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Quantitative Changes of Plant Defense Enzymes in Biocontrol of Pepper (*Capsicium annuum* L.) Late Blight by Antagonistic *Bacillus subtilis* HJ927

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Abstract To investigate plant protection, pathogenesis-related (PR) proteins and plant defense enzymes related to cell wall lignification were studied in pepper plants inoculated with antagonistic *Bacillus subtilis* HJ927 and pathogenic strain *Phytophthora capsici*. Phytophthora blight disease was reduced by 53% in pepper roots when preinoculated with *B. subtilis* HJ927 against *P. capsici*. The activities of PR proteins (chitinase and β-1,3,-glucanase) and defense-related enzymes (peroxidase, polyphenoloxidase, and phenylalanine ammonia lyase) decreased in roots of *B. subtilis*+*P. capsici*-treated plants, but increased in leaves with time. The decrease and increase were much greater in *P. capsici*-treated plants than in *B. subtilis* HJ927+*P. capsici*-treated plants, although *P. capsici*-treated plants had more severe damage. Therefore, changes of enzyme activities do not seem to be directly related to plant protection. We suggest that the change of these enzymes in pathogen-treated plants may be related to plant response rather than to resistance against pathogen attacks.

Key words: PR-protein, late blight, chitinase producing bacteria

Plant disease resistance is known to be achieved by inducing PR proteins, which can hydrolyze fungal cell wall or liberate compounds to elicit a defense reaction. Earlier studies indicated a transient increase of PR protein, such as chitinase and β-1,3-glucanase, suggesting an early defense response of the plant to the invading fungus [27]. Induction of chitinase and β-1,3-glucanase by the pathogen *P. capsici* was detected in pepper tissues by Jung *et al.* [15] and Hong *et al.* [13], respectively. Contrary to the above, however, there are some suggestions that plant protection is not associated with accumulation of PR proteins [11, 26] although the role of plant PR proteins in degrading fungal structure has generally been known for many years [1, 4].

On the other hand, plants are able to defend themselves against pathogen by producing defense enzymes involved in cell wall lignification [18, 22, 28]. Hammerschmidt and Kúc [9] detected lignin deposition with the resistance of cucumber to *Cladosporium cucumerinum* and suggested lignification as the underlying mechanism induced by systemic resistance [10]. Phenylalanine ammonia lyase (PAL) is a key enzyme in the phenylpropanoid biosynthetic pathway that plays important roles in flavonoid productions and lignin biosynthesis [5]. Studies with several different species of plants have shown that the activity of PAL is increased by environmental factors such as fungal infection. Chen *et al.* [2] reported that high levels of PAL were induced in cucumber roots when inoculated with *P. aphanidermatum*, and Paul and Sharma [25] demonstrated that the activity of PAL was increased in barley leaves during suppression leaf stripe disease. Oxidative enzymes such as peroxidase (POX) and polyphenol oxidase (PPO) catalyze the formation of lignin and other oxidative phenols, thereby contributing to the formation of defense barriers by reinforcing the cell structure during the defense reaction against pathogenic agents. Mohammadi and Kazemi [22] reported that specific activities of PPO and POX were significantly increased in wheat heads of resistant and susceptible cultivars, respectively, following the inoculation with *F. graminearum* conidia.

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Certain beneficial microorganisms such as PGPR or antagonistics have recently been shown to induce systemic resistance [27, 29]. It was previously shown that pretreatment with *Pseudomonas fluorescens* protected radish through induction of systemic resistance not only against the fungal root pathogen *Fusarium oxysporum f.* sp. *raphani*, but also against the avirulent bacterial leaf pathogen *Pseudomonas syringae pv. tomato* and fungal leaf pathogen *Alternaria brassicicola* [12]. In particular, certain microorganisms such as *Streptomyces*, *Pseudomonas*, and *Trichoderma* species have been reported to be an effective biocontrol agent against phythophthora blight on pepper plants [14, 19]. However, although the potential of the PGPR to colonize plant root and trigger plant defense reactions has been established, the biochemistry of their interaction has been poorly understood.

The present study was undertaken to measure specific activities of defense enzymes involved in reinforcement of cell walls and pathogenesis-related (PR) proteins in leaves and roots of pepper plants preinoculated with *B. subtilis* HJ927, following infection of *P. capsici*, and to obtain better insight into the possible relationships between induction of defense enzymes and induced disease resistance.

Material and Method

Selection of Antagonist Isolates

Soil samples were collected from a pepper field severely infested by *P. capsici*. HC agar media containing the following compositions were inoculated with serially diluted soil samples; dried *P. capsici* hypae 0.5%, colloidal chitin 0.5%, Na_2HPO_4 0.2%, KH_2PO_4 0.1%, NaCl 0.05%, NH_4Cl 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2 \cdot 2H_2O$ 0.05%, yeast extract 0.05%, and agar 1.5%. They were then incubated at 30°C for 5 day. Colonies bigger than 5 mm diameter were selected and successively examined for antifungal activity against *P. capsici* on CP agar plate containing colloidal chitin 0.5%, Na_2HPO_4 0.1%, KH_2PO_4 0.05%, NaCl 0.025%, NH_4Cl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.025%, $CaCl_2 \cdot 2H_2O$ 0.025%, yeast extract 0.025%, agar 0.75%, and potato dextrose agar 0.5%. One strain having strong antifungal activity was selected for further use and kept in 50% glycerol solution at −70°C. Based on its 16s ribosomal RNA gene sequence, this strain was identified as *Bacillus subtilis* and named as *B. subtilis* HJ927.

Preparation of Inoculum

Bacterial inoculum (*Bacillus subtilis* HJ927) was cultured at 30°C in CP broth, containing 0.2% colloidal chitin, for 3 days. The cultures were diluted in sterile distilled water to 4×10^6 colony/ml. A virulent pathogen, *Phytophthora capsici* (KACC 40480), was obtained from Korean Agricultural Culture Collection and grown on V8 juice agar in the dark at 30°C for 3 days. A chopped medium containing hypae was flooded with sterile deionized water and incubated under continuous fluorescent light for 5 days at 30°C for sporangia production. After 5 days of incubation, it was chilled at 4°C for 30 min to release zoospores. Mycelia and sporangial debris were removed from the zoospore suspension by filtration through sterile cheesecloth, and the filtrate was diluted with sterile water to 2×10^6 zoospores/ml [16].

Plant Growth

Three pepper seeds (*Capsicum annuum* L.) were sterilized with alcohol and then planted in the 600-ml pot containing autoclaved soil mixture (soil: quartz sand: vermiculite, 2:1:1/v:v:v). The chemical characteristics of the soil were 0.45% of organic matter, 72 ppm of P_2O_5, 0.34 $cmol_c$ of K, 11.10 $cmol_c$ of Ca, 4.68 $cmol_c$ of Mg, 12.67 $cmol_c$ of cation exchangeable capacity, pH 6.29, and 0.65 dsm^{-1} of electronic condition. Peppers were grown at 24°C in an artificially illuminated room (12,000 lux at plant height) with a 16-h photoperiod and thinned to one in each pot at 2 weeks after planting.

One-hundred-fifty ml of bacteria inoculum (4×10^6 colony/ml) were inoculated three times (each 50 ml) by pouring into each pot at 5, 6, and 7 weeks after planting. Control plants received broth culture without bacteria. At 7 weeks after planting, the half of both bacteria-inoculated plants and non-treated plants were infected with 50 ml of *P. capsici* zoospore solution (2×10^6 zoospores/ml). Plants were watered with nutrient solution [KNO_3, 2.57 mM; $MgSO_4 \cdot 7H_2O$, 1.01 mM; $Ca(NO_3)_2 \cdot 4H_2O$, 3.6 mM; KH_2PO_4, 0.51 mM; $ZnSO_4 \cdot 7H_2O$, 0.11 μM; H_3BO_3, 4.58 μM; $MnCl_2 \cdot 4H_2O$, 1.01 μM; $CuSO_4 \cdot 5H_2O$, 0.08 μM; $Na_2MoO_4 \cdot 2H_2O$, 0.05 μM; $C_{10}H_{12}FeN_2NaO_8$, 62.7 μM] during the growth period of the plant [17]. Plants were harvested at 0, 1, 2, 3, 6, and 9 days after infection of *P. capsici* zoospore. Plants were then carefully washed with running tap and distilled water, and and weight of fresh roots and shoots was measured.

Root Mortality

Root mortality was measured by the method of Liu and Huang [21]. Fresh root (0.5 g) was incubated in 5 ml of 50 mM phosphate buffer (pH 7.4) containing 0.6% 2,3,5-triphenyltetrazolium chloride for 24 h in the dark at 30°C. Roots were then rinsed thoroughly with distilled water. Formazan was extracted twice from the roots with 95% ethanol at 70°C for 4 h. Combined extracts from the two extractions were adjusted to a final volume of 30 ml with 95% ethanol. The extracted solution was spectrophotometrically measured at 490 nm. A standard curve was prepared using different proportions of living roots and roots killed in an autoclave. Root mortality was expressed as percentage of dead root fresh weight vs. total root fresh weight.

Enzyme Activities

Frozen roots and leaves were ground at 4°C in an ice-chilled mortar with liquid nitrogen, and the resulting powder was suspended in 100 mM potassium phosphate buffer, pH 7.0 (2:2.5, w/v). Crude homogenates were centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatants were kept frozen at -20°C until use.

β-1,3-Glucanase was assayed based on the release of reducing sugars from laminarin, as described by Liang *et al.* [20]. Enzyme extract (50 μl) was incubated with 50 μl of laminarin (10 mg/ml) in 50 mM sodium acetate buffer, pH 5.0, at 37°C for 1 h. The reaction was terminated by adding 1.5 ml of DNS reagent and by boiling the mixture in a water bath: The mixture was heated in a 100°C bath for 5 min. Reducing sugar equivalents were measured by a UV spectrophotometer at 550 nm. One unit of β-1,3-glucanase activity was defined as the amount of enzyme required to liberate 1 μmol of glucose equivalent at 37°C for 1 h.

Chitinase activity was assayed by measuring the release of N-acetyl-glucosamin from prepared colloidal chitin [8], as described by Singh *et al.* [28]. A reaction mixture containing 10 μl of supernatant, 500 μl of 0.5% colloidal chitin, and 490 μl of 50 mM sodium acetate buffer (pH 5.0) was incubated in a water bath for 1 h at 37°C, and 200 μl of NaOH was added to stop the reaction and the reaction mixture was centrifuged at 12,000 rpm for 1 min. The supernatant (750 μl) was mixed with Schale's reagent and boiled for 15 min. The amount of N-acetyl-glucosamin produced was measured at 470 nm with a spectrophotometer. One unit of chitinase activity was defined as the amount of enzyme required to liberate 1 μmol of N-acetyl-D-glucosamin equivalent at 37°C for 1 h.

PAL activity was measured using a modification of the procedure described by El-Shora [5]. The mixture containing 1.9 ml of 100 mM Tris-HCl buffer (pH 8.5), 1 ml of 15 mM L-phenylalanine, and 100 μl of enzyme extract was incubated at 30°C for 15 min. The reaction was terminated by the addition of 200 μl of 6 N HCl, and absorbance of solution was measured at 290 nm by a UV spectrophotometer. One unit represents the conversion of 1 μmol L-phenylalanine to cinnamic acid per min.

PPO activity was assayed according to the method of Park [24]. The assay mixtures contained 20 μl of enzyme extract, 0.5 ml of distilled water, and 0.5 ml of assay solution (7.1 g Na_2HPO_4+5.25 g citrate+2.76 g catechol/250 ml), which was added before measurement. PPO activity was presented as the change in unit of absorbance at 420 nm per g of fresh weight per min.

For POX, the oxidation of guaiacol was measured, as described by Fu and Huang [7]. Fifty μl of enzyme extract was added to 2.85 ml of 100 mM phosphate buffer (pH 7.0) and mixed with 50 μl of 20 mM guaiacol. The reaction was started by the addition of 20 μl of 40 mM H_2O_2 to the mixture, and the initial rate of increase in absorbance was measured at 470 nm over 1 min. One unit of activity was defined as a change in absorbance of 0.001/min.

RESULTS

Plant Growth

The changes of plant fresh weight are shown in Fig. 1. Fresh weights of root and shoot were the highest in *B. subtilis* HJ927-treated plants, followed by non-treated, *B. subtilis* HJ927+*P. capsici*-treated, and *P. capsici*-treated plants at 9 days after *P. capsici* infection. Infection of *P. capsici* induced sudden wilt of entire plants, which was caused by rotting of the stems near the soil surface, root decay, and leave abscission. Progressive development of disease in *P. capsici*-treated plants eventually led to asudden decrease of leaves fresh weight at 6 days after infection. However, *B. subtilis* HJ927 protected plants from *P. capsici*, as shown in its fresh weight and root mortality (Figs. 1 and 2).

Root Mortality

Until 3 days, root mortality was almost constant in all treatments. However, root mortality in *P. capsici*-treated plants markedly increased thereafter with 78% of maximal value at the 9th day. In *B. subtilis* HJ927+*P. capsici*-treated plants, there was no change in root mortality for 6 days, but slightly increased to 25% at the 9th day (Fig. 2).

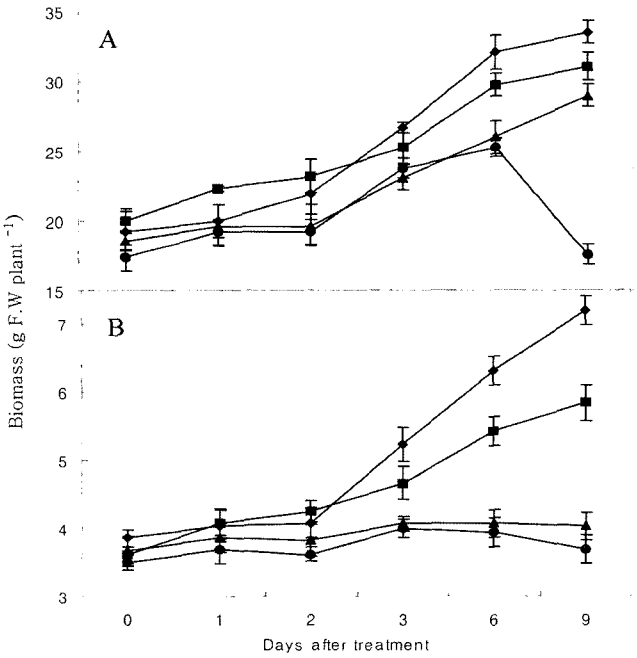

Fig. 1. Changes in biomass of pepper leaves (A) and roots (B), as influenced by control (-■-), *B. subtilis* HJ927 (-◆-)-, *B. subtilis* HJ927+*P. capsici* (-▲-)-, and *P. capsici* (-●-)-treated plants. Mean values were 6 replicates. Bars represent standard error.

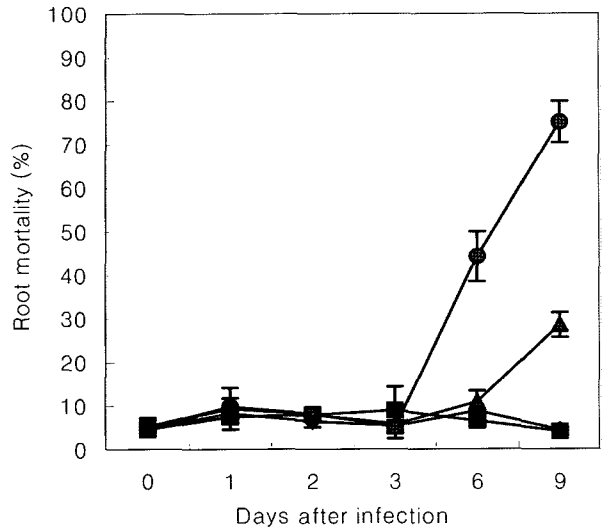

Fig. 2. Changes of root mortality in response to *P. capsici* infection, as influenced by control (-■-), *B. subtilis* HJ927 (-◆-)-, *B. subtilis* HJ927+*P. capsici* (-▲-)-, and *P. capsici* (-●-)-treated plants.
Mean values were 6 replicates. Bars represent standard error.

Enzyme Activities

As shown in Figs. 3 and 4, β-1,3-glucanase and chitinase activities had similar trend in each treatment plant. At 6 days after infection, β-1,3-glucanase and chitinase activities in leaves of *P. capsici*-treated plants were increased, while they decreased in the roots. Significant difference of PR protein changes in both roots and leaves was not observed between control plants and *B. subtilis* HJ927-treated plants during the experimental period. That means that *B. subtilis* HJ927 alleviated the effects on PR protein contents in *B. subtilis* HJ927+*P. capsici*-treated plants.

Activities of PAL, POX, and PPO also had a trend similar to those of PR proteins, as shown in Figs. 5, 6, and 7. PAL, POX, and PPO activities were increased in leaves of *P. capsici*-treated plants at 6 days after infection, but decreased in roots. However, the increase and decrease of these enzymes by *P. capsici* infection was alleviated in *B. subtilis*+*P. capsici*-treated plants.

DISCUSSION

In this study, we quantitatively investigated the changes of PR proteins in plant disease suppression. Previous studies on induced resistance indicated that all forms of induced systemic resistance (ISR) are associated with accumulation of pathogenesis-related protein [1, 4]. However, in the present study, the increase of PR proteins was not found to be directly associated with disease suppression. *B. subtilis* HJ927 alleviated the increase of PR protein in leaves of *B. subtilis* HJ927+*P. capsici*-treated plants, compared with that of *P. capsici*-treated plants, and reduced disease symptom. In other words, even though PR proteins were

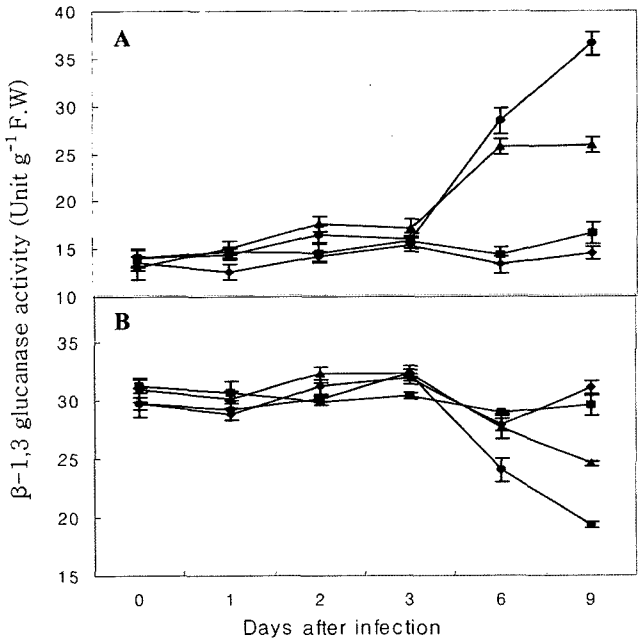

Fig. 3. Changes of β-1,3-glucanase in response to *P. capsici* infection, as influenced by control (-■-), *B. subtilis* HJ927 (-◆-)-, *B. subtilis* HJ927+*P. capsici* (-▲-)-, and *P. capsici* (-●-)-treated plant leaves (A) and roots (B).
Mean values were 6 replicates. Bars represent standard error.

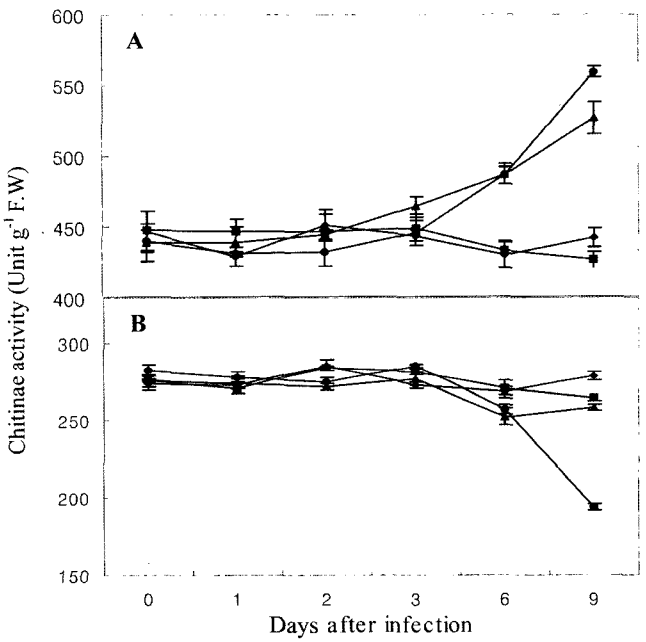

Fig. 4. Changes of chitinase in response to *P. capsici* infection, as influenced by control (-■-), *B. subtilis* HJ927 (-◆-)-, *B. subtilis* HJ927+*P. capsici* (-▲-)-, and *P. capsici* (-●-)-treated plant leaves (A) and roots (B).
Mean values were 6 replicates. Bars represent standard error.

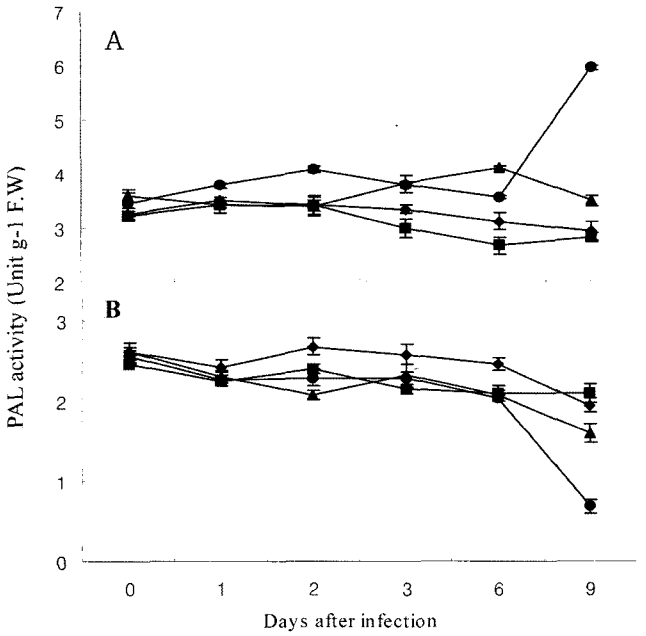

Fig. 5. Changes of PAL in response to *P. capsici* infection, as influenced by control (-■-), *B. subtilis* HJ927 (-◆-)-, *B. subtilis* HJ927+*P. capsici* (-▲-)-, and *P. capsici* (-●-)-treated plant leaves (A) and roots (B).
Mean values were 6 replicates. Bars represent standard error.

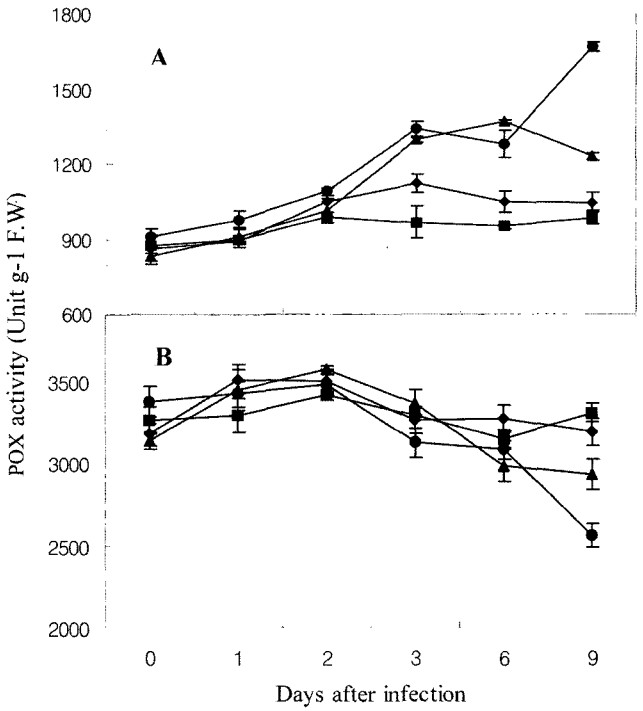

Fig. 6. Changes of POX in response to *P. capsici* infection, as influenced by control (-■-), *B. subtilis* HJ927 (-◆-)-, *B. subtilis* HJ927+*P. capsici* (-▲-)-, and *P. capsici* (-●-)-treated plant leaves (A) and roots (B).
Mean values were 6 replicates. Bars represent standard error.

more in *P. capsici*-treated plants than in *B. subtilis* HJ927+*P. capsici*-treated plants, the plants were not protected from *P. capsici* and finally died (Fig. 2).

These results are consistent with our previous findings that, although disease was reduced by *G. intraradics* [30], mycorrhizae *Glomus intraradics* alleviated the increase or decrease of both PR proteins and antioxidative enzymes in pepper plants preinoculated with *G. intraradics*+*P. capsici*, compared with *P. capsici*.

These results are consistent with the result of Hoffland *et al.* [11, 12], who demonstrated that *P. fluorescens* WCS 417r-mediated ISR against *F. oxysporum* was not associated with PR protein in roots and leaves of radish. Pieterse *et al.* [26] indicated that *P. fluorescence* WCS 417r induced systemic resistance in arabidopsis without activating PR gene expression and subsequent accumulation of PR proteins. Similar results have also been observed in earlier studies [3, 6, 30]. All these data suggested that there is no close quantitative or qualitative relationship between the PR protein and the acquired systemic resistance. Therefore, the role of PR proteins as defense mechanisms in pepper remains questionable.

On the other hand, plants are able to defend themselves from phytopathogenic agents by producing a wide spectrum of antimicrobial compounds, among which the oxidoreductive enzymes such as POX and PPO have been implicated in cellular protection and disease resistance [22]. Indeed, we observed that disease symptoms caused by infection of *P.*

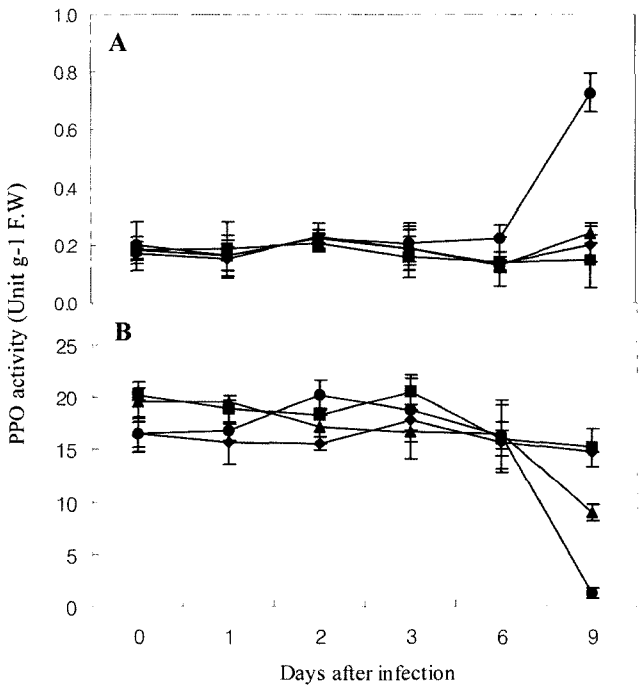

Fig. 7. Changes of PPO in response to *P. capsici* infection, as influenced by control (-■-), *B. subtilis* HJ927 (-◆-)-, *B. subtilis* HJ927+*P. capsici* (-▲-)-, and *P. capsici* (-●-)-treated plant leaves (A) and roots (B).
Mean values were 6 replicates. Bars represent standard error.

capsici were less severe in *B. subtilis* HJ927+*P. capsici*-treated plants than in *P. capsici*-treated plants, in which PR proteins and enzymes of phenylpropanoid metabolisms were less activated. POX, PPO, and PAL activities in roots of *B. subtilis* HJ927+*P. capsici*-treated plants were decreased, but increased in leaves. However, the extents of decrease and increase were much greater in *P. capsici*-treated plants than those in *B. subtilis* HJ927+*P. capsici*-treated plants, although *P. capsici*-treated plants were severely damaged. This agrees with Mozzetti *et al.* [23], who demonstrated that more PAL, PPO, and POX were induced in the susceptible pepper plant than in the resistant ones after inoculation with *P. capsici*, although there was greater plant protection in the resistant pepper plants. We suggest that changes of these enzyme activities as a consequence of inoculation are generally associated with disease symptom rather than resistance.

Changes of induced enzyme activities in biocontrol of late blight in pepper by *Bacillus subtilis* HJ927 might be associated with plant response or disease severity rather than resistance against pathogen attack.

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