Scientific Bulletin, Series F, Biotechnologies, Vol. XVI, 2012
ISSN Online 2285-5521, ISSN-L 2285-1364

DEFENSE RESPONSE INDUCED IN TOMATO PLANTS (*LYCOPERSICON ESCULENTUM*) BY MICROBIAL INFECTIONS

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Abstract

Most of the microorganisms associated with plants are pathogens so plants generally respond to pathogen infections by using their defensive system. Plant pathogen-related (PR) proteins including peroxidase, β-1,3-glucanase, phenylalanine ammonia-lyase and chitinase are proteins that are induced as result of pathogen infections. Chitinases and β-1,3-glucanase can degrade the cell walls of some plant pathogens and that may play a role in the host plant's defensive system. In our experiments we used Bacillus licheniformis designated as Bw, that shown antagonistic activities against various fungal pathogens including Rhizoctonia solani that cause Damping–Off of tomato. Soil application on tomato seedling roots (var. Marmade) of R. solani alone and B. licheniformis in combination with R. solani induced a significant increase in the activities of β-1,3-glucanase and phenylalanine ammonia-lyase (PAL). Peroxidase activity in both fungal and bacterial treatments was also significantly stimulated. In contrast, peroxidase activity was reduced upon inoculation of plants with a mixture of B. licheniformis- R. solani. Results indicated that PR proteins were induced in all treatments with pathogenic or non-pathogenic microorganisms.

Key words: *Bacillus licheniformis,* β-1,3-glucanase, *Lycopersicum esculentum,* peroxidase, phenylalanine ammonia-lyase, *Rhizoctonia solani*.

INTRODUCTION

Plant defence related enzymes, including PR-proteins, are generally induced by plant pathogens or by mechanical injuries. Treatment with various types of biotic and abiotic elicitors leads to increased activities of peroxidase (PO), PAL, PPO, β-1,3-glucanase and chitinase. Peroxidases have been implicated in a number of physiological functions that may contribute to systemic resistance induction, including phenol oxidation, lignification [18] and in deposition of phenolic compounds into plant cell walls during interaction [10]. Enzymes like β-1,3-glucanase and chitinase have been reported to induce systemic resistance and differential expression of defense related genes by pathogen agents and other elicitors, in several crops [20], [12], [13]. This defense proteins have the potential to hydrolyse the major components of fungal cell walls like chitin and β-1,3-glucans respectively [11]. PAL plays an important role in the biosynthesis phenolic phythoalexins [7] and like PO and PPO catalyse the last step in the biosynthesis of lignin or other oxidative phenols. Phenolic compounds may be fungitoxic and may increase the mechanical strength of the host cell wall. In the last decades, many studies have been carried out on the antagonistic capacity of several species of fungi and bacteria (*Trichoderma, Pseudomonas, Bacillus*). Soil application of *P. fluorescens* increased the level of enzymes involved in the phenyl propanoid pathway and pathogenesis related proteins (PR- proteins) in response to *Fusarium oxysporum* f. sp.*lycopersici* causing wilt of tomato and *Colletotrichum capsici* causing fruit rot of pepper [18]. *Bacillus subtilis* is known to control diseases caused by the fungi such as *Rhizoctonia solani* and *Fusarium solani* [1].

In the present study we have evaluated *Bacillus licheniformis* Bw for its ability to control *Rhizoctonia solani* which is the causal agent of damping off of tomato seedlings. Induction of defense-related enzymes in tomato plants was investigated after soil application of different treatments.

MATERIALS AND METHODS

Microbial strains and culture conditions. *Bacillus licheniformis* BW strain, selected in previous experiment for its antifungal activity was used in this work. The fungal pathogen *Rhizoctonia solani* was kindly provided by dr. Maria Oprea from Institute for Plant Protection- Bucharest. The isolates were maintained on slants of potato-dextrose-agar (PDA) (*R. solani*) and nutrient agar (NB) (*B. licheniformis*).

Plant materials and experimental treatments. Tomato seeds (*Lycopersicum esculentum* cv. Marmande), after sterilization and drying, were grown in pots containing sterilized soil (500 g /pot). 14 days after, uniform seedling were selected and transplanted to pots divided into 5 groups (15 seedlings/ group) and treated as the following:

- Plants without any treatments witch serve as healthy control;
- Plants infected with *Bacillus licheniformis* (antagonistic control);
- Plants infected with *Rhizoctonia solani* (infected control);
- Plants infected firstly with *Rhizoctonia solani* at the time of transplanting, and than with *Bacillus licheniformis* Bw suspension.

Induction of defense responses. For the pots inoculated with *R. solani* alone, discs (5 mm) of the pathogen were cut from the edge of 3-days-old culture and incorporated in the root zone of the each tomato seedling. Bacterial inoculum containing $9x10^9$ cfu / ml was used as biocontrol agent against *R. solani*. Plants samples were collected 10 days after treatments application. Fresh plant samples were homogenizated with liquid nitrogen and stored at – 18°C.

Enzymatic assays

Plant tissue (0,5 g) was crushed in a mortar under liquid nitrogen. Soluble proteins were extracted in 50 mM potassium phosphate buffer (pH 7,0. 0,1 mM EDTA, 4 % polyvinylpirolidone (PVP) and 0,2 mM ascorbic acid. After centrifugation at 10000 x for 10 min. at 4°C, the supernatant was used for enzime activity assay.

Estimation of peroxidase (PO) activity. For PO extraction sodium acetate buffer 0,2M (pH 6.5) containing 1% PVP, 15 mM/l β-mercaptoethanol and 0.25% Triton x100 was used.

The reaction mixture contained enzyme extract 0,1 ml, 30 mM guaiacol, 1 ml sodium acetate buffer 0.1 M (pH 5.4), and 0.05 H_2O_2 3 %. Absorption was measured at 470 nm for 5 min at intervals of 60s. Enzyme activity was expressed in units/ ml enzyme. One unit of enzymatic activity represents the changes in D.O. with 0.1 units/ min.

Estimation of β-1,3-glucanase activity. Leaf samples (0,5 g) were extracted with 2 ml sodium acetate buffer 0.05 M (pH 5.0) and centrifugated at 12000 g for 15 min at 4°C. The activity of β-1,3-glucanase was determined by measuring the release of reducing sugars using laminarin dinitrosalicylate method [17]. The reaction mixture contained 62.5 µl laminarin 4% and 62.5 µl enzyme extract was incubated at 40°C for 30 min. The reaction was stopped by adding 375 µl dinitrosalicylic acid reagent (preparated by adding 300 ml NaOH 4,5% to 880 ml containing 8,8 g of dinitrosalicylic acid and 22.5 g K Na tartrate) with subsequent heating for 5 min in a boiling water bath. The resulting solution was dilued with 4,5 ml distilled water and vortexed. Absorption was measured at 500 nm. Enzyme activity was expressed as 1 nmol of reducing substances/ min/ ml enzyme.

Estimation of phenylalanine-ammonia-lyase (PAL) activity. Activity of PAL was evaluated according to the method of Assis et al. (2001) [2]. 0,4 ml enzyme extract was incubated with 0.5 ml of borate buffer 0.1 M (pH 8.8) and 1ml L-phenylalanine (20 mM) for 30 min at 37 °C. The reaction was stopped with 1ml HCl 1N. The assay mixture was

extracted with 3ml toluene by vortexing for 30 sec. The absorbance of toluene phase containing trans- cinnamic acid was measured at 290nm. The amount of trans- cinnamic acid formed was calculated using its extinction coefficient of 9630 M^{-1} [8]. One unit is defined as the formation of μmol trans-cinnamic acid / min/ ml enzyme.

Isolation of total RNA

Total RNA from both treated and untreated tomato cultures were isolated using SV Total RNA Isolation System kitt (Promega Corporation, USA) according to the manufacturer's instructions.

RT-PCR (Reverse Transcription- PCR) assay

For RT-PCR, first strand cDNA synthesis and PCR reactions were performed using the Access RT-PCR System kitt (Promega Corporation, USA) according to the manufacturer's indications. For cDNA amplification, 1 μg of RNA was added to 50 μl PCR mixture. PCR amplification was performed using peroxidase specific primers:

Forward: 5` - ATA CTT GCC CGA ACG TCA CCA GC -3`

Reverse: 5`- CAT CCA ATG ACA AAG CAG TCG TGG-3`

The mixtures were amplified with the following cycling parameters: 45°C for 3 min at first cycle, 94°C for 2 min, 1 cycle, 94°C for 30 sec., 60°C for 1 min; 68 °C - 2 min for 45 cycles with a final extinction at 68°C, 7 min. The RT-PCR products was determined by 1,2 % (w/v) agarose gel electrophoresis.

RESULTS AND DISCUSSIONS

Change in activities of peroxidase, β-1,3-glucanase**,** and phenylalanine ammonia lyase was recorded 10 days after treatments application.

Peroxidase (PO) activity. The production of reactive oxygen species is one of the earliest cellular responses following pathogen recognition [9]. As shown in figure 1, in contrast with results obtained from control sample (13,25 U/ml), infection by *Rhizoctonia solani* produced an increased levels in peroxidase activity in tomato leaves (28,35 U/ml). Significantly increased levels in PO activity (37,74 U/ml) were recorded in the plant inoculated with *Rhizoctonia solani* and *Bacillus licheniformis* (fig. 1). High levels of PO activity in tomato leaves is an important element of desease resistance mechanism which are involved directly or indirectly in restriction of pathogen spreading.

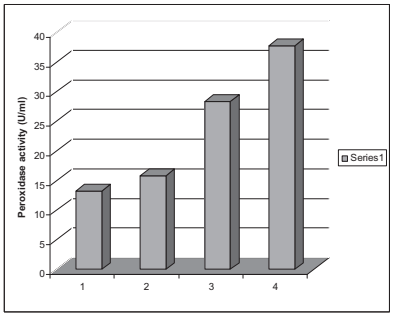

Fig. 1. Induction of peroxidase (PO) activity in the leaves of tomato: (1) Control; (2) Plants infected with *Bacillus licheniformis*; (3) Plants infected with *Rhizoctonia solani*; (4) Plants infected with *Rhizoctonia solani + Bacillus licheniformis*.

Induction of β-1,3-glucanase activity. Plant β-1,3-glucanase are pathogenesis- related proteins which are implicated in plant defense responses against pathogen infection or other stimuli [6], [15], [20]. Induction of β-1,3-glucanase in response to various pathogen and their elicitors has been well investigated in plants. In this study β-1,3-glucanase activity was significantly increased both in the harvested pods of plant infected with *Rhizoctonia solani* alone and *R. solani* in combination with *Bacillus licheniformis*. This activity was higher in the treated plants with *R. solani* (3,988 U/ml) (fig. 2). Plant β-1,3-glucanase can directly inhibit the fungal growth *in vitro* by catalyzing the hydrolysis of β-1,3-glucan which is a major component of the cell walls of many pathogenic fungi.

Induction of phenylalanine ammonia - lyase (PAL) activity. Soil aplication of *B. licheniformis* as biocontrol agent and *B.licheniformis - R. solani* in combination caused plants to synthesize PAL .This activity ranged from 0,3572 U/ml (*B. licheniformis*) to 0,1570 U/ml (*B. licheniformis - R. solani*) respectivly. Phenylalanine ammonia-lyase (PAL) catalyses the first step in the biosynthesis of phenylpropanoids, which form a wide variety of plant secondary products.

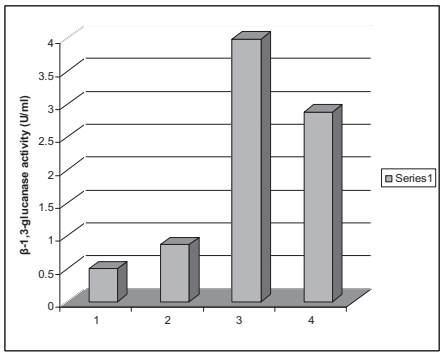

Fig. 2. Induction of β-1,3-glucanase activity in the leaves of tomato: (1) Control; (2) Plants infected with *Bacillus licheniformis*; (3) Plants infected with *Rhizoctonia solani*; (4) Plants infected with *Rhizoctonia solani* + *Bacillus licheniformis*.

The transcription of PAL is regulated in response to various factors that induce the accumulation of flavonoids, lignin and compounds thought to be involved in plant defence reactions [3]. Lignin is the major product of phenylpropanoid metabolism and deposition of lignin to reinforce plant cell in a inducible defense mechanism used for protection against pathogen invasion [14]. Phenylpropanoids are also known to act as molecular signals during recognition processes in the interaction between some soil born bacteria and their host plants [16]. PAL activity is often correlated with changes in the rate of acumulation of phenylpropanoids. The induction of PAL is influenced by a variety of stimuli including plant hormones, mechanical injuries, light and disease which can be involved in PAL inactivation system [4], [21]. In our experiments the activity of PAL was increased significantly in healthy plants (control) and in plants treated whit *R. solani* alone (fig. 3). The time required to activate the defense mechanisms is important for the supression of the pathogen. It seems that the activity of PAL reached a maximum levels on the first days after treatments and once time the PAL activity decreased dramaticaly.

Determination of mRNA transcript level by reverse transcription (RT)- PCR. To investigate the transcriptional expression of the *POX* gene in tissues treated with different types of biotic stresses, we isolated total RNA from leaf tissues and carried out RT-PCR analysis using the specific PCR primers. As shown in figure 4, the RT-PCR products from tomato treated plants were relieved that *POX* gene expression was highly induced in plants subjected to various types of treatment: *B. licheniformis* (lane 2), *R. solani* (lane 3) and *R. solani* in combination with *B. licheniformis* (lane 4).

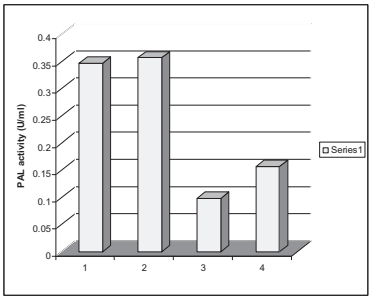

Fig. 3. Induction of phenylalanine ammonia - lyase (PAL) activity: (1) Control; (2) Plants infected with *Bacillus licheniformis*; (3) Plants infected with *Rhizoctonia solani*; (4) Plants infected with *Rhizoctonia solani* + *Bacillus licheniformis*.

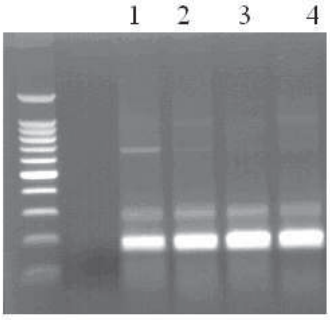

Fig. 4. Expression of transcript level for *POX* gene in tomato plants: (1) Control; (2) Plants infected with *Rhizoctonia solani*; (3) Plants infected with *Bacillus licheniformis*; (4) Plants infected with *Rhizoctonia solani* + *Bacillus licheniformis*.

CONCLUSIONS

In the present study, soil application of *Bacillus licheniformis* as a biocontrol agent against *Rhizoctonia solani* increased the activity of defense related enzymes in tomato seedlings 10 days after treatments application. Plant treatments with *Rhizoctonia solani* or *Rhizoctonia solani* in combination with *Bacillus licheniformis* produced significantly increased levels in peroxidase (PO) and β-1,3-

glucanase activities in tomato leaves. Peroxidases, which generate H_2 O_2 and oxidize phenols, are important in lignin biosynthesis. The expression of *POX* gene in tissues treated with different types of biotic stresses, was investigated by RT-PCR analysis using the specific PCR primers. RT-PCR products from tomato treated plants were relieved that *POX* gene expression was highly induced. However, in all cases there was a strong correlation between induction of mRNA activity and enzyme activity.

Phenylalanine ammonia-lyase (PAL) activity was positively correlated with the plants infected by *Bacillus licheniformis*. However, the highest levels in PAL activity was recorded in the control plants.

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