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Defensive-related enzyme response in plants treated with a mixture of *Bacillus* strains (IN937a and IN937b) against different pathogens

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Abstract

In previous research, a mixture of *Bacillus amyloliquefaciens* strain IN937a and *Bacillus pumilus* strain IN937b consistently provided systemic protection against multiple diseases in various crops. The objective of this study was to investigate defense-related enzyme responses in plants induced by a mixture of IN937a and IN937b against different pathosystems. Four plant/pathosystems, tomato with *Sclerotium rolfsii* and *Ralstonia solanacearum* and pepper with *S. rolfsii* and *Colletotrichum gloeosporioides*, were used to test the efficacy of the mixture in greenhouse assays. Treatments consisted of non-challenged healthy control, nonbacterized pathogen control, and bacterized with a mixture of IN937a and IN937b and challenged later with pathogens. Total superoxide dismutase (SOD) and peroxidase (PO) activities were investigated. Before pathogen inoculation, higher levels of SOD and PO activities were observed in plants treated with a mixture of IN937a and IN937b compared with non-challenged healthy and nonbacterized pathogen controls. After challenge with all pathogens, plants treated with the bacterial mixture had SOD and PO activity levels 25–30% greater than the nonbacterized pathogen control. Additionally, significant disease protection in each plant pathosystem was observed with the bacterial mixture. Low levels of natural SOD and PO activities in the non-challenged healthy control occurred during the assay. In conclusion, a mixture of IN937a and IN937b induced similar responses of SOD and PO activities against different pathogens, and these physiological changes were associated with disease protection with all the tested pathogens.
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Keywords: Defensive-related enzymes; Different pathosystems; Induced systemic resistance; Plant growth-promoting rhizobacteria; Peroxidase; Superoxide dismutase

1. Introduction

In general, plant growth-promoting rhizobacteria (PGPR) have the capacity to provide protection against diverse pathogens (van Loon et al., 1998). This phenomenon has been demonstrated by many greenhouse and field experiments (Hoffland et al., 1996; Raupach and Kloepper, 1998; Jetiyanon and Kloepper, 2002; Jetiyanon et al., 2003; Silva et al., 2004).

Recently, defensive-related mechanisms associated with PGPR-mediated induced systemic resistance (ISR) against incoming pathogen have been thoroughly investigated. In contrast to systemic acquired resistance (SAR), PGPR-mediated ISR is generally independent of salicylic acid accumulation and not associated with induction of genes encoding pathogenesis-related proteins (Hoffland et al., 1995; Pieterse et al., 1996; van Wees et al., 1999). However, certain metabolic changes have been demonstrated in association with ISR (Jetiyanon et al., 1997; Benhamou et al., 2000; Ramamoorthy et al., 2002a, 2002b).

Reactive oxygen species (ROS) including superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) are often detected in plant–pathogen interactions and are associated with symptom development. Plants have acquired the relevant protective defense mechanisms to maintain the lowest possible levels of ROS inside the cell

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during stress circumstance (Wojtaszek, 1997). During early plant response to pathogen infection, the O_2^- generated is usually rapidly dismutated via superoxide dismutase (SOD) catalysis to H_2O_2. Newly formed H_2O_2 could be directly toxic to microbes (Peng and Kúc, 1992), dismutated by catalase to form water and oxygen, detoxified by ascorbate peroxidase, or may contribute to the structural reinforcement of plant cell wall via peroxidase activity (Bolwell et al., 1995) thus rapidly making the plant cell wall more resistant to microbial penetration and enzymatic degradation (Bradley et al., 1992; Brisson et al., 1994).

Several studies of beneficial rhizobacteria have reported that SOD and/or PO enzymes are associated with ISR (Jetiyanon et al., 1997; Ramamoorthy et al., 2002a, 2002b; Silva et al., 2004). SOD (EC 1.15.1.1) is part of a group of antioxidative enzymes that are most important in amelioration the damage caused by oxidative stress (Alscher et al., 2002; De Gara et al., 2003). Additionally, the role of PO (EC 1.11.1.7), which is associated with the macromolecular assembly of the poly(phenolic) domain of lignifying/suberizing tissues, has been intensively studied in solanaceous and cucurbits plants (Hammerschmidt and Kuc, 1982; Hammerschmidt et al., 1982, 1984; Borg-Olivier and Monties, 1993; Bernards et al., 1995, 1999). However, most studies of rhizobacteria-mediated ISR have focused on investigating the presence or the greater extent of those enzymes against either soil-borne or air-borne pathogens. Furthermore, little evidence has been demonstrated for defensive-related enzymes in plants induced by the same PGPR strain against both soil-borne and air-borne pathogens, especially in different biological systems assayed.

Previous investigations demonstrated that a mixture of PGPR strains (*Bacillus amyloliquefaciens* strain IN937a and *Bacillus pumilus* strain IN937b) consistently provided a broad spectrum of disease protection against both soil- and air-borne pathogens, including cucumber mosaic virus, *Colletotrichum gloeosporioides, Ralstonia solanacearum*, *Rhizoctonia solani*, and *Sclerotium rolfsii* (Jetiyanon and Kloepper, 2002; Jetiyanon et al., 2003). However, it is unclear whether the observed disease protection relates to SOD and PO enzymes. The objective of this study was to investigate the defensive-related enzyme responses (i.e. SOD and PO) of plants treated with a mixture of IN937a and IN937b against one air-borne pathogen (*C. gloeosporioides*) and two soil-borne pathogens (*S. rolfsii* and *R. solanacearum*), especially after pathogen inoculation.

2. Materials and methods

2.1. Source of bacterial cultures

Bacillus amyloliquefaciens strain IN937a and *B. pumilus* strain IN937b were obtained from the culture collection of the phytobacteriology laboratory of Auburn University (Auburn, AL, USA). The bacteria were maintained in tryptic soy broth (TSB) (Becton Dickinson, Sparks, MD, USA) supplemented with 20% glycerol at −80 °C for long-term storage. For experimental use, each bacterial strain was transferred onto tryptic soy agar (TSA; Becton Dickinson) and incubated at 28–30 °C for 24 h. The bacterial cells were harvested and suspended in 10 ml autoclaved double distilled water (ddH_2O). Each bacterial concentration was then adjusted to 10^5 or 10^4 CFU/ml in autoclaved ddH_2O. A mixture of IN937a and IN937b was prepared by combining equal proportion of each strain prior to application to seed (10^{10} CFU/ml of final concentration) or root (10^8 CFU/ml of final concentration) treatment.

2.2. Source of pathogens and inoculum preparation

Ralstonia solanacearum (Smith) race 1 biovar 3 the causal agent of bacterial wilt disease of tomato, *C. gloeosporioides* (Penz.) Penz. and Sacc. the causal agent of anthracnose disease of pepper, and *S. rolfsii* Sacc. the causal agent of southern blight disease were obtained from the Plant Pathology and Microbiology Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand.

Spore suspensions of *C. gloeosporioides* and bacterial suspensions of *R. solanacearum* were maintained in cryovials containing TSB supplemented with 20% glycerol and kept at −80 °C for long term storage. Mycelia of *S. rolfsii* were maintained on potato dextrose agar (PDA; Becton Dickinson) and stored at room temperature. For experimental use, pathogens were produced by transferring *C. gloeosporioides* on PDA and incubated at 30 °C for 7–10 days, *R. solanacearum* in TSB and incubated in an orbital incubator shaker (100 rpm, Gyromax 707, Amerex Instrument, Lafayette, CA) at 30 °C for 24 h, and *S. rolfsii* on PDA and incubated at 30 °C for 4–5 days.

2.3. Greenhouse assays

Four experimental assays were conducted under greenhouse conditions. They were long cayenne pepper against *C. gloeosporioides*, tomato against *R. solanacearum*, tomato against *S. rolfsii*, and long cayenne pepper against *S. rolfsii* were tested. Each assay was conducted two times. In each assay, the experimental design was a factorial with randomized complete block. There were three treatments in each assay including a non-challenged healthy control, a nonbacterized pathogen control, and bacterized with a mixture of IN937a and IN937b and challenged later with pathogens. The bacterized treatment alone was not included in the assay, since this study was focused on investigating the defensive-related enzyme responses of plants after pathogen inoculation in different plant/pathosystems. The factors were sampling times before and after challenge with the pathogen and the time of disease rating. In assays of tomato against *R. solanacearum* and *S. rolfsii*, and long cayenne pepper against *S. rolfsii*, 12 plants (pots) per treatment were used as replicates in each sampling time and the time of disease rating. Each pot contained one plant. Five plants (pots) per treatment in each sampling time and the

time of disease rating were used as replicates in the assay of long cayenne pepper against *C. gloeosporioides*.

2.4. Source of seeds and treatment with bacteria

In bacterized treatment, seeds of tomato (*Lycopersicon esculentum* Mill cv. Srida; obtained from CHIA TAI Co., Ltd., Thailand) and long cayenne pepper (*Capsicum annuum* L. var. *acuminatum* Fingerh cv. 111 CHANYA; obtained from Known-You Seed Company, Thailand) were soaked in bacterial suspensions (10^{10} CFU/ml) maintained in 250-ml flasks and were then incubated in the orbital incubator shaker (100 rpm, Gyromax 707, Amerex Instrument, Lafayette, CA) at 30 °C for 60 min. They were planted in a polyetyrene seedling tray (Thai Charoen Thong Karntor Co., Ltd., Thailand) containing sterile soilless peat-based medium (Klasmann–Deilmann GmbH, Geeste-Groß Hesepe, Germany). Seeds of nonbacterized and non-challenge controls were soaked in autoclaved ddH_2O. Seven days after seeding, tomato and long cayenne pepper plants were transplanted into 10-cm-diam. plastic pots containing sterile soilless peat-based medium. Each pot contained one plant.

Fifteen days after seeding, each pot of tomato and pepper in bacterized treatment was drenched with 100 ml of bacterial suspension (10^8 CFU/ml). One hundred milliliters of autoclaved ddH_2O was drenched in each pot of nonbacterized and non-challenge controls. The temperature in the greenhouse was 33 °C during the day and 27 °C at night. The humidity was approximately RH 80–85%.

2.5. Southern blight disease

For challenge inoculation, a PDA culture of *S. rolfsii* (completely cover the plate) was cut into pieces (1.5×1.5 cm^2). Each piece was placed adjacent to plant's stem base 15 days after soil drench. A piece of PDA without the pathogenic fungus was placed adjacent to plant's stem base of non-challenge healthy control. Plant stems (3-cm long above the infection site) in each treatment were collected at different time intervals consisting of 0, 12, 24, 36, and 48 h after challenge (HAC) with the pathogen. Samples were immediately transferred to ultra freezer (−80 °C) for further biochemical analysis.

Disease incidence was observed over time and was rated 6 days after challenge. Plants were observed for another 14 days. Plants showing either external (yellowing and wilting) or internal symptoms (browning of the vascular tissue) were scored as diseased.

2.6. Bacterial wilt disease

Fifteen days after soil drench, plant roots were cut with a sterile scissors and the cut roots were drenched with *R. solanacearum* (10^8 CFU/ml; 100 ml per pot). The non-challenged healthy control received drenches of 100 ml of autoclaved ddH_2O per pot. Plants were kept in a dark moist chamber for 22 h and were transferred to the greenhouse. Plant stems (3-cm long above the soilless peat-base medium line) in each treatment were collected at different time intervals consisting of 0, 24, 96, 168, and 240 HAC with the pathogen. Samples were immediately transferred to the ultra freezer (−80 °C) for further biochemical analysis.

Disease incidence was monitored daily and recorded 10 days after challenge. Plants were observed for another 14 days. Plants showing either external (wilting) or internal symptoms (browning of the vascular tissue) were scored as diseased.

2.7. Anthracnose disease

A fertilizer (15–15–15; N–P–K) was applied 30 days after planting. When the pepper fruits were setting, a fertilizer (13–13–21; N–P–K) was applied. Plants were challenged 60 days after planting. At this stage, the fruits were fully developed having 3–4 fruits per plant. For challenge inoculation, pepper fruits were drenched with fungal spore suspension of *C. gloeosporioides* (10^5 spores/ml) supplemented with 0.05% of Triton X-100 (Fluka, Switzerland). An autoclaved ddH_2O supplemented with 0.05% of Triton X-100 was used for drenching fruit in the non-challenged healthy control. The plants were then maintained in a moist chamber for 24 h and were transferred to a greenhouse. Two pepper fruits were randomly collected from each plant at different time intervals consisting of 0, 24, 96, 168, and 240 HAC. When the disease appeared on the fruit surface, only healthy tissues approximately 1 cm width around the diseased area were cut and kept in −80 °C for further biochemical analysis.

Disease severity was observed daily and rated as described by Jetiyanon and Kloepper (2002) 14 days after challenge.

2.8. Enzyme activity assay

Samples (1 g) were flash-frozen in liquid N_2, crushed into a fine powder in a mortar with a pestle. They were homogenized in 2 ml of a pre-chilled 0.1 M Tris–HCl buffer, pH 7.0 containing 1% polyvinyl-polypyrrolidone (Sigma–Aldrich, Inc., MO, USA). The homogenate was centrifuged at 10,000*g* in a refrigerated tabletop centrifuge (SORVALL® Biofuge Stratos, Kendro Laboratoty Products, Germany) for 10 min at 4 °C. The supernatant to be used for the enzymatic activity assay was transferred to a 1.5 ml vial and stored at −20 °C. A colorimetric assay for enzyme activity was performed with a DR/4000U Spectrophotometer (HACH Company, CO, USA). The reaction rates were linear and proportional to the enzyme or protein concentration added. The standard Bradford assay (1976) was employed to test the protein concentration for plant extracts in each sample.

2.8.1. Superoxide dismutase activity (SOD)

All extracts were tested for SOD activity using the riboflavin/methionine system (Beauchamp and Fridovich,

1971). The 1 ml reaction mixture in 3 ml tube contained 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 μM nitroblue tetrazolium, 2 μM riboflavin, and the enzyme extract. Riboflavin was added last. The tubes were stirred and the reaction was initiated by placing the tubes under two 18 W fluorescent lamps. After 10 min, the reaction was terminated by switching off the light. Nonilluminated tubes served as blanks. The tubes were stirred and the reaction mixture was then poured into 1.5 ml cuvettes. Total SOD activity was assayed spectrophotometrically at 560 nm. One unit of SOD activity is defined as the amount that inhibits nitroblue tetrazolium photoreduction by 50% under the assay conditions. All chemicals were purchased from Sigma–Aldrich, Inc.

2.8.2. Peroxidase activity (PO)

All extracts were tested for peroxidase activity using guaiacol as the hydrogen donor. Procedures were modified from Hammerschmidt et al. (1982). The 1 ml reaction mixture in 1.5 ml cuvette contained 0.25% (v/v) guaiacol in 0.01 M sodium phosphate buffer (pH 6.0), enzyme extract, and 0.1 M H_2O_2. Enzyme extract was added last to initiate the reaction. The changes in absorbance at 470 nm were recorded at 30 s intervals for 3 min. The enzyme activity was expressed as changes in the absorbance min^{-1} mg $protein^{-1}$. All chemicals were purchased from Sigma–Aldrich, Inc.

2.9. Statistical analysis

All data were analyzed by analysis of variance (ANOVA) and the treatment means were separated by using Fisher's protected least significant difference (LSD) test $P \leqslant 0.05$ using SAS software (SAS Institute, Gary, NC, USA).

3. Results

3.1. Disease protection in four different plant/pathosystems

3.1.1. Tomato against S. rolfsii

Four days after challenge, the leaves began to turn yellow, wilt, or die from the tips downward in the nonbacterized pathogen control and some plants of the bacterized treatment. The fungus grew upward in the plant and covered the tomato stem with a cotton-like, white mycelial mass. Invaded stem tissues were pale brown. A mixture of IN937a and IN937b resulted in significant ($P \leqslant 0.05$) disease reduction of 46% compared with nonbacterized pathogen control (Table 1). The number of diseased plants in bacterized treatment did not increase over time after the initial disease rating.

3.1.2. Long cayenne pepper against Sclerotium rolfsii

Three days after challenge, lower leaves of most nonbacterized pathogen control began drooping, turned yellow, and then wilted. Leaf stalks, especially at the bottom, also detached from the main stem. Invaded stem tissues were pale brown. A mixture of IN937a and IN937b elicited significant ($P \leqslant 0.05$) disease suppression of 46% compared with nonbacterized pathogen control (Fig. 1A and Table 1). The number of diseased plants in the bacterized treatment did not increase over time after the initial disease rating.

Table 1
Efficacy of a mixture of IN937a and IN937b for protection against four different plant/pathogen systems under greenhouse condition[a]

Treatment	Number of symptomatic[c] plant in tomato/*S. rolfsii*	Number of symptomatic[d] plant in pepper/*S. rolfsii*	Number of symptomatic[e] plant in tomato/*R. solanacearum*	Disease severity of infected[f] pepper fruits in pepper/*C. gloeosporioides*
Nonbacterized pathogen control	12.0 a[g]	12.0 a	12.0 a	66.25 a
Mixture of IN937a and IN937b[b]	6.5 b	6.5 b	6.0 b	38.75 b
Non-challenged healthy control	0.0 c	0.0 c	0.0 c	0.00 c
$LSD_{0.05}$	4.05	4.05	3.51	10.32

[a] Each assay consisted of 3 treatments (non-challenged healthy control, nonbacterized pathogen control, and bacterized with a mixture of IN937a and IN937b and challenged later with pathogens). In assays of tomato against *R. solanacearum* and *S. rolfsii*, and long cayenne pepper against *S. rolfsii*, twelve plants (pots) per treatment were used as replicates. Only five plants (pots) per treatment were used as replicates in the assay of long cayenne pepper against *C. gloeosporioides*. Each assay was examined independently twice. The data shown in the table is the mean from two assays.

[b] IN937a = *Bacillus amyloliquefaciens* strain, IN937a = *Bacillus pumilus* strain IN937b.

[c] Symptomatic plants were recorded 6 days after challenge with *Sclerotium rolfsii* by determining the incidence of leaves showing yellowing and wilting.

[d] Symptomatic plants were recorded 6 days after challenge with *Sclerotium rolfsii* by determining the incidence of leaves showing yellowing and wilting.

[e] Symptomatic plants were recorded 10 days after challenge with *Ralstonia solanacearum* by determining the incidence of leaves showing wilting.

[f] Disease severity was rated 14 days after challenge with *Colletotrichum gloeosporioides* by determining percentage of fruit area covered with lesions. (0 = all fruits were healthy, 10 = 10% of fruit area was destroyed, 25 = 25% of fruit area was destroyed, 50 = 50% of fruit area was destroyed, 75 = 75% of fruit area was destroyed, and 100 = 100% of fruit area was destroyed).

[g] Numbers with different letter show significant differences at $P \leqslant 0.05$ according to least significant difference (LSD) test.

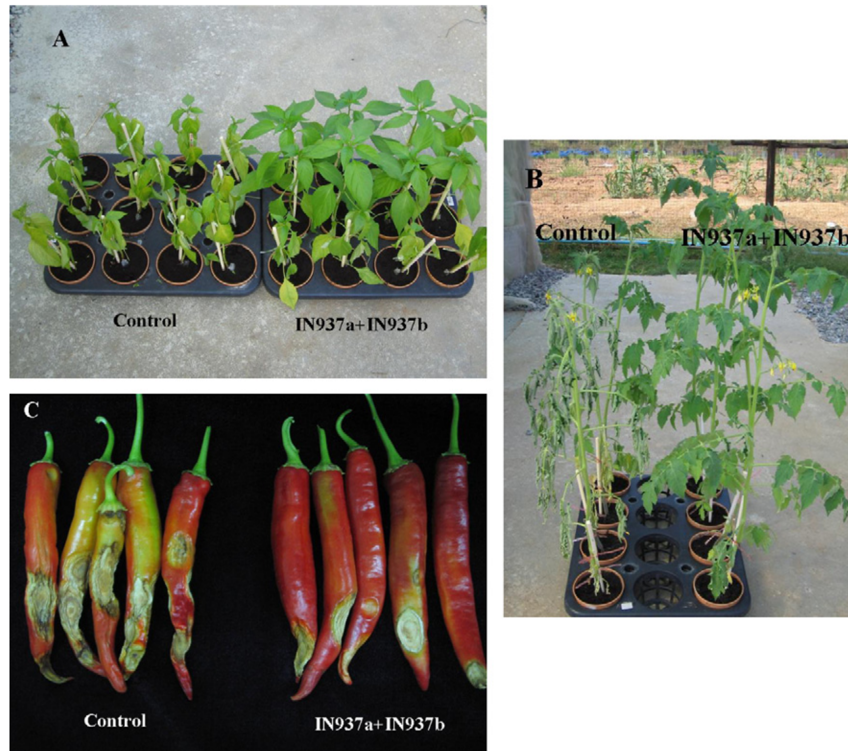

Fig. 1. The ability of a mixture of IN937a and IN937b for induced systemic protection against southern blight (A), bacterial wilt (B), and anthracnose (C).

3.1.3. Tomato against *R. solanacearum*

Four days after challenge, susceptible plants showed initial symptom as one-sided wilting during day time and recovering at night time. Then, the wilt gradually developed throughout the whole plant resulting in a permanent wilting. All plants in the nonbacterized pathogen control reached the permanent wilting stage 10 days after challenge. Bacterized treatment provided significant ($P \leqslant 0.05$) disease suppression of 50% compared with the nonbacterized pathogen control (Fig. 1B and Table 1). The number of diseased plants in the bacterized treatment did not increase over time after the initial disease rating.

3.1.4. Long cayenne pepper against *C. gloeosporioides*

Several small lesions first appeared at the infection site of susceptible fruits 4 days after challenge. Each lesion quickly developed as a sunken area, expanded and merged into a larger patch. Fruit distortion also occurred on some fruits. However, lesions on pepper fruits induced by a mixture of IN937a and IN937b developed slower, leading to smaller lesions than those on the nonbacterized pathogen control. Fourteen days after challenge, the mixture of IN937a and IN937b showed significant reduction in disease severity of 42% compared with the nonbacterized pathogen control (Fig. 1C and Table 1).

3.2. Induction of SOD activity in four different plant/pathosystems

At 0 HAC, total SOD activity in the mixture of IN937a and IN937b was generally higher than those in the nonbacterized pathogen control and non-challenged healthy control. Some amount of natural SOD activity in non-challenged healthy control was consistently presented throughout the assays (Fig. 2A–D). In the assay of tomato against *S. rolfsii*, the enzyme activity in both the nonbacterized pathogen control and the mixture of IN937a and IN937b increased overtime after challenge. During 12–48 HAC, mixture of IN937a and IN937b resulted in significant increase ($P \leqslant 0.05$) of enzyme activity ranging from 27–37% comparing to nonbacterized pathogen control (Fig. 2A).

In the assay of pepper against *S. rolfsii*, after challenge inoculation, the pattern of enzyme activity in the nonbacterized pathogen control and the mixture of IN937a and IN937b was similar to the tomato system. The level of an increased enzyme activity in both treatments was approximately 50% less than in the tomato system; however, a similar significant difference of enzyme activity was also observed with the mixture of IN937a and IN937b compared to the nonbacterized pathogen control (Fig. 2B).

In the assay of tomato against *R. solanacearum*, the enzyme activity in both the nonbacterized pathogen control and the mixture of IN937a and IN937b increased over time after challenge until reaching the highest level at 168 HAC and then declined from 168–240 HAC. During 24–240 HAC, the mixture of IN937a and IN937b gave significant difference of enzyme activity ranging from 20–32% compared with the nonbacterized pathogen control (Fig. 2C). In the assay of pepper against *C. gloeosporioides*, the level of SOD activity in all treatments was the least compared with the other three plant/pathosystems

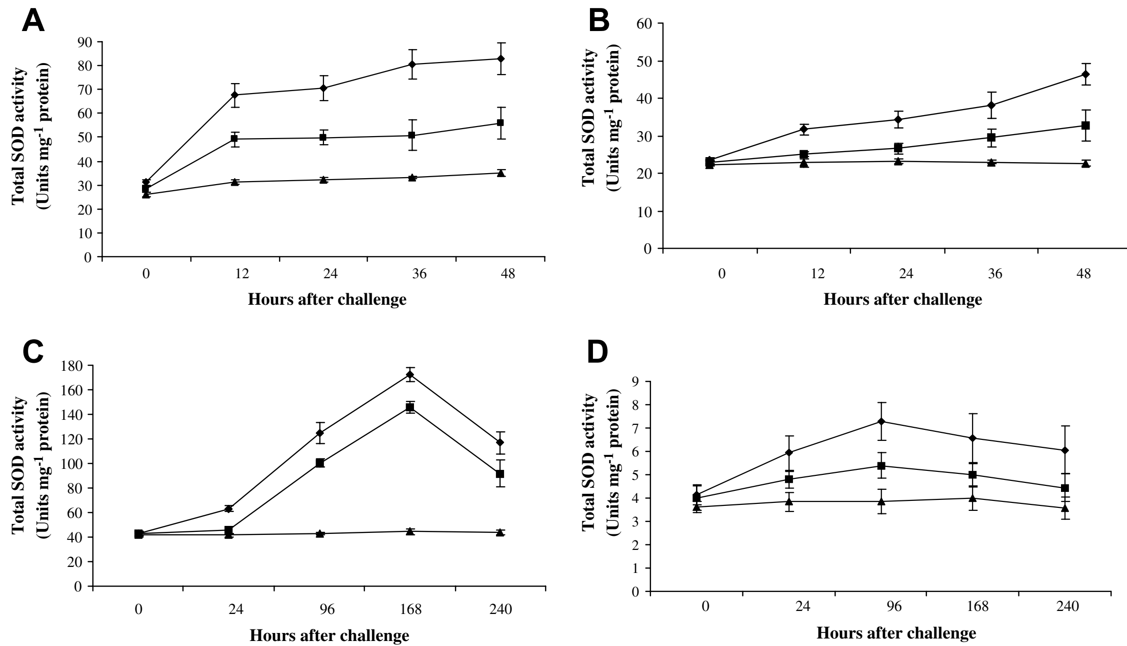

Fig. 2. Influence of a mixture of IN937a and IN937b on total superoxide dismutase (SOD) activity before and after challenge in four different plant/pathosystems: (A) tomato/*Sclerotium rolfsii* (B) long cayenne pepper/*Sclerotium rolfsii* (C) tomato/*Ralstonia solanacearum* and (D) and long cayenne pepper/*Colletotrichum gloeosporioides*. Non-challenged healthy control (▲), nonbacterized pathogen control (■), and a mixture of IN937a and IN937b and challenged later with pathogens (♦). In each plant/pathosystem, each value at particular sampling time is the mean from two independent experiments. Vertical bars represent standard deviations from mean of four replications.

(Fig. 2D). After challenge inoculation, the pattern of an increased enzyme activity in the nonbacterized pathogen control and mixture of IN937a and IN937b was similar to the tomato against *R. solanacearum*, but the highest level was reached earlier at 96 HAC. During 24–168 HAC, mixture of IN937a and IN937b also provided significant difference of enzyme activity ranging from of 20–36% compared with the nonbacterized pathogen control.

3.3. Induction of PO activity in four different plant/pathosystems

Generally, at 0 HAC, the mixture of IN937a and IN937b had higher total PO activity than the nonbacterized pathogen control and the non-challenged healthy control. Some amount of natural PO activity in the non-challenged healthy control was consistently expressed throughout the assays (Fig. 3A–D). In the assay of tomato against *S. rolfsii*, the PO activity in both the nonbacterized pathogen control and mixture of IN937a and IN937b slightly increased during 12–24 HAC and then substantially inclined during 36–48 HAC. A mixture of IN937a and IN937b showed significant increase in enzyme activity of 47% at 36 HAC and 26% at 48 HAC compared with the nonbacterized pathogen control (Fig. 3A). In the assay of pepper against *S. rolfsii*, after pathogen inoculation, the pattern of PO activity in mixture IN937a + IN937b and the nonbacterized pathogen control was similar to SOD activity. During 24–48 HAC, the mixture of IN937a and IN937b provided significant increase in enzyme activity ranging from 31% to 36% compared with the nonbacterized pathogen control (Fig. 3B).

In the assay of tomato against *R. solanacearum*, during 24–240 HAC, the percentage of significant difference of PO activity in the mixture of IN937a and IN937b was 17–31% greater than the nonbacterized pathogen control (Fig. 3C). The pattern of PO activity in both treatments was similar to the SOD activity, but the highest level of PO activity was reached earlier at 96 HAC. During 96–240 HAC, the decline in activity of enzyme in the mixture of IN937a and IN937b was slower than in the nonbacterized pathogen control. In the assay of pepper against *C. gloeosporioides*, the PO activity in both the nonbacterized pathogen control and the mixture of IN937a and IN937b increased overtime after challenge (Fig. 3D). During 24–240 HAC, mixture of IN937a and IN937b showed significant increase in enzyme activity ranging from 39–53% compared with the nonbacterized control.

4. Discussion

The results presented here indicate that a mixture of two bacteria previously shown to elicit induced systemic resistance induces activity of SOD and PO before inoculation with pathogens. This finding is in agreement with the suggestion by M'Piga et al. (1997) that biotic inducers may evoke transcriptional activation of plant defense genes. Similarly, several studies have reported that biocontrol agents can stimulate other plant defense-related enzymes, callose, or phenolic compounds (Zdor and Anderson,

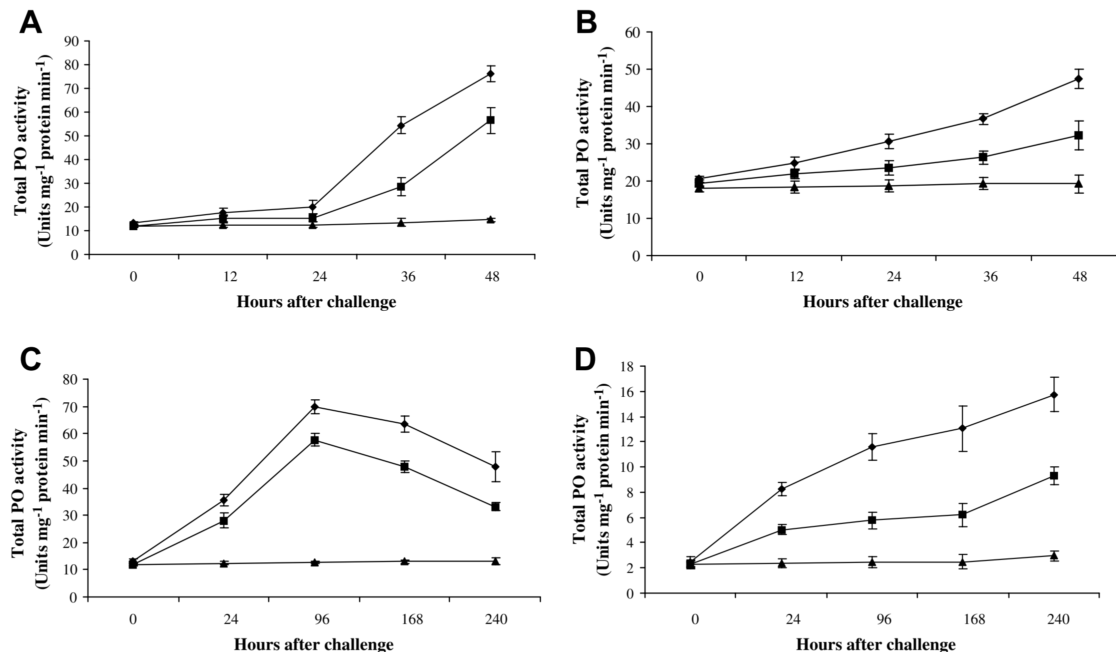

Fig. 3. Influence of mixture of IN937a and IN937b on total peroxidase (PO) activity before and after challenge in four different plant/pathosystems: (A) tomato/*Sclerotium rolfsii* (B) long cayenne pepper/*Sclerotium rolfsii* (C) tomato/*Ralstonia solanacearum* and (D) long cayenne pepper/*Colletotrichum gloeosporioides*. Non-challenged healthy control (▲), nonbacterized pathogen control (■), and a mixture of IN937a and IN937b and challenged later with pathogens (♦). In each plant/pathosystem, each value at particular sampling time is the mean from two independent experiments. Vertical bars represent standard deviations from mean of four replications.

1992; Yedidia et al., 1999; Chen et al., 2000; Bargabus et al., 2002).

In previous studies, rhizobacteria were found to induce increased activity of SOD (Jetiyanon et al., 1997) and PO (Jetiyanon et al., 1997; Ramamoorthy et al., 2002a, 2002b; Silva et al., 2004) after pathogen challenge. In the present study, a mixture of two rhizobacteria was also found to elicit increased activity of SOD and PO in plants 48 h after challenge compared to nonbacterized pathogen control. The SOD and PO activities in nonbacterized pathogen control plants increased after challenge, indicating that some increased enzyme activity is a natural response of susceptible infected plants to pathogen attack. However, the level of increase was too low to overcome pathogen invasion, and disease resulted.

The percentage of significant difference in SOD and PO activity between the mixture of IN937a and IN937b and the nonbacterized pathogen control was similar among the four tested pathosystems. The SOD response (Fig. 2) in induced plants in all pathosystems was approximately 25% greater than in nonbacterized pathogen control, while the PO response in most pathosystems (Fig. 3) was approximately 30%. Furthermore, similar enzyme responses in induced plants were also correlated with the same level of disease protection against different pathogens (Table 1). In the system of tomato and pepper against *Pythium aphanidermatum* (Ramamoorthy et al., 2002a), tomatoes treated with *Pseudomonas fluorescens* isolate Pf1 expressed a greater percentage of enhanced PO activity, compared to the nonbacterized pathogen control, than did peppers . In addition, bacterial-treated tomato had higher disease suppression than pepper. These findings suggest that disease protection is proportional to the amount of enhanced PO enzyme activity.

The higher level of SOD activity elicited by the mixture of IN937a and IN937b found in the present study may play an important role in ameliorating damage to plant cells caused by pathogens, which would partially account for the observed delay in symptom development with bacterial treatment. In nonbacterized pathogen control plants, SOD activity increased at a slower rate, while symptoms developed faster. Increased PO activity in induced tomato and pepper plants, observed in the present study upon treatment with the bacterial mixture, may lead to accumulation of lignin which is another important physical barrier to impede pathogen invasion. Thus, increases in both SOD and PO activity elicited by the bacterial mixture could explain the observed disease suppression in all the tested pathosystems.

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References

Alscher, R.G., Erturk, N., Heath, L.S., 2002. Role of superoxide dismutase (SODs) in controlling oxidative stress in plants. Journal of Experimental Botany 53, 1331–1341.

Bargabus, L.R., Zidack, N., Sherwood, J.E., Jacobson, B.J., 2002. Characterization of systemic resistance in sugar beet elicited by a non-pathogenic, phyllosphere-colonizing *Bacillus mycoides*, biological control agent. Physiological and Molecular Plant Pathology 61, 289–298.

Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assays and assay applicable to acrylamide gels. Analytical Biochemistry 44, 276–287.

Benhamou, N., Gagné, S., Quéré, D.L., Dehbi, L., 2000. Bacterial-mediated induced resistance in cucumber: beneficial effect of the endophytic bacterium *Serratia plymuthica* on the protection against infection by *Pythium ultimum*. Phytopathology 90, 45–56.

Bernards, M.A., Lopez, M.L., Zajicek, J., Lewis, N.G., 1995. Hydroxycinnamic acid-derived polymers constitute the polyaromatic domain of suberin. The Journal of Biological Chemistry 270, 7382–7386.

Bernards, M.A., Fleming, W.D., Llewellyn, D.B., Priefer, R., Yang, X., Sabatino, A., Plourde, G.L., 1999. Biochemical characterization of the suberization-associated anodic peroxidase of potato. Plant Physiology 121, 135–145.

Bolwell, G.P., Butt, V.S., Davies, D.R., Zimmerlin, A., 1995. The origin of the oxidative burst in plants. Free Radical Research 23, 517–532.

Borg-Olivier, O., Monties, B., 1993. Lignin, suberin, phenolic acids and tyramine in the suberized, wound-induced potato periderm. Phytochemistry 32, 601–606.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248–254.

Bradley, D.J., Kjellbom, P., Lamb, C.J., 1992. Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. Cell 70, 21–30.

Brisson, L.F., Tenhaken, R., Lamb, C.J., 1994. Function of oxidative cross-linking of cell wall structural proteins in plant disease resistance. The Plant Cell 6, 1703–1712.

Chen, C., Bélanger, R., Benhamou, N., Paulitz, T.C., 2000. Defense enzymes induced in cucumber roots by treatment with plant growth-promoting rhizobacteria (PGPR) and *Pythium aphanidermatum*. Physiological and Molecular Plant Pathology 56, 13–23.

De Gara, L., de Pinto, M.C., Tommasi, F., 2003. The antioxidant systems vis-à-vis reactive oxygen species during plant-pathogen interaction. Plant Physiology and Biochemistry 41, 863–870.

Hammerschmidt, R., Kuć, J., 1982. Lignification as mechanism for induced systemic resistance in cucumber. Physiological Plant Pathology 20, 61–71.

Hammerschmidt, R., Nuckles, E.M., Kuć, J., 1982. Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. Physiological Plant Pathology 20, 73–82.

Hammerschmidt, R., Lamport, D.T.A., Muldoon, E.P., 1984. Cell wall hydroxyproline enhancement and lignin deposition as an early event in the resistance of cucumber to *Cladosporium cucumerinum*. Physiological Plant Pathology 24, 43–47.

Hoffland, E., Bakker, P.A.H.M., Van Loon, L.C., 1996. Comparison of systemic resistance induced by avirulent and nonpathogenic *Pseudomonas* species. Phytopathology 86, 757–762.

Hoffland, E., Pieterse, C.M.J., Bik, L., Van Pelt, J.A., 1995. Induced systemic resistance in radish is not associated with accumulation of pathogenesis-related proteins. Physiological and Molecular Plant Pathology 46, 309–320.

Jetiyanon, K., Tuzun, S., Kloepper, J.W., 1997. Lignification, peroxidase and superoxide dismutases as early plant defense reactions associated with PGPR-mediated induced systemic resistance. In: Ogoshi, A., Kobayashi, K., Homma, Y., Kodama, F., Kondo, N., Akino, S. (Eds.), Plant Growth-promoting Rhizobacteria: Present Status and Future Prospects. Sapporo, Japan, pp. 265–268.

Jetiyanon, K., Kloepper, J.W., 2002. Mixtures of plant growth promoting rhizobacteria for induction of resistance against multiple plant diseases. Biological Control 24, 285–291.

Jetiyanon, K., Fowler, W.D., Kloepper, J.W., 2003. Broad-spectrum protection against several pathogens by PGPR mixtures under field conditions in Thailand. Plant Disease 87, 1390–1394.

M'Piga, P., Bélanger, R.R., Paulitz, T.C., Benhamou, N., 1997. Increased resistance to *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomato plants treated with the endo-phytic bacterium *Pseudomonas fluorescens* strain 63–28. Physiological and Molecular Plant Pathology 50, 301–320.

Peng, M., Kúc, J., 1992. Peroxidase-generated hydrogen peroxide as a source of antifungal activity *in vitro* and on tobacco leaf disks. Phytopathology 82, 696–699.

Pieterse, C.M.J., van Wees, S.C.M., Hoffland, E., van Pelt, J.A., van Loon, L.C., 1996. Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. The Plant Cell 8, 1225–1237.

Ramamoorthy, V., Raguchander, T., Samiyappan, R., 2002a. Enhancing resistance of tomato and hot pepper to *Pythium* diseases by seed treatment with fluorescent *pseudomonads*. European Journal of Plant Pathology 108, 429–441.

Ramamoorthy, V., Raguchander, T., Samiyappan, R., 2002b. Induction of defense-related proteins in tomato roots treated with *Pseudomonas fluorescens* Pf1 and *Fusarium oxysporum* f. sp. *lycopersici*. Plant and Soil 239, 55–68.

Raupach, G.S., Kloepper, J.W., 1998. Mixtures of plant growth promoting rhizobacteria enhance biological control of multiple cucumber pathogens. Phytopathology 88, 1158–1164.

Silva, H.S.A., Romeiro, R. da S., Macagnan, D., Halfeld-Vieira, B. de A., Pereira, M.C.B., Mounteer, A., 2004. Rhizobacterial induction of systemic resistance in tomato plants non-specific protection and increase in enzyme activities. Biological Control 29, 288–295.

van Loon, L.C., Bakker, P.A.H.M., Pieterse, C.M.J., 1998. Systemic resistance induced by rhizosphere bacteria. Annual Review of Phytopathology 36, 453–483.

van Wees, S.C.M., Luijendijk, M., Smoorenburg, I., van Loon, L.C., Pieterse, C.M.J., 1999. Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* is not associated with a direct effect on expression of known defense-related genes but stimulates the expression of the jasmonate-inducible gene Atvsp upon challenge. Plant Molecular Biology 41, 537–549.

Wojtaszek, P., 1997. Oxidative burst: an early plant response to pathogen infection. Biochemical Journal 322, 681–692.

Yedidia, I., Benhamou, N., Chet, I., 1999. Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by biocontrol agent *Trichoderma harzianum*. Applied and Environmental Microbiology 65, 1061–1070.

Zdor, R.E., Anderson, A.J., 1992. Influence of root colonizing bacteria on the defence responses in bean. Plant and Soil 140, 99–107.