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Changes in pathogenesis-related proteins in pepper plants with regard to biological control of phytophthora blight with *Paenibacillus illinoisensis*

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Abstract. To evaluate the biocontrol effectiveness of chitinase-producing bacterium, *Paenibacillus illinoisensis* strain KJA-424 against pathogenic strain of *Phytophthora capsici* in pepper plants, growth response and kinetics of pathogen related (PR) proteins were estimated after inoculation with *P. capsici* (P), and with a combination of *P. capsici* and strain KJA-424 cell culture (P + A). Fresh weight and chlorophyll content in shoots at P + A-treated plants significantly increased by 23.4 and 34.2%, respectively after 7 days of inoculation, compared to P-treated plants. Root mortality in P + A-treated plants was significantly reduced compared to P-treated plants. Seven days after inoculation, the activities of β-1,3-glucanase, cellulase and chitinase in P-treated roots had decreased by 54.8, 36.5 and 52.8%, respectively, compared to P + A-treated roots, while those in P-treated leaves increased by 22.8, 36.3 and 23.8%, respectively, compared to those in P + A-treated leaves. The activities of β-1,3-glucanase, cellulase and chitinase in roots are negatively correlated with root mortality. All these results suggest that the inoculation of an antagonist, *P. illinoisensis* alleviates root mortality, reduction of PR proteins in roots, and activates of PR proteins in leaves infected by *P. capsici*.

Key words: biocontrol agents, *Paenibacillus illinoisensis*, phytophthora blight, *Phytophthora capsici*, PR proteins, root mortality

Introduction

The common defense response of plants includes the production of peroxidases and phenolic compounds (Spanu and Bonafante-Fasolo, 1988), the deposition of structural polymers, such as lignin and callose (Campbell and Ellis, 1992; Benhamou and Nicole, 1999), and the

accumulation of pathogenesis-related (PR) proteins, such as β-1,3-glucanases and chitinases with antimicrobial potential (Dalisay and Kuc, 1995; van Loon, 1997).

Phytophthora blight has a destructive phase at the crown of the stem that results in wilting and the development of a hypersensitive response (HR) as a defence response. Kim et al. (1994, 1997) reported that pepper stems with infection of pathogenic strain *P. capsici* induced β-1,3-glucanase and chitinase, which can protect plants by degrading pathogenic fungal cell walls. The accumulation of these PR proteins in infected stem tissues became much more pronounced in the resistant than in the susceptible cultivar (Egea et al., 1999). In addition, Pozo et al. (2002) suggested that tomato roots infected with *P. parasitica* resulted in the early accumulation of β-1,3-glucanase, chitinase and other PR proteins, which were associated with induced systemic resistance (ISR). Also, the increase of the chitinase activity was positively correlated with ISR to viral bacterial, and fungal pathogens in cucumber plants (Metraux and Boller, 1986). PR proteins accumulated in tomato roots infected with *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Benhamou et al., 1990) and in tomato infected with *Phytophthora infestans* (Christ and Mosinger, 1989). Some results were inconsistent with general response in pathogen-challenged radish (Hoffland et al., 1996) and tobacco (Maurhofer et al., 1994; Beffa et al., 1996), which were severely damaged and/or even died although PR proteins were higher than in non-infected plants.

However, to our knowledge, the kinetics of PR proteins activity in relation to stress intensity with time of pathogen infection, has not yet been studied, although the invasion of pepper by *P. capsici* involving in PR proteins accumulation have been largely accessed under resistance pepper (García-Pérez et al., 1998) and susceptible pepper cultivars (Alcázar et al., 1995).

The objective of the present work was to investigate changes of PR proteins induced in pepper plants after challenge with a phytopathogen, *P. capsici*, with the antifungal bacterium, *Paenibacillus illinoisensis* KJA-424 having a potential antagonist effectiveness against *Rhizoctonia solani* in our previous studies (Jung et al., 2003).

Materials and methods

Plant material and growth condition

The surface of pepper (*Capsicum annuum* L. Chungok) seeds was sterilized in 70% ethanol for 2 min, and thoroughly washed with sterile distilled

water. The seeds were sown three in each pot and then thinned to one per pot after 1 week. The seedlings were grown in a greenhouse for 2 months. Plant growth medium consisted of modified Johnson solution (1957) containing 8 mM NH_4NO_3 and 0.2 mM KH_2PO_4. Natural light was supplemented by Silvania incandescent and cool-white lamps, 400 μmol photons m^{-2} s^{-1} with 16/8 h of light/dark photo period, 27/20 °C of thermoperiod and 60–70% relative humidity. Soils were steam sterilized and mixed with sterilized quartz sand and vermiculate in a ratio of 1:2:1 (v/v/v) and placed into 500 ml pots. Chemical characteristics of the soils prepared consisted of 0.41% of organic metter, 0.11% of nitrogen, 32.7 ppm of P_2O_5, 107.6 ppm of SiO_4, 0.24 Cmol kg^{-1} of K, 3.89 Cmol kg^{-1} of Ca, 1.10 Cmol kg^{-1} of Mg, 2.63 me kg^{-1} of cation exchangeable capacity (CEC), and 0.62 ds m^{-1} of electronic conductivity.

In vivo *assays with antagonist formulations*

To prepare inoculum of the pathogen, potato dextrose broth (50 ml) was placed in a 250-ml Erlenmeyer flask and autoclaved, and then inoculated with PDA plugs (0.8 cm in diameter) of *P. capsici* KACC-40483 obtained from the Korean Agricultural Culture Collection (KACC). The *P. capsici* was grown on a V8 juice agar medium at 25 °C in darkness. Ten milliliter (average of 10^5 ml^{-1} zoospores) of *P. capsici* cultured in the medium for 4 days was used as the inoculum.

P. illinoisensis strain KJA-424 as an antagonist (A) was isolated from soil in the west coast of Korea as previously described by Jung et al. (2002). KJA-424 was grown at 30 °C in a 1-l Erlenmeyer flask containing 500 ml of 0.2% colloidal chitin on a rotary shaker at 180 rpm min^{-1} for 3 days. Twenty milliliters (average of 2.7×10^9 cfu ml^{-1}) of KJA-424 was applied directly into each pot soil. Pepper plants received no inoculation (C), *P. capsici* inoculation (P) and dual inoculation of *P. capsici* and *P. illinosensis* KJA-424 (P + A). The experiments were replicated twice with same treatment. The design was composed with two pots having one plant for the first experiment and three pots for the second. The results of all measurements are given as pooled mean of five separate pots. On two months disease incidence was expressed as the degree of root infection showing typical symptoms caused by *P. capsici*, which included lesions of roots.

Chlorophyll concentration

Chlorophyll was extracted by soaking 100 mg of leaves in 10 ml dimethyl sulfoxide (DMSO) at 60 °C for 60 min. Chlorophyll

concentration was measured at 663 and 645 nm using a spectrophotometer (U-1100 HITACHI, Japan) by Mackinney method (1941).

Root mortality

Root mortality was measured using the modified method of Knievel (1973). About 500 mg fresh roots were incubated with 10 ml of 0.6% 2,3,5-triphenyltetrazolium chloride in 0.05 M phosphate buffer (pH 7.4) for 24 h in the dark at 30 °C. Roots were then rinsed twice with deionized water. Formazan was extracted twice from the roots with 95% ethanol at 70 °C for 4 h. Combined extracts were adjusted to a final volume of 50 ml with 95% ethanol. Absorbance was read at 490 nm. A standard curve was made using different proportions of living roots and killed roots to calculate root mortality. Root mortality was expressed as percentage dead root dry weight (DW) of the total root DW.

Protein extraction

Seedlings were divided into leaves and roots. The sample was washed under running tap water and dried gently and then ground with a mortar and pestle under liquid nitrogen. The ground sample was homogenized in 50 mM Tris buffer (pH 6.7). The homogenate was centrifuged at $10{,}000 \times g$ and 4 °C, and the supernatant was collected and kept at −20 °C. Protein concentration was determined using the method of Bradford (1976).

Measurement of pathogenesis-related enzyme activities

β-1,3-Glucanase activity (EC 3.2.1.6) was assayed by measuring the amount of the reducing end group, glucose, produced from laminarin (Yedidia et al., 2000). The assay mixture consisted of 100 μl of enzyme solution, 25 μl of 1% laminarin, and 375 μl of 50 mM sodium citrate buffer (pH 5.0). After incubation at 37 °C for 1 h, 1.5 ml of 3-amino-5-nitrosalicylic acid (DNS) was added, and then heated in boiling water for 5 min. The absorbance was immediately measured at 550 nm using a spectrophotometer. The reducing sugar was calculated from a standard curve obtained from known concentrations of glucose. One unit of β-1,3-glucanase activity was defined as the amount of enzyme that liberated 1 μmol of glucose per hour.

Cellulase activity (EC 3.2.1.4) was assayed by measuring the amount of the reducing end group, glucose, produced from carboxymethyl cellulose (Yedidia et al., 2000). The reaction mixture (0.5 ml) contained

250 μl crude enzyme preparation and 250 μl 1% CMC stock solution dissolved in 50 mM sodium acetate buffer (pH 5.0). After incubation at 37 °C for 1 h, 1.5 ml of DNS was added, and then heated in boiling water for 5 min. The absorbance was immediately measured at 550 nm using a spectrophotometer. The reducing sugar was calculated from a standard curve obtained from known concentrations of glucose. One unit was defined as the amount of enzyme that liberated 1 μmol of glucose per hour.

Chitinase activity (EC 3.2.1.14) was assayed by measuring the amount of the reducing end group, GlcNAc (*N*-acetyl-β-D-glucosamine), produced from colloidal chitin, as described by Lingappa and Lockwood (1962). The assay mixture consisted of 20 μl of enzyme extract, 230 μl of distilled water, 500 μl of 1.0% colloidal chitin in 0.1 M sodium acetate buffer (pH 5.0). After incubation at 37 °C for 2 h, 200 μl of 1 N NaOH was added. After brief centrifugation (10,000 × *g*, 10 min), 750 μl supernatant was mixed with 1 ml of Schales' reagent (0.5 M sodium carbonate + 1.5 mM potassium ferricynide), and then heated in boiling water for 15 min. The absorbance was immediately measured at 420 nm using a spectrophotometer. The activity was calculated from a standard curve obtained from known concentrations of *N*-acetyl-β-D-glucosamine. One unit of chitinase activity was defined as the amount of enzyme that liberated 1 μmol of GlcNAc per hour.

Statistical analysis

Treatment effects were determined by analysis of variance (one-way ANOVA) according to the general linear model procedure of the Statistical Analysis System 8.1 (SAS Institute, 2000). Means were separated with Tukey's Studentized Range Test at $p = 0.05$. Regression analysis was also undertaken to determine the closeness of the relationship between the measured variables, between β-1,3-glucanase, cellulase, and chitinase activity and root mortality.

Results

Growth characteristics

Plant biomass and chlorophyll content measured after 7 days of dual inoculation with pathogen (*P. capsici*) and antagonist (*P. illinoisensis* KJA-424) are shown in Table 1. Total shoot biomass was the lowest (6.99 g plant^{-1}) in the P-treated plants. Fresh weight in shoots

Table 1. Effects of dual inoculation of pathogenic *Phytophthora capsici* and antagonistic *Paenibacillus illinoisensis* KJA-424 on the growth of pepper (after 7 day of treatment)

Treatments	Plant biomass (plant^{-1})		Chlorophyll (mg/g F.W)
	Fresh wt. shoot (g)	Dry wt. root (g)	
C	9.77 ± 1.01a	0.86 ± 0.12a	1.358 ± 0.077a
P	6.99 ± 0.71b	0.41 ± 0.14c	0.765 ± 0.096c
P + A	9.12 ± 0.98a	0.70 ± 0.14b	1.162 ± 0.050b

* Pepper seedlings were grown in greenhouse at 27/20 °C (light/dark) of thermo period for 7 days after inoculation *P. capsici* (zoospore, 10^5 ml^{-1}) with or without *P. illinoisensis* (average of 2.7×10^9 cfu ml^{-1}). C; Control (Non-inoculation of pathogen or antagonist), P; Pathogen, P + A; Pathogen + *P. illinoisensis*. Mean (±SE) within a column followed by the same letter(s) were not significantly different at $p < 0.05$; Tukey's Studentized Range Test.

significantly increased by 28.5 and 23.4% in control (C) plants, pathogen + antagonist (P + A) treated plants, respectively, compared with P-treated plants. Chlorophyll content was the lowest (0.765 mg·g^{-1}) in leaves of the P-treated plants. Chlorophyll content increased by 43.7 and 34.2% in C plants and P + A-treated plants, respectively, compared with P-treated plants. The late blight caused by *P. capsici* was significantly suppressed by the co-treatment of the antagonist, *P. illinoisensis* KJA-424. On day 7, P-treated plants not only appeared to be yellowing and wilting but also reached to an advanced stage of root decay. However, P + A-treated plants looked healthy, possibly resulting from a significant protection against phytophthora blight.

Root mortality

Root mortality of P-treated plants drastically increased after 5 days of inoculation but almost completely suppressed by co-inoculation of antagonistic KJA-424 (Figure 1). Little difference of root mortality was observed between control and P + A.

PR protein activities

Enzyme activities of β-1,3-glucanase, cellulase and chitinase in pepper roots were assayed using laminarin, carboxymethyl cellulose and colloidal chitin as substrates, respectively. The activities were measured at 1, 3, 5 and 7 days after inoculation of *P. capsici* and/or *P. illinoisensis* KJA-424.

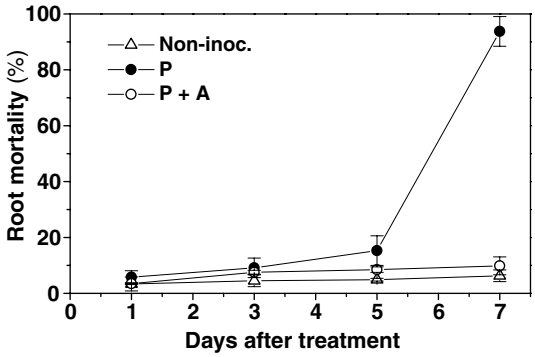

Figure 1. Root mortality of pepper plants as affected by dual inoculation of pathogenic *P. capsici* and antagonistic *P. illinoisensis* KJA-424. Mortality of pepper roots was measured at 1, 3, 5 and 7 days after inoculation of *P. capsici* and/or *P. illinoisensis* KJA-424. Non-inoculated peppers (△), peppers treated with *P. capsici* (●) and peppers treated with *P. capsici* + *P. illinoisensis* (○). Each value is the mean ± S.E. for n = 5.

PR protein activities were not significantly different in both control and P + A-treated roots throughout experimental period (Figure 2). PR protein activities were the highest in P-treated roots on the first day after treatment and then rapidly decreased. By the seventh day, the activities of β-1,3-glucanase, cellulase and chitinase in P-treated roots significantly decreased by 54.8, 36.5 and 52.8%, respectively, compared to P + A-treated roots.

Activities of β-1,3-glucanase, cellulase and chitinase were also assayed in leaves of pepper. PR protein activities were not significantly different in both control and P + A-treated leaves throughout the experimental period (Figure 3). PR protein activities were the highest in P-treated leaves on day 7 after treatment. At the same time, the activities of β-1,3-glucanase, cellulase and chitinase in P-treated leaves significantly increased by 22.8, 36.3 and 23.8%, respectively, compared with P + A-treated leaves.

Interrelationship of PR protein activity with root mortality

To estimate a relationship between PR proteins and root mortality, scatter plots were used for each treatment in pepper roots (Figure 4). Scatter plots showed inverse relationship between PR protein activities and mortality in roots.

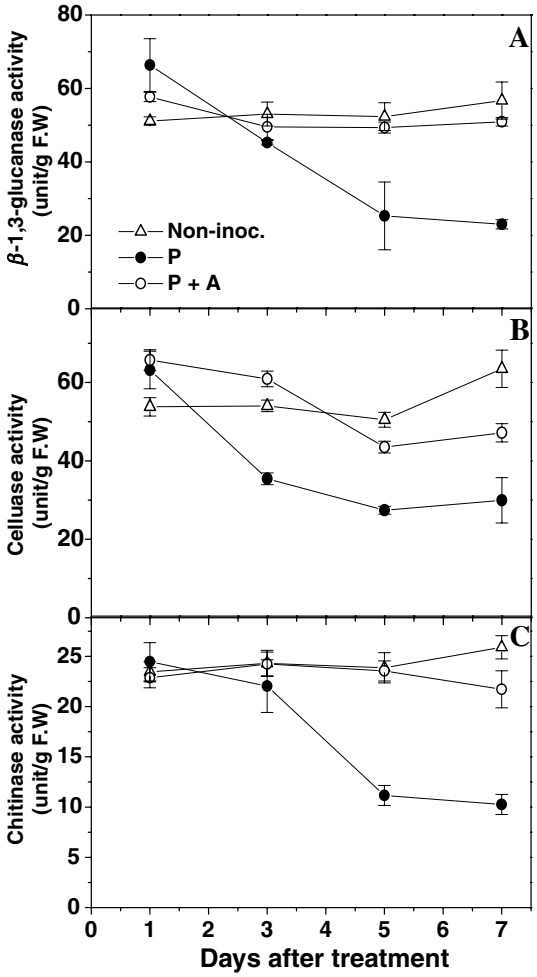

Figure 2. β-1,3-glucanase (A), cellulase (B) and chitinase activity (C) in pepper roots upon *P. capsici* and/or *P. illinoisensis* KJA-424 inoculation. Non-inoculated peppers (△), peppers treated with *P. capsici* (●) and peppers treated with *P. capsici* + *P. illinoisensis* (○). Each value is the mean ± S.E. for n = 5.

Discussion

Biological control of soil-borne pathogens is a multifaceted process in which antibiosis and hydrolytic enzyme activities are the most recognized mechanisms (Haran et al., 1995). Different PR protein families are known to have antifungal properties, and thought to play an important role in plant defense by restricting the growth and development of pathogens (Boller, 1992). Our work has paid attention to the activities

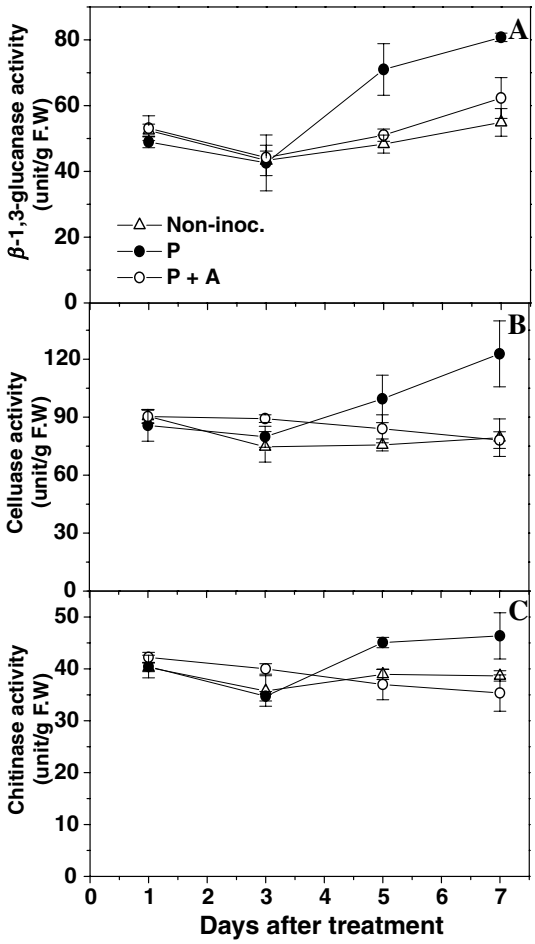

Figure 3. β-1,3-Glucanase (A) cellulase (B) and chitinase activity (C) in pepper leaves upon *P. capsici* and/or *P. illinoisensis* KJA-424 inoculation. Non-inoculated peppers (△), peppers treated with *P. capsici* (●) and peppers treated with *P. capsici* + *P. illinoisensis* (○). Each value is the mean ± S.E. for n = 5.

of PR proteins in host plants as affected by an antagonist (*P. illinoisensis* KJA-424) and a pathogen (*P. capsici*), co-inoculated to investigate antagonist effectiveness.

After pathogen challenge, P-treated plants showed the highest damage on growth condition (Table 1). Also, root mortality of plants highly increased in P-treated roots (Figure 1). Symptoms of disease mainly are presented by falling leaves, the reduction of leaves elongation and rotten stem. Plant roots might be damaged by penetration of *P. capsici* zoospores into cell tissues. However, P-treated plants with

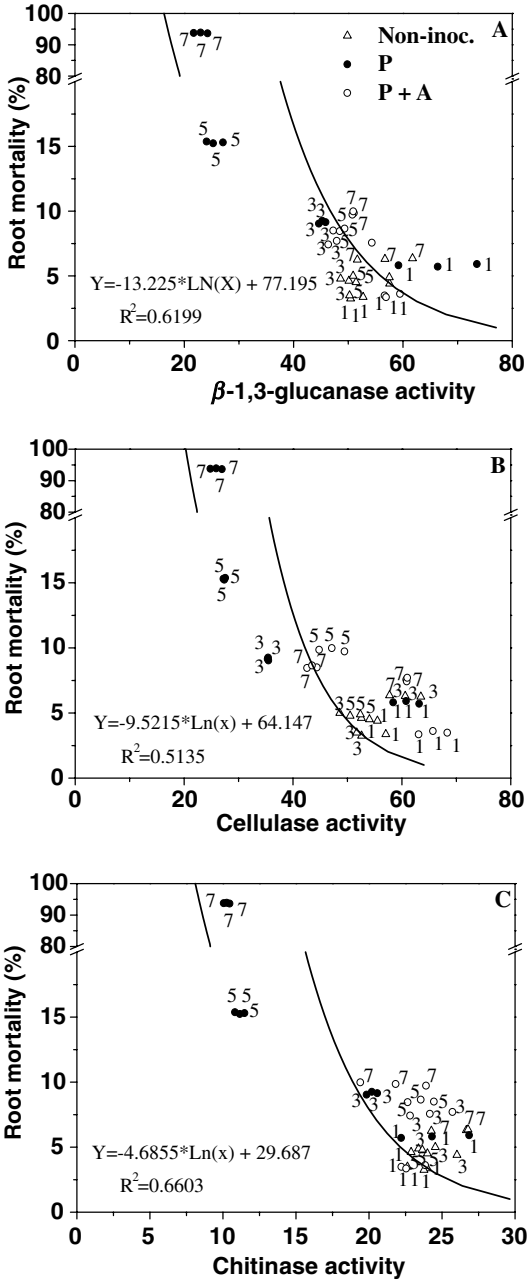

Figure 4. Scatter plot of β-1,3-glucanase (A) cellulase (B) and chitinase activity (C) in pepper roots upon *P. capsici* and/or *P. illinoisensis* KJA-424 inoculation. Non-inoculated peppers (△), peppers treated with *P. capsici* (●) and peppers treated with *P. capsici* + *P. illinosensis* (○).

antagonist (*P. illinoisensis*) are almost protected from disease by blight for the experimental period (Table 1 and Figure 1). This result indicates that the co-inoculation of *P. illinoisensis* KJA-424 has a protective effect against *P. capsici* challenge.

In pathogen-host study, the present study, β-1,3-glucanase, cellulase and chitinase activity of P-treated plants decreased in roots at 7 days after treatment, while they increased in leaves (Figures 2 and 3). These results are consistent with Lawrence et al. (1996), who demonstrated that chitinase and β-1,3-glucanase isozymes were induced in all genotypes upon challenge with *Alternaria solani* infection to tomato leaves. In other pathogen–host study, the invasion of pepper by *P. capsici* depends on the intensity of capsidiol accumulation and induction of PR proteins (García-Pérez et al., 1998) causes an increase in peroxidase activity (Alcázar et al., 1995). In antagonist–host experimental design, Yedidia et al. (2000) suggested that PR proteins highly accumulated in *T. harzianum* treatment in cucumber roots. In pathogen–antagonist–host combination, the non-pathogenic *Pseudomonas fluorescens* strain WCS417 in radish roots was able to induce resistance against *Fusarium oxysporum* f. sp. *raphani* without detectable accumulation of PR proteins (Hoffland et al., 1995, 1996).

These results suggested that the infection by *P. capsici* of roots does not lead to PR protein activation in the root, but does have a systemic effect causing increases in β-1,3-glucanase, chitinase and cellulase in leaves. However, PR protein activities of P + A-treated roots and leaves maintained rather constant and same levels as those of control plants for experimental period (Figures 2 and 3). This clearly indicated that co-inoculation of KJA-424 with *P. capsici* reduces PR protein activity of pepper plants from biochemical aberration such as the drastic decrease and increase of PR proteins in roots and leaves, respectively. Our result was not consistent with that of Dassi et al. (1998) who showed that PR protein expression was clearly enhanced by *Phytophthora parasitica*-infection in tomato roots.

PR protein activity in pepper roots was not significantly different for the first 3 days, showing the resistance to pathogen infection, thereafter PR protein activity rapidly decreased with increasing root mortality caused by root rot (Figures 2A, C and 4). However, PR proteins of pepper roots with antagonist, *P. illinoisensis* might be activated by antibiotic production. It was noteworthy that, under pathogen infection, PR proteins in roots were closely associated with the increase in root mortality, as evidenced by a negative correlation ($r = 0.7873$) between root mortality and β-1,3-glucanase activity (Figure 4A). This negative correlation permits us to hypothesize that PR proteins in leaves

might be induced by the mechanism of a long-distance systemic resistance through signal molecules that activate defense-related genes, although mechanisms for the different regulation of PR proteins in roots and leaves of a single plant are not clear.

All these results suggest that the inoculation of an antagonist, *P. illinoisensis* alleviates root mortality, reduction of PR proteins in roots, but activates of PR proteins in leaves infected by *P. capsici*.

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