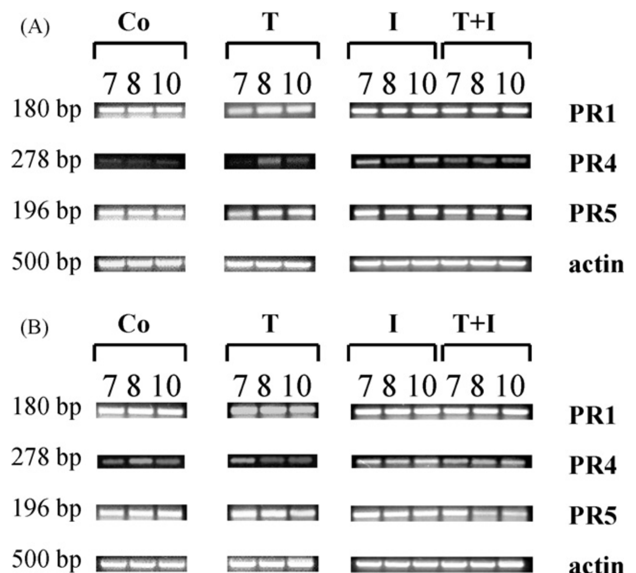

Fig. 5. Time course expression of wheat PR genes in response to *F. culmorum* infection (I), CR-treatment (T) and *F. culmorum* infection on CR-treated seeds (T + I). Coleoptiles (A) and roots (B) were harvested 7, 8 and 10 days after each treatment as well as from control seeds (Co). RT-PCR analyses were performed using specific primers. As RT-PCR internal control, actin mRNA was also amplified.

tions, PR genes belonging to the group 4 are inducible by antagonist and pathogen fungi, whereas those belonging to the groups 1 and 5 are constitutively expressed at high levels in both young shoots and roots.

4. Conclusion

The present research indicates an active participation of the host plant in the biocontrol exerted by *C. rosea*. Therefore, the *C. rosea* biocontrol strain used in this study, in addition to its recognised mycoparasitic nature, may reduce *F. culmorum* disease through an association with roots and activation of the plant defence system. Furthermore, the influence of *C. rosea* and *F. culmorum* on wheat seeds either alone or in combination resulted in enhanced expression of some peroxidase and chitinase isoforms as well as PR4 proteins. However, it is worthwhile to point out that there is abundant chitinase and peroxidase activity in coleoptiles and roots and their involvement in protection of the host is far from clear. Still, we could argue that the induced chitinase and peroxidase isoforms may play a role in protection of the host from pathogen invasion. In the three-component system pathogen–antagonist–wheat, it seems that CR-treatment by itself promotes plant growth and reduces the expression of some peroxidase likely to maintain toxic compounds below phytotoxic levels. In addition, the presence of the antagonist seems to influence the expression of some chitinase isoforms that could be involved in the reduction of pathogen growth by disruption of the cell wall. Moreover, both antagonist and pathogen induced the expression of PR4 proteins that are endowed with ribonuclease activity as previously reported [22]. We could figure out that they probably exert their role on the invading microorganisms by a translation-inhibitory process ascribed to their enzymatic activity.

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