Induction of plant antioxidant system by interaction with beneficial
and/or pathogenic microorganisms

Florența Elena Helepciuă, Monica Elena Mitoiu*, Anca Manole-Păunescu, Florentina Aldea, Aurelia Brezeanu, Călina Petruța Cornea

Department of Plant and Animal Cytobiology, Institute of Biology Bucharest of
Romanian Academy, 296 Splaiul Independenței, 060031 Bucharest, P.O. Box 56-53, Romania

Department of Biotechnology, University of Agronomical Sciences and Veterinary
Medicine, Bucharest, Romania

Corresponding author: MONICA ELENA MITOI
E-mail: mcarasan@yahoo.com

Abstract

The interaction of plant with both pathogenic and non-pathogenic microorganisms can trigger active
defense reactions in plant. The defense response of Cucumis sativus L. plants treated with beneficial
bacteria and phytopathogenic fungi was evaluated. Cucumber plantlets treated with bacterial isolates
having antifungal activity (Bacillus licheniformis B40, Bacillus amyloliquefaciens Bw, Bacillus sp. Bw1,
Pseudomonas aeruginosa P14) and fungal (Pythium debaryanum Hesse) suspensions were examined for
antioxidant system activation. Potential alteration in superoxide dismutases, catalases, guaiacol
peroxidases and lignin production was approached using biochemical and histochemical methods.
Bacterial inoculation increased the number of superoxide dismutase and catalase isoforms, and also the
enzymatic activity of these antioxidants. Reinforcement of epidermal and cortical cell walls through lignin
deposition was observed using histochemical methods. These results showed that antioxidant enzymes
activity and lignin production were intensified in treated variants, indicating the activation of some
defense mechanisms, and a potential increase of plant resistance to pathogen attack.

Keywords: defense response, antioxidant enzymes, antagonistic bacteria, Cucumis
sativus, Pythium debaryanum.

Introduction

Plant rhizosphere is colonized by numerous microorganisms, which are attracted by plant
root exudates. Some of the microorganisms from the rhizosphere induce plant systemic
resistance, which is associated with activation of plant defense mechanisms. Some defense
mechanisms have a structural nature and block the microorganisms to enter and spread
through the plant, like the case of lignin deposition. Also, as a result of plant-microbe
interaction, in plants are activated molecular mechanisms which refer to rapid generation of
high amount of reactive oxygen species (ROS), production of lytic enzymes or other
chemicals with antimicrobial activity [1, 2]. ROS production is common in healthy plants,
and they were initially considered just toxic by-products of different metabolic pathways [3].
However, it is now shown their role in establishment of defense mechanisms (papillae
formation and the assembly of structural barriers, programmed cell death and hypersensitivity
response), but also in signaling by synergistic interaction between themselves and other
signaling molecules such as salicylic acid, jasmonate and ethylene [4]. An important role as
Induction of plant antioxidant system by interaction with beneficial and/or pathogenic microorganisms signals in other critical plant processes like growth and development is attributed to these highly reactive molecules [2]. Despite these activities, ROS react easily with various biomolecules, causing irreversible damage and leading to necrosis and plant death [5]. To overcome ROS toxicity, plants produce enzymatic and nonenzymatic antioxidants that scavenge oxygen species. The first line in antioxidant defence is enzymatic antioxidant system, which neutralizes superoxid anion (\( \text{O}_2^{\cdot -} \)) and hydrogen peroxide (\( \text{H}_2\text{O}_2 \)). ROS enzymatic neutralization is ensured by antioxidant enzymes, like superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and guaiacol peroxidase (GPX, EC 1.11.1.7). SOD catalyses \( \text{O}_2^{\cdot -} \) dismutation and is considered the most effective intracellular enzymatic antioxidant being present in all subcellular compartments prone to ROS mediated oxidative stress [6]. The \( \text{H}_2\text{O}_2 \) resulted from this reaction is subsequently converted to \( \text{H}_2\text{O} \) and \( \text{O}_2 \) by CAT, another indispensible enzyme for ROS neutralization. \( \text{H}_2\text{O}_2 \) can also be degraded by GPX activity, which has an additional role in structural cell wall modifications and production of secondary metabolites with antimicrobial activity. In this study we evaluated the induction of structural and enzymatic defense response by new bacterial strains with antifungal activities.

Materials and Methods

Plant and microbial cultures and growth conditions
Cucumber seeds (Marketer cultivar) were surface sterilized with a solution of 1% sodium hypochlorite for one hour, rinsed and then aseptically transferred in culture vessels on sterile filter paper with 10 ml of water. The seeds were germinated in glass jars at 25ºC, with a photoperiod of 16 h light and eight hours dark. Three weeks old cucumber plantlets were treated with five ml of \( 10^8 \text{ CFU ml}^{-1} \) bacterial suspension and/or five ml of three days fungal suspension. The microbial strains used in this study are presented in Table 1.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics</th>
<th>Origin</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus amyloliquefaciens Bw</td>
<td>antifungal activity</td>
<td>Isolated from soil, Faculty of Biotechnology, UASVM, Bucharest</td>
<td>[7]</td>
</tr>
<tr>
<td>Bacillus sp.Bw1</td>
<td>antifungal activity</td>
<td>Isolated from soil, Faculty of Biotechnology, UASVM, Bucharest</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus licheniformis B40</td>
<td>( \alpha ) amylase, antifungal activity, indole 3-acetic acid production</td>
<td>Collection of Faculty of Biotechnology, UASVM, Bucharest</td>
<td>[8,9,10]</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa P14</td>
<td>antifungal activity, indole 3-acetic acid production</td>
<td>Isolated from soil, Faculty of Biotechnology, UASVM, Bucharest</td>
<td>[11,9,10]</td>
</tr>
<tr>
<td>Pythium debaryanum</td>
<td>plant pathogen</td>
<td>Collection of RDIPP, Bucharest</td>
<td>-</td>
</tr>
</tbody>
</table>
Bacterial strains were grown on LB broth on a rotary shaker, 150 rpm at 28ºC, while fungal suspension was obtained by inoculating 30 ml of PD (potato dextrose) broth with 3 x 1cm² of actively growing *P. debaryanum* mycelium. The treatment consisted of 5 ml of bacterial, fungal or mixed suspension and was applied on plant root. The bacterial suspensions were maintained on cucumber roots, removed after 24 hours, when the fungal suspension was added. After 24 hours cucumber plantlets were grinded and total protein extract was obtained.

**Isoenzymes detection**

Total protein extract was obtained by grinding whole cucumber plantlets and adding 1:1 (w/v) extraction buffer (0.05 M phosphate buffer, pH 7, 2 mM EDTANa₂ and 4% (w/v) PVP). The homogenate was maintained at 4°C overnight, centrifuged at 15000 rpm for 15 minutes, and the supernatant was used for quantification and electrophoretic analyses. Isoenzymes detection was performed using native polyacrylamide gel electrophoresis (PAGE) technique. The activity of peroxidase isoforms was detected according to a modified method of Kavitha & Umesha [12]. The gel was incubated in 0.5 M acetate buffer (pH 5) containing 0.08% benzidine, 180 μl of glacial acid acetic and several drops of H₂O₂ (total volume 30 ml) until brown bands appeared on violet background.

For catalase staining the method of Weydert & Cullen [13] was used, with some modifications. The gel was first washed 3 times for 15 min in distilled water, incubated in phosphate buffer 0.01 M, pH 7, supplemented with 0.003% hydrogen peroxide, and then immersed in a mix of 2% FeCl₃ and 2% K₂Fe(CN)₆ in equal proportions. After staining yellow bands appear and the gel is colored in light green.

Superoxide dismutases were stained according to Beauchamp & Fridovich [14], in a solution of phosphate buffer 0.036M (pH 7.8), 2.4 mM NBT, 0.028 M TEMED, and 27.9 μM riboflavin, until white bands appear.

**Enzyme activity quantification**

Quantification of superoxide dismutase activity was performed according to Beauchamp & Fridovich [14]. The volume of enzyme extract required to inhibit NBT reduction by 50% was defined as one unit of SOD activity.

Catalase activity was assayed at 240 nm by measuring the rate of H₂O₂ degradation from the reaction mixture, using the method described by Garcia-Limones & al. [15]. One enzymatic unit was defined as the volume of enzymatic extract that decomposes one μmol of H₂O₂ in one minute at 25°C.

Guaiacol peroxidase enzymatic activity was assayed by measuring the absorbance at 470 nm for 5 min. The method was described by Garcia-Limones & al. [15]. One unit of enzyme activity represented the amount of enzyme catalyzing the oxidation of 1 μmol of guaiacol in 1 minute.

Statistical analysis was performed using SPSS (Statistical Package for Social Sciences). The significance of the differences between the samples was tested by employing T-test (independent samples two-tailed). The results with *p*<0.05 were considered statistically significant.

**Histochemical analyses**

The treatment procedure for histochemical analyses was done as described before, except that for lignin detection the plants were used 12 days after fungal inoculation. Histochemical staining of peroxidase was performed using 3,3’-diaminobenzidine (DAB) as substrate, following the producer recommendations (Carl Roth GmbH). Fresh handmade cross-sections through hypocotyl were placed in an incubation solution (0.05M Tris-HCl buffer, pH 7, 0.01M imidazole and 0.1%DAB) for five minutes. The sections were then immersed in the staining solution (0.05M Tris-HCl buffer, pH 7, 0.01M imidazole, 0.1% DAB and 0.003%
H$_2$O$_2$). The sections were maintained in this solution for two minutes, and the reaction was stopped with distilled water. Brown colored tissues indicate enzymatic activity of POX. Wiesner test was used for lignin localization employing the method described by Fernandez-Garcia & al. [16], with some modifications. Sections were immersed in 1% phloroglucinol dissolved in a mixture of 3:1 ethanol:HCl (95% ethanol and 20% HCl) for two minutes. Positive lignin staining is indicated by areas colored in red-violet. All stained sections were visualized using bright-field light microscopy under a Nikon Eclipse E200 microscope and micrographs were recorded using a Nikon Coolpix 5400 digital camera.

Results

Superoxide dismutase activity increased in all treated variants, except for plants treated with P14 strain (Figure 1 a). The highest activity was detected in plants treated with B40 bacterial strain. Differences in SOD electrophoretic patterns were observed in microbial treated plants comparing to control, which presented only three isoforms with SOD activity (1, 2 and 6) (Figure 1 b).

Figure 1. Enzymatic activity (a) and native PAGE analysis (b) of SOD from cucumber non-infected plants (C), and plants treated with: B. amyloliquefaciens (Bw), B. amyloliquefaciens – P. debaryanum (Bw-Py), Bacillus sp. (Bw1), Bacillus sp. – P. debaryanum (Bw1-Py), B. licheniformis (B40), B. licheniformis – P. debaryanum (B40-Py), P. aeruginosa (P14), P. aeruginosa – P. debaryanum (P14-Py). Asterisks indicate significant differences between control and treated variants according to the $t$-test at level $p<0.05$ (*).

The results obtained with SOD isoforms allowed the detection of maximum six isoforms/variant. The highest number of bands was detected in Bw-Py and Bw1-Py variants. In all treated variants SOD 3 isoform was present, while in control was not expressed. Treatment with B40 and P14 bacterial strains induced the expression of a supplementary isoform (SOD 4) comparing with Bw and Bw1 variants. Colonization with both bacteria and fungi induced the expression of some characteristic bands, SOD 4 and 5, except for B40-Py variant.
**Catalase** activity was higher in treated variants comparing with the control (Figure 2a). Generally, mixed treatment induced a slight increase of enzymatic activity comparing with bacterial treatment. However, the highest CAT activity was detected in plants treated with P14 strain. The electrophoretic pattern of CAT isoenzymes (Figure 2b) showed the presence of supplementary isoforms in variants that received mixed treatment (CAT5 for Bw1-Py, CAT5 and CAT6 for Bw-Py and B40-Py, CAT4 for P14-Py). CAT3 was present only in P14, while the treatment with Bw bacterial strain induced the expression of CAT7.

![Image](Figure 2a: Enzymatic activity (a) and native PAGE analysis (b) of CAT from cucumber non-infected plants (C), and plants treated with: *B. amyloliquefaciens* (Bw), *B. amyloliquefaciens* – *P. debaryanum* (Bw-Py), Bacillus sp. (Bw1), Bacillus sp. – *P. debaryanum* (Bw1-Py), *B. licheniformis* (B40), *B. licheniformis* – *P. debaryanum* (B40-Py), *P. aeruginosa* (P14), *P. aeruginosa* - *P. debaryanum* (P14-Py). Asterisks indicate significant differences between control and treated variants according to the t-test at level $p<0.05$ (*).]

**Guaiacol peroxidase** activity was detected in all variants, with the highest values in plants treated with Bw1 and P14 strains (Figure 3). Mixed treatment (bacteria and fungal pathogen) allowed the detection of increased GPX activity only in Bw-Py and P14-Py variants, comparing with control and *Pythium debaryanum* treatment. No significant qualitative differences between variants in GPX isofoms electrophoretic pattern were observed. However, more intensified bands were detected in some samples (data not shown).
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Figure 3. Enzymatic activity of GPX in cucumber non-infected plants (C), and plants treated with: *B. amyloliquefaciens* (Bw), *B. amyloliquefaciens – P. debaryanum* (Bw-Py), *Bacillus* sp. (Bw1), *Bacillus sp. – P. debaryanum* (Bw1-Py), *B. licheniformis* (B40), *B. licheniformis – P. debaryanum* (B40-Py), *P. aeruginosa* (P14), *P. aeruginosa – P. debaryanum* (P14-Py). Asterisks indicate significant differences between control and treated variants according to the t-test at level $p<0.05$ (*).

**Histochemical analyses** were performed in order to detect possible cellular differences in plants subjected to treatments with beneficial bacteria, pathogen or combined treatments. The peroxidase activity (Figure 4 b, c) increased compared with control variant (Figure 4 a) in epidermal and subjacent cortex but also in vascular bundles especially in cambial and xylem tissues, and in fiber cap (sclerenchyma).

**Lignin deposition**

Comparing with control (Figure 4 d), in variants treated with bacteria it was observed an increased lignin accumulation in epidermal cell walls of and also in xylem vessels (Figure 4 e, f). An irregular lignin deposition was also obvious in some cortical cells, prefiguring an angular collenchyma.

Figure 4. Histochemical staining of POX (A, B, C – brown coloration; ep – epidermis, phl v – phloem vessels, cb – cambium, xv – xylem vessels) and lignin deposition (D, E, F – red-violet coloration, see arrows) in cucumber hypocotil sections: A – control variant POX; B - plants treated with Bw strain; C – plants treated with Bw1 strain; D – control variant lignin; E – plants treated with Bw strain; F – plants treated with Bw1 strain. Scale bars = 100 μm.
Discussions

It was shown by numerous authors that microorganisms induce various plant defense mechanisms [17, 18, 19, 20, 21] or promote plant growth and development [22]. However, in case of studies regarding the impact of beneficial bacteria on plant antioxidant system differences were recorded among both plant species and microorganisms used. In this order, the aim of this study was the evaluation of plant response, mainly the activation of cucumber antioxidant system as a result of interaction with pathogenic and/or beneficial microorganisms. Three types of antioxidant enzymes were examined: superoxide dismutases, catalases and guaiacol peroxidases.

Within a cell, the superoxide dismutase is considered a key enzyme in regulation of intracellular levels of ROS [23], as it constitutes the first line of antioxidant system defence against ROS [24, 6], and is capable to scavenge the superoxide radical with the formation of hydrogen peroxide. The hydrogen peroxide molecules formed as a result of superoxide dismutases activity are subsequently degraded by catalases and peroxidases to H2O and O2.

The isosuperoxide dismutase expression was different between treated variants and the control (SOD 3), and also according to the treatment applied (SOD 4 is present in B40 and P14, but not in Bw and Bw1 variants). Mixed treatment generally conducted to expression of SOD 4 and SOD 5 in case of Bw-Py and Bw1-Py and SOD 5 in case of P14-Py. Only in case of B40-Py it was observed the absence of both SOD 4 and 5 isoforms, although treatment with B40 triggers the expression of SOD 4. From the total of six isosuperoxid dismutases, only three are present in control (SOD 1, SOD 2 and SOD 6), suggesting that the interaction with beneficial bacteria and phytopathogenic fungi activate the production of other three isoforms (3, 4 and 5). An early response in plant-microbe interaction is the formation of superoxide radicals and plants have to eliminate these radicals using superoxide dismutases or other antioxidants. The presence of SOD 3, SOD 4 and SOD 5 can be attributed to the plant necessity to activate new isoforms in order to counteract the excessive accumulation of this free radical. As a result of SOD activity, superoxide radicals are transformed in hydrogen peroxide, which is also a highly reactive molecule and can cause important damages. Hydrogen peroxide can be degraded both by the action of catalases and peroxidases. Our results showed that catalases presented a significant increased activity in treated variants comparing with the control. Similar to isosuperoxide dismutase electrophoretic pattern, some new catalase isoforms appear as a result of interaction between the microorganisms used and cucumber plants. Moreover, treatment-characteristic bands appear in three situations: CAT 3 for P14, CAT 4 for P14-Py and CAT 7 for Bw.

Although a significant increase in peroxidase activity in plant-microbe interactions have been previously reported [25, 26, 18, 19], we did not observed significant modifications in GPX enzymatic activity except for three variants. Also, unlike SOD and CAT, the electrophoretic spectrum of guiaiacol POX did not showed qualitative differences among the samples treated with bacteria or with mixtures of beneficial bacteria and phytopathogenic fungi. The relatively uniform presence of the guaiacol POX isoforms can be attributed to the implication of these enzymes in different plant processes like formation of diferulic bonds or the cross bonds between cell wall proteins, in oxidizing cinnamic alcohols during the process of lignification and suberization, but also in processes of oxidation of phenolic compounds during the hypersensitive response [27]. Moreover, maintaining of peroxidase activity at the level of the control samples could be explained by the involvement of catalases in H2O2 degradation. Catalase is unique among H2O2–degrading enzymes, as it not requires a reductant in order to catalyze H2O2 dismutation, the reaction of H2O2 removal being performed using a very energy-efficient mechanism [28, 29]. Although they have low substrate affinities at low substrate concentrations [30], catalases have a very fast turnover rate and a very high
specificity for H$_2$O$_2$, characteristics that distinguish them from other peroxide-metabolizing enzymes [29].

The different affinities of peroxidases and catalases for H$_2$O$_2$ suggest that the function of these two different classes of H$_2$O$_2$-scavenging enzymes differs, catalases being involved in the removal of H$_2$O$_2$ excess, whereas peroxidase would be mainly involved in fine modulation of ROS [30, 31].

Thus, catalase appears to be the first enzyme involved in H$_2$O$_2$ detoxification, and only in systems where catalase is no longer sufficient, H$_2$O$_2$ or its derivatives such as organic peroxides must primarily be metabolized by peroxidases, which all require reductants [32, 29].

Histochemical peroxidase staining also indicate that in the system used by us peroxidase activity increased in plants subjected to bacterial treatment, mostly in metabolic active tissues (cambium and xylem). In case of lignin, an increased lignification of protective tissues (epiderma and collenchima) of treated plants was observed, that could prevent the microbial spread through the plant. The accumulation of lignin could also limit the diffusion of microbial enzymes and toxins, and also the uptake of water and nutrients by the microorganisms [33]. Lignification represents thus an important structural defense mechanism used by plants against microorganisms.

**Conclusions**

Plants treatment with beneficial bacteria leads to expression of new SOD and CAT isoforms, while mixed treatment increases even more the number of isoforms. This shows that the treatments with the selected beneficial bacteria activate plant enzymatic antioxidant system, and presumably increases plant capacity to resist pathogen attack. Moreover, the fortification of protective tissues shows an improved structural defense capacity of treated plants. We can conclude that prior treatment with non-pathogenic bacteria activates some components of the structural and biochemical defense systems in cucumber, this indicating an improved resistance to potential phytopathogen attack. Induction of plant antioxidant system by antagonistic bacteria is of great interest because plant treatment with bioproducts based on such bacteria could enhance plant protection against fungal pathogens.

**References**

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