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SYSTEMIC GENE EXPRESSION OF DEFENSE-RELATED ENZYMES INDUCED BY PLANT GROWTH-PROMOTING RHIZOBACTERIA

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Abstract

Plant growth-promoting rhizobacteria can induce both local and systemic plant defense responses. As in the case of local resistance, systemic resistance is associated with the activation of antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD), and peroxidase (POX), and the synthesis of other defense-related enzymes, such as phenylalanine ammonia-lyase (PAL). This study aimed to evaluate the effect of bacterial treatment on transcriptional changes of genes encoding five defense-related proteins in distal tissues of Cucumis sativus L. plantlets. Suspensions of Pythium debaryanum Hesse (Py) and antagonistic bacteria, such as Bacillus licheniformis (B40), Bacillus amyloliquefaciens (Bw), Bacillus sp. (Bw1) and Pseudomonas aeruginosa (P14) were applied on cucumber plantlets. Total RNA was isolated, and reverse transcription-PCR (RT-PCR) was performed. A semi-quantitative method was used to determine alterations in the expression of five defense-related genes in treated plants. Plants pretreated with the selected bacterial strains showed overexpression of genes encoding two defense-related enzymes: one involved in the primary antioxidant system and the other a key enzyme in the phenylpropanoid pathway.

Key words*: plant defence-related enzymes, systemic gene expression, beneficial microorganisms.*

INTRODUCTION

In their natural environment, plants interact with multiple types of stressors, and to cope with these diverse and constant challenges, plants have evolved diverse defense strategies. Besides preformed defense mechanisms, which are passive innate characteristics (Ding et al., 2022; Flors et al., 2024), plants have also developed inducible defense strategies. Plant growth promoting rhizobacteria (PGPR) represent a group of valuable microorganisms that contribute to plant growth stimulation but also to defense system activation (Shoresh et al., 2005; Yu et al., 2022; Kour et al., 2024). Induced resistance is achieved through an active, highly energy consuming process and is defined by substantial gene regulation changes with significant impact on both primary and secondary metabolism. As a result of changes in gene regulation, plant-induced defense reactions can be manifested both locally and systemically. Systemic resistance is characterized by the triggering of defense signals in distal areas from the treatment site in response to various elicitors, whereas local resistance involves the induction of defense reactions primarily at the site of application.

Plant induced resistance relies on two main pathways: one dependent on salicylic acid (SA), and another on jasmonic acid (JA) and ethylene (Et). Although it was previously considered that resistance induced by beneficial microorganisms is solely dependent on the JA/Et pathway, subsequent studies have shown that both SA and JA/Et are activated during resistance induction in plant-microbe interaction (Yu et al., 2022; Jung et al., 2024).

Systemic resistance is achieved through a very complex and diverse set of signaling pathways, being associated with the systemic synthesis of enzymatic and non-enzymatic antioxidants, antimicrobials, pathogenesys related proteins (PR) and signaling molecules in healthy plant tissues (Pieterse et al., 2014; Yang et al., 2023). In the case of a further pathogen attack, plants manifest accelerated and stronger structural defense responses, such as cell wall

reinforcement and the obturation of intercellular spaces (Helepciuc et al., 2013), and biochemical defense responses, such as the induction of phenylalanine ammonia-lyase (PAL) and the synthesis of antioxidant enzymes (Mauch-Mani et al., 2017; Jung et al., 2024).

In the current study, we aimed to induce a systemic defense response in cucumber plants treated with previously tested beneficial bacterial strains and pathogenic fungi. These bacterial strains were tested as candidates for PGPR and proved to synthesise indole acetic acid (Helepciuc et al., 2008), have antifungal activity (Mitoi et al., 2012), activate different types of structural and biochemical defense responses in plants (Helepciuc et al., 2013; Helepciuc et al., 2014), but also managed to colonize cucumber roots (Helepciuc et al., 2019). In this study, the systemic expression of genes encoding antioxidant enzymes (superoxide dismutase, peroxidase, and catalase) and phenylalanine ammonia-lyase was tested to evaluate the plant defense response.

MATERIALS AND METHODS

The **biological material** consisted of *C. sativus* var. Marketer plantlets, the fungal phytopathogen *Pythium debaryanum*, and suspension cultures of the bacterial strains *Bacillus licheniformis* (B40), *Bacillus amyloliquefaciens* (Bw), *Bacillus* sp. (Bw1), and *Pseudomonas aeruginosa* (P14) (Collection of the Faculty of Biotechnology, USAMV of Bucharest).The seeds were sterilised with 1% sodium hypochlorite for 1h, washed three times, and aseptically germinated in glass culture dishes (13/8 cm) on wet filter paper at 25ºC with a 16/8 h photoperiod.

To obtain a fungal suspension, potato dextrose (PD) liquid medium was inoculated with 3 pieces of 1 cm^2 of actively growing *P. debaryanum* mycelium and cultured at 28ºC for three days.

Bacterial suspensions were obtained by cultivating the bacterial strains on LB broth at 28ºC, 150 rpm for 24 h.

Plant treatment

The three weeks old plantlets were treated with 10^8 CFU/ml bacterial suspension or with three days old fungal suspension. Also, in some variants, mixed treatment was applied, consisting of both bacterial and fungal suspension.

Plant roots were treated by application on the filter paper from culture dishes of 5 ml of fungal pathogen for the Py variant or each bacterial suspension for B40, P14, Bw and, respectively, BW1 variants. The mixed treatment was performed by applying the bacterial suspension followed by infection with the pathogenic fungus after 24 h for B40-Py, P14-Py, Bw-Py and Bw1-Py experimental variants.

RNA isolation

After 24 h from fungal infection and 48 h from bacterial suspension treatment, cucumber leaves were collected and used for total RNA extraction with the InuuPREP Plant RNA Kit (AnalytikJena). The purity and total RNA concentration were estimated using a NanoDrop 1000 spectrophotometer (Thermo Scientific).

Reverse transcription-PCR amplification

RT-PCR amplifications were performed in a 25 µl reaction volume containing 5 µl AMN/Tfl reaction buffer, 0.5 µl AMV reverse transcriptase, 0.5 µl Tfl DNA polymerase, a concentration of 1 µM for each of the forward and reverse primers, 1mM Mg SO_4, 0.2 mM dNTP Mix (Promega, USA) and 4 µl of 125 ng/µl total RNA.

The five sets of primers were documented from the literature: Cu/Zn-superoxide dismutase (EF121763) and Mn-superoxide dismutase (EF203086) (Gao et al., 2009), peroxidase (M91374) (Shoresh et al., 2005), catalase (AY274258) (Xia et al., 2009) and phenylalanine ammonia-lyase (AF529240) (Bradley, 2009).

The cycling conditions were as follows: reverse transcription for 45 min at 45°C, reverse transcriptase inactivation and RNA primer denaturation for 2 min at 94°C, followed by 45 cycles of 30 s at 94°C (denaturation), 1 min at the specific annealing temperature (Table 1), 2 min at 68°C (extension), and 1 cycle for 7 min final extension step at 68°C. The amplifications were performed in an Eppendorf Mastercycler gradient (Germany).

Table 1. The specific tested primer sequences and annealing temperatures for mARN of the studiedgenes

Interest gene	RT-amplified fragment (bp)	Forward and reverse primer sequences	T_a (°C)
CAT		5'-ATGCTGGAAGAGGAGGCTAT-3'	58
Xia et al., 2009	732	5'-ATGGTGAGGACATTTGGGAG-3'	
Cu/Zn-SOD		5´-GACTGGGCCACATTTCAACC-3´	55.8
Gao et al., 2009	297	5´-GCCTTGCCATCTTCACCAA-3´	
Mn-SOD		5´-CAATGGCGGAGGTCACATTA-3´	55.8
Gao et al., 2009	330	5´-AGAGCAAGCCACACCCATC-3´	
POX		5′-AGAGCAACAAGGTCGGTTTCA-3′	60
Shoresh et al., 2005	1135	5′-GTGCCGACATCCTAGCTCAAG-3′	
PAL		5'-AGTTGCATGAAATGGATCCTC-3'	54
Bradley, 2009	468	5'-TTATGTTGCTCGGCACTTTG- 3'	

Electrophoretic separation

Amplicons were separated on 1.5% agarose gel, and multiple molecular weight and concentration markers, such as GeneRuler 50 bp DNA Ladder (Thermo Scientific), Gene Ruler Low Range DNA Ladder (Thermo Scientific), and DNA Standard 100 bp DNA Ladder (Serva) were used. For DNA band visualization, a 0.01% SYBR Safe solution was used.

Estimation of amplicons molecular weight and concentration

The gels were visualized using GENi (Syngene), equipment, and the DNA molecular weight and DNA concentrations were estimated using the GeneTools (Syngene) software, having as reference the above-mentioned molecular weight and concentration markers.

RESULTS AND DISCUSSIONS

In the current research, the systemic expression of cucumber enzymes Cu/Zn superoxide dismutase (*Cu/Zn-SOD*), Mn superoxide dismutase (*Mn-SOD*), peroxidase (*POX*), catalase (*CAT*), and phenylalanine ammonia-lyase (*PAL*) was analysed. In the case of superoxide dismutases and peroxidase, the separation of the amplification mixture on an agarose gel evidenced multiple reaction products, indicating low specificity of the primers used. In the case of some studied genes, the interest RT-PCR products are found among the multitude of amplified fragments, such as the 330 bp amplicon of *Mn-SOD* (Gao et al., 2009), 297 bp amplicon of *Cu/Zn-SOD* (Gao et al., 2009). In case of *POX* (Shoresh et al., 2005), the interest amplicon of 1135 bp, was not identified. Of the five defense-related genes tested, only *CAT* and *PAL* were considered for further analyses, as the amplified products had an appropriate molecular weight.

Catalase gene expression

The reverse transcription of the catalase gene using the set of primers mentioned by Xia et al. (2009) showed a single amplification product with a molecular weight between 433 and 461 bp (Figure 1), the entire sequence as presented in the GeneBank database having 732 bp.

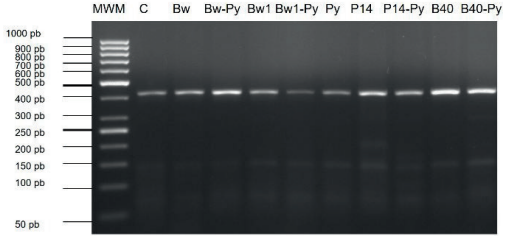

Figure 1. The UV visulization of CAT RT-product on 1.5% agarose gel electrophoresis: MWM - molecular weight marker; C - untreated control variant; Bw, Bw1, P14, B40 - experimental variants treated with beneficial bacterial suspensions; Py - infected variant with *P. debaryanum* phytopathogen; Bw-Py, Bw1-Py, P14-Py, B40-Py - experimental variants treated both with beneficial bacterial strains and phytopathogen

The treatments with different types of bacterial suspensions, such as B40, Bw, Bw1, and P14, induced an increase in CAT transcript concentrations compared to the control (Figure 1). The best stimulation was produced by B40 strain (7.001 ng cDNA/µl), followed by P14 and Bw, while the Bw1 strain had the weakest induction effect (3.78 ng cDNA/µl). The application of the phytopathogen determined a decrease in the expression of this CAT gene for all infected experimental variants, with the exception of the Bw-Py variant, in which pathogen administration caused an increase in this *CAT* gene expression (Table 2). Higher

CAT amplicon concentrations than in the control were observed in all treated variants, except for the Py variants Bw1-Py and (Table 2). The variants treated only with the pathogen (Py) and Bw1-Py variant, which was stimulated with the least inductive bacterial strain Bw1, registered the lowest CAT amplicon concentrations. The highest concentrations were recorded in B40- treated variants (B40, respectively B40-Py).

Table 2. The estimation of CAT cDNA amplicon concentrations in leaves of cucumber plantlets treated with beneficial bacterial suspensions and/or infected with *P. debarianum* phytopathogen

Experimental variant	CAT RT-amplicon concentration (ng/µl)
C	3.149
Bw	4.325
Bw-Py	5.926
Bw1	3.780
Bw1-Py	1.153
Py	2.921
P14	5.644
P14-Py	4.853
B40	7.001
B40-Py	6.001

The concentration of ROS in plants rises rapidly during plant-microbe interaction, reaching significantly higher concentrations than in normal conditions (Ding et al., 2022). The better expression of CAT amplicon shows the enzymatic antioxidant system, which is involved in counteracting the abundance of ROS in plant cells as a result of plant-microbe interaction. In a previous work, we also showed an increased catalase activity compared with other H_2O_2-degrading enzymes (guaiacol peroxidase) in cucumber plants treated with both beneficial and pathogenic microorganisms (Helepciuc et al., 2014). Similar results were obtained in a long-term experiment: maize embryos that showed increased pathogen resistance had a higher catalase activity (Magbanua et al., 2007). However, other studies reported a decrease in antioxidant enzymes, for example, in *in vitro* strawberry regenerants treated with anatoxinic sera obtained from different phytopathogenic *Botrytis cinerea* or non-phytopathogenic *Trichoderma viride* and *Penicillium chrysogenum* fungal strains (Cogălniceanu et al., 2010). Besides their role in excess H_2O_2 neutralization, catalases can also regulate various defense signaling pathways, influencing the (Riseh et al., 2024). Catalase expression is particularly important in plant-microbe interaction due to its dual role in plant protection. First, catalase is a highly efficient hydrogen peroxide neutralizer (Scandalios et al., 1997; Mhamdi et al., 2010; Rao et al., 2025) being directly involved in maintaining the balance between reducing reactions associated with defense responses and oxidizing reactions responsible for oxidative damage within cells. Besides cellular redox homeostasis, catalases are also involved in defense signaling pathways, interfering with both jasmonic acid and salicylic acid signaling pathways and shaping the plant's response in beneficial and pathogenic interactions (Riseh et al., 2024).

Phenylalanine ammonia-lyase gene expression

In the case of the phenylalanine ammonia-lyase gene (Bradley, 2009) it was also obtained a main reversetranscription product with a molecular weight of 437-520 bp (Figure 2) comparing with the 533 bp sequence shown in the GenBank database.

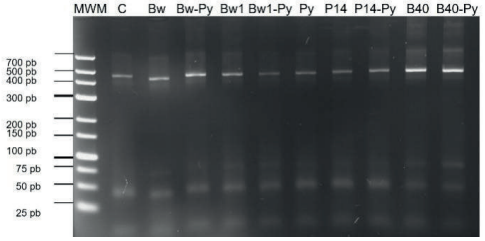

Figure 2. The UV visualization of PAL RT-product on 1.5% agarose gel electrophoresis: MWM - molecular weight marker; C - untreated control variant; Bw, Bw1, P14, B40 - variants treated with beneficial bacterial suspensions; Py - infected variant with *P. debaryanum* phytopathogen; Bw-Py, Bw1-Py, P14-Py, B40-Py - variants treated with both beneficial bacterial strains and phytopathogen

The concentration of PAL amplicon increased in all bacterial-treated variants compared with the control, except for P14 (Figure 2). The best stimulatory effect was observed in the variant treated with the B40 strain, followed by Bw and Bw1, while P14 inhibited PAL expression (Table 3). Also, infection with *P. debaryanum* decreased the PAL concentration in the variant untreated with beneficial bacteria. The amplicon

corresponding to the PAL gene had the highest concentration in the infected B40-Py (4.910 ng/µl), while the lowest concentration was registered in Bw1-Py variant (0.864 ng/µl) (Table 3).

Table 3. The estimation of PAL cDNA amplicon concentrations in leaves of cucumber plantlets treated with beneficial bacterial suspensions and/or infected with *P. debarianum* phytopathogen

Experimental variant	PAL RT-amplicon concentration (ng/µl)
C	1.825
Bw	3.651
Bw-Py	4.413
Bw1	2.794
Bw1-Py	0.864
Py	1.151
P14	1.098
P14-Py	1.744
B40	4.659
B40-Py	4.910

Except for Bw1-Py variant, the concentration of PAL amplicon increases in variants with mixed treatment (beneficial bacteria and fungal phytopathogen) compared with bacterial pretreated variants or with the pathogen-treated variant (Py) (Table 3). These results suggest that in the case of pretreatments with beneficial bacteria, the *PAL* gene is overexpressed in subsequent interaction with the pathogen.

A previous study also reported that overexpression of a *PAL* gene (*OsPAL1*) induces resistance in rice to both *Magnaporthe oryzae* and *Xanthomonas oryzae* pv *oryzae* (Zhou et al., 2018). Also, in soybean, PAL positively regulates plant resistance to *Phytophtora sojae* (Zhang et al., 2017).

PAL is involved in the first step of the phenylpropanoid pathway, catalyzing the non-oxidative L-phenylalanine deamination with the formation of trans-cinnamic acid (Liu et al., 2024, Amjad et al., 2024). Trans-cinnamic acid is a crucial player in plant defense responses, as it is the precursor of multiple defense-related compounds such as lignin, suberin, monolignols, flavonoids, coumarins, phytoalexins, phenolic acids, and salicylic acid (Zhang et al., 2017; You et al., 2020; Liu et al., 2024). In plants, these compounds can have structural roles; they can act as antioxidants and antimicrobial compounds, but they can also play major roles as signaling molecules in plant defense pathways. The variety of defense-related compounds highlights the crucial role of PAL in plant biotic stress responses. The enhanced expression of *PAL* was correlated with an increase in lignin content in pathogen-resistant rice, pointing out the involvement of PAL in lignin biosynthesis, an important non-specific structural defense mechanism which can confer resistance to a wide range of pathogens (Zhou et al., 2018). In addition to its role in structural compounds, antimicrobials, and antioxidants, PAL also plays a major role in the plant hormone salicylic acid biosynthesis through the phenylpropanoid pathway, an alternative to the well described isochorismate synthase pathway (Liu et al., 2024; Wang et al., 2025).

Various studies have documented phenylalanine-derived salicylic acid biosynthesis in different plant species, highlighting, for example, its importance in *Brachypodium distachyon* resistance to *Panicum mosaic virus* (PMV) and its satellite virus (SPMV) (Pant et al., 2021). Also, Ullah et al. (2023) mentioned the reliance of *Populus* sp.'s on phenylalanine-derived salicylic acid biosynthesis for both constitutive and induced pathways.

In this study, the bacterial treatment managed to activate different components of the cucumber plants' defense system. First, the catalase gene shows the activation of the enzymatic antioxidant system to neutralize the excess of reactive oxygen species generated in plants, which is perhaps one of the earliest defense responses in plant-microbe interactions. Additionally, the overexpression of phenylalanine ammonia-lyase, a key enzyme in the phenylpropanoid pathway, primarily creates the premises for the synthesis of important defense-related compounds with structural, antimicrobial, and antioxidant properties. Furthermore, the initiation of the phenylpropanoid pathway enables the production of important signaling molecules, such as salicylic acid, compared with the untreated variant.

CONCLUSIONS

RT-PCR analysis showed a differential response in pretreated plants with bacterial suspensions compared with the untreated variant. Plants challenged with the selected bacterial strains showed overexpression of genes encoding CAT and PAL, but in the case of further infection, *CAT* transcript concentrations decrease while *PAL* expression increases. These findings indicate a variable and complex response of plants, which can ensure better protection against pathogen attack and a differentiate priming effect conferred by beneficial bacterial strains.

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