ANTIOXIDANT RESPONSE IN SOYBEAN CELL SUSPENSIONS TREATED WITH FUNGAL ELICITORS

HELEPCIUC Florenţa-Elena, MITOI Monica Elena, ALDEA Florentina, MATEI Sorin, MATEI Gabi-Mirela, COGĂLNICEANU Gina

Abstract. Soybean, *Glycine max* (Linnaeus 1737, Merrill 1917), is a cultivated plant with major economic value, so it is very important to maintain this crop-yield and in the same time to minimize the environmental impact, using environmental friendly technologies. The aim of this study was to describe the antioxidant response induced in soybean cell suspensions by fungal elicitors obtained from *Trichoderma viride*, *Trichoderma harzianum*, *Penicillium chrysogenum* and *Botrytis cinerea* strains. We used six experimental variants, represented by various combinations of the fungal filtrates, obtained using different procedures (E1, E2, E3, E4, PD-E1, PD-E4, each with 1%, 5% and 10% concentrations (w/v)). Soybean cell suspensions were treated for 24 hrs with the corresponding fungal filtrates, and after 24 hrs, quantitative and qualitative determinations of antioxidant enzymes were performed. Among the tested antioxidant enzymes, the peroxidases (POX) activity was the only one increasing in all experimental variants and for all elicitor concentrations used, except for 10% concentration of E1 variant. In case of catalases (CAT), the enzymatic activity increased only in E2 variant at 1% fungal filtrate concentration and E4 at 1% and 5% fungal filtrate concentration, the other variants presenting a decrease of enzymatic activity compared to the control. Superoxide dismutase (SOD) activity was generally higher in treated variants, with a maximum for 5% fungal filtrate concentrations in E1 and E4 variants. Concluding, some defense mechanisms were activated by fungal filtrate administration, and the E4 variant was the most effective in the activation of soybean cell suspension enzymatic antioxidant system by increasing the activities of the three major enzymes involved in reactive oxygen species detoxification, POX, CAT and SOD, at 1 and 5% concentration.

Keywords: fungal elicitors, cell suspension, soybean, antioxidant enzymes.

Rezumat. Răspunsul antioxidant al suspensiilor celulare de soia tratate cu elicitori fungici. Soia, *Glycine max* (Linnaeus 1737, Merrill 1917), este o plantă de cultură cu o valoare economică deosebită. Astfel, este foarte importantă menţinerea productivităţii acestei culturi şi în acelaşi timp minimizarea impactului asupra mediului înconjurător prin utilizarea unor tehnologii nepoluante. Scopul prezentului studiu este de a descrie modificările biochimice în suspensiile celulare de soia, induse de tratamentul cu filtrate fungice obtinute din tulpini de *Trichoderma viride*, *Trichoderma harzianum*, *Penicillium chrysogenum* şi *Botrytis cinerea.* Au fost utilizate şase variante experimentale, reprezentate de diferite amestecuri de filtrate fungice, obţinute utilizând diferite proceduri (E1, E2, E3, E4, PD-E1, PD-E4, fiecare variantă în concentraţie de 1%, 5% şi 10%). Suspensiile celulare de soia au fost tratate timp de 24h cu filtratele fungice în concentraţiile amintite iar după 24h au fost realizate analize calitative şi cantitative ale unor enzime antioxidate. Dintre enzimele testate, activitatea peroxidazelor (POX) a crescut la toate variantele testate, la toate concentraţiile de elicitori utilizate, cu excepţia concentraţiei de 10% a variantei E1. În cazul catalazelor (CAT), activitatea enzimatică a crescut la plantele tratate cu filtratul fungic corespunzător variantei E2 la concentraţia 1% şi la varianta E4 concentraţiile 1% şi 5%. Celelalte variante au prezentat o descreştere a activităţii enzimatice în comparaţie cu varianta martor. Activitatea enzimatică a superoxid dismutazelor (SOD) a fost în general mai ridicată la variantele tratate, înregistrând o valoare maximă la concentraţia de 5% a variantelor E1 şi E4. În concluzie, unele mecanisme de apărare ale celulelor de soia au foste activate în urma administrării filtratelor fungice. De asemenea, administrarea filtratului fungic din varianta E4, având concentraţiile de 1% şi 5%, a fost cea mai eficientă pentru activarea sistemului antioxidant enzimatic la suspensii celulare de soia, prin creşterea activităţii enzimatice ale enzimelor implicate în neutralizarea speciilor reactive de oxigen, POX, CAT şi SOD.

Cuvinte cheie: elicitori fungici, suspensii celulare, soia, enzime antioxidante.

INTRODUCTION

Plant cell suspensions are ideal experimental systems for the study of morphogenetic processes, secondary metabolism and the response of cells towards aggressive environmental agents, including pathogen attack. Cellular suspensions are relatively homogenous systems, axenic, with cells deprived of cuticle, with reduced needs for intercellular transport and almost all are metabolically active.

G. max is a cultivated plant with major economic value, soybean seed being the world main source of vegetable protein and oil. This valuable plant species has numerous pathogens, which can cause significant crop losses, so it appeared the necessity to develop new technologies to improve disease resistance and implicitly to prevent crop losses.

This goal can be achieved by activation of plant defense system, enhancing basal resistance to pathogens. Plants resistance to pathogens can be enhanced by both biotic and abiotic factors (elicitors). Among biotic resistance inducers, microbial cultures were often used to activate different defense responses in plants (TON et al., 2002, BENHAMOU et al., 2003, SIDDIQUI & MEON, 2009, AN et al., 2010). Also, it was studied the utilization of elicitors, both exogenous (SHIMIZU et al., 2013) and endogenous (MOHARAM, 2013) for defense activation against pathogens. Although different aspects of disease resistance activation in plants were intensely studied, there are no reports regarding fungal filtrates utilization for defense induction in soybean cell suspensions. The goal of the present study is to describe the biochemical response of soybean cell suspensions to the treatment with fungal elicitors. The elicitors were represented by a mixture of fungal filtrates obtained from strains of antagonistic species *Trichoderma viride* and *Trichoderma harzianum*, a non-pathogenic species *Penicillium chrysogenum* and a pathogenic species, *Botrytis cinerea.* The elicitors from these microorganisms can activate plant defense responses dependent on various signalling molecules, like

salicylic acid, jasmonic acid or ethene. In our study, combining fungal filtrates from these four different species we intended to activate different defense signalling pathways and to induce defense-specific reactions in our *in vitro* system.

MATERIALS AND METHODS

Soybean cell suspension preparation

We used two-weeks old plantlets obtained from aseptic germinated seeds of the Romanian cultivar Daciana (provided by the NARDI Fundulea). Explants of 3-5 mm from cotyledon, epicotyl, hypocotyl and leaf fragment were cultivated on callusogenesis culture medium. Cell suspensions were obtained on S2-4 liquid medium, from fragments of callus induced on the same growth medium supplemented with agar. The (growth) callusogenesis medium contains essential nutrients, micronutrients and vitamin B5 GAMBORG (1968), to which we added 1 mg/l 2,4D, 0.1 mg/l BAP, 0.3 g/l hydrolysed casein, 7 g/l agar and 30 g/l sucrose. The callus was grown in dark conditions, at a temperature of 25ºC ± 2ºC.

Treatment protocol

The seven variants used by us were represented by: M - control (no treatment), E1 - treatment with fungal filtrate obtained from *Botrytis cinerea* strains F1 (BcF1), E2 - treatment with fungal filtrate obtained from *Botrytis cinerea* strains Bc F1, Bc F7, Bc S1, Bc P2, E3 - treatment with fungal filtrate from *Trichoderma viride* strains P 456 (TvP456), TvP1, *Trichoderma harzianum* ThP8 and *Penicillium chrysogenum* A2 (Pc A2), E4 - treatment with fungal filtrate obtained from strains Bc F1, Bc F7, Bc S1, Bc P2, TvP456, TvP1, ThP8, Pc A2. PD1-E4 and PD2-E1 represented the technological variants of E4 and E1 fungal filtrate, which differed in concentration of initial fungal inoculums. Soybean cell suspensions were treated for 24 hrs with the six fungal filtrates in 1%, 5% and 10% concentrations (w/v), each with three replicates, and were maintained in dark, on an orbital shaker at 80 rpm. Each variant was.

Total protein extract preparation

The samples used for this experiment consisted of both cellular suspension soybean cells and cellular suspension filtrate. Total cytosolic proteins were extracted by grinding the cells with quartz sand in phosphate buffer 0.05M, pH7, with 2 mM Na_2EDTA and 4% (w/v) PVP, at 40°C. Extracellular proteins were obtained by suspension cell filtration. After centrifugation at 15,000 rpm, for 15 min, the supernatant was stored at 4ºC, and the total protein content was measured according to BRADFORD (1976).

Enzyme quantification and electrophoretic analysis of isoenzymes

The quantification of enzymatic activity of peroxidases, catalases and superoxide dismutases was done as described before (HELEPCIUC et al., 2014 – in press).

Isozyme electrophoresis was done in native polyacrylamide gel (10% polyacrylamide for POX and SOD, 8% polyacrylamide for CAT) in 50mM Tris-glycine buffer, pH 7, at 10 mA for migration and 15 mA for concentration for 2 hrs. POX were stained using the method with benzidine in acetate buffer pH 5 (WANG & WANG, 1989). Superoxide dismutase (SOD) was stained with Nitro Blue Tetrazolium according to BEAUCHAMP & FRIDOVICH (1971), and catalase (CAT) was stained according to the method described by IORDĂCHESCU & DUMITRU (1988). For quantification of each enzyme activity we have used three replicates.

RESULTS AND DISCUSSIONS

Callus and cell suspension particularities

In order to obtain cellular suspensions, it is important that the inoculum, represented by callus, to be friable. However, we have obtained a compact, proliferative, non-morphogenic and very adherent callus, characteristics that allowed us only to obtain a cell suspension formed of cell aggregates with a 1-2 mm diameter, rich in chlorophyll. The structure of the obtained callus is probably a result of a very active secondary metabolism and a high content of proteins involved in adhesion (KITABAKE & FUJITA, 2000). Optic microscopy analysis of this callus on squash samples identified small cells, with a weakly developed vacuome and a high rate of division, and of elongated cells, with well-developed vacuoles and a high metabolic rate (Fig 1). In addition, we observed xylematic cells that facilitate the transport of nutrients towards the interior of cell clusters (Fig. 1).

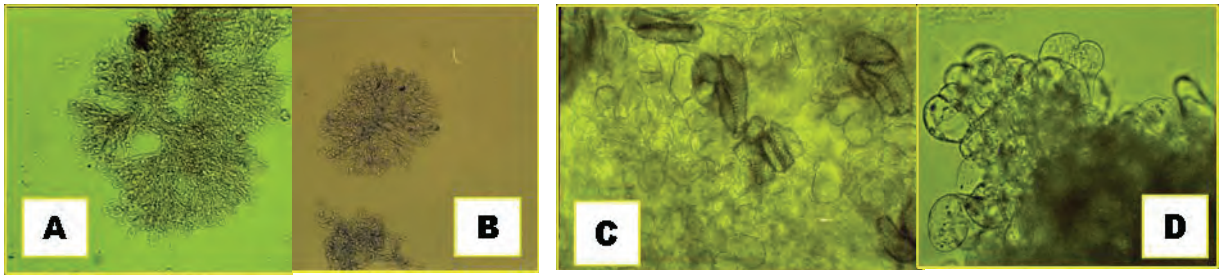

Figure 1. Light microscope observations of soybean cell suspensions on squash preparates: A, B – suspension cell aggregates with a 1-2 mm diameter; C – (xylematic) tracheids (cells) differentiation; D – small proliferating cells at the periphery of a cell aggregate.

Cell suspension response to fungal elicitors

Administration of fungal elicitors did not induce either visually or microscopically changes in the treated variants comparing with the control, but at biochemical level, the analysis of enzymes involved in oxidative stress control, like POX, CAT and SOD indicated a defense response of the soybean cells suspension. SOD activity increased compared to the control, but the response was not uniform, in several experimental variants the enzyme activity decreasing at higher concentrations of the elicitor. The highest SOD activity value was recorded for the 5% elicitor concentration in E1 experimental variant, while in 10% concentration variant the enzyme activity decreased (Fig. 2). The superoxide dismutase spectrum (Fig. 3) reveals five SOD isoenzymes in all variants except for E1 at 5% and 10% concentrations, in these two variants only two isoforms being expressed. The SOD isozymes are not correlated with enzyme activity measurements.

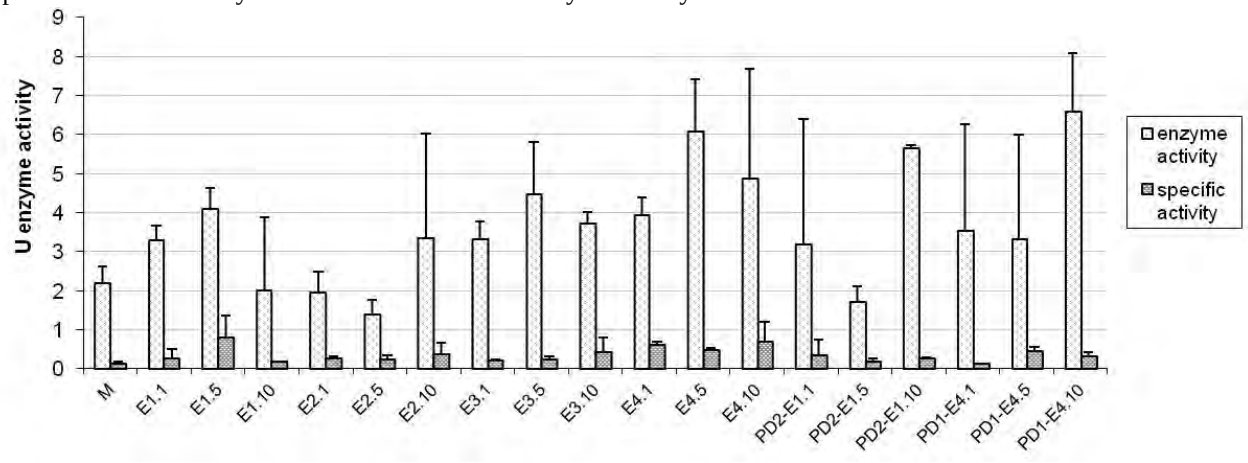

Figure 2. SOD activity in untreated cell suspensions (M) and in treated cell suspensions: E1, E2, E3, E4, PD1-E4 and PD2-E1. The second digit of each sample represents the concentration of the fungal filtrate 1%, 5% or 10%.

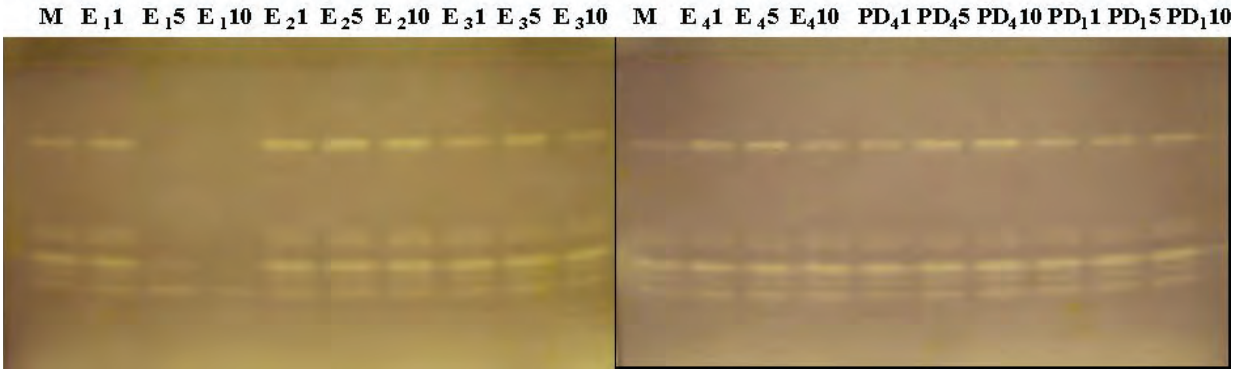

Figure 3. SOD electrophoretic spectra of untreated soybean cell suspension (M) and in treated cell suspensions: E1, E2, E3, E4, PD1-E4 and PD2-E1. The second digit of each sample represents the concentration of the fungal filtrate 1%, 5% or 10%.

The treatment with fungal filtrates induced an increase in POX activity independent of concentration and the type of elicitor used (Fig. 4). The highest POX activity level was observed for the 1% concentration of the experimental variant E4. The treatments with the lowest concentration of fungal filtrate (i.e. 1%) induced an increase in POX activity in variants E2 and E4.

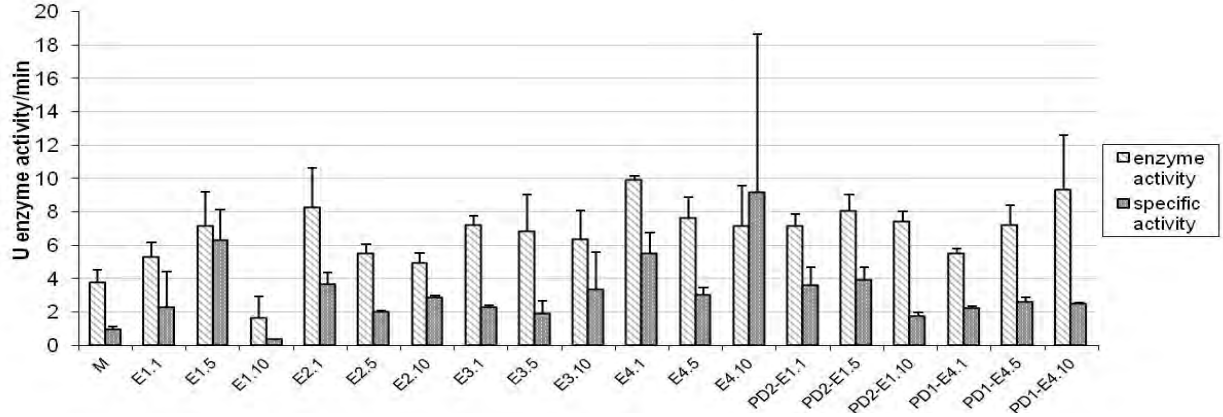

Figure 4. POX activity in untreated cell suspensions (M) and in treated cell suspensions: E1, E2, E3, E4, PD1-E4 and PD2-E1. The second digit of each sample represents the concentration of the fungal filtrate 1%, 5% or 10%.

Electrophoresis of POX showed differences in the activities of the isozymes, generally correlated with the dynamics of overall POX activity. POX isozymes are concentrated in the slow-migration zone and their activity increases in the E4 experimental variant. In case of E4 variant, new isoforms of POX are present in the samples treated with high concentrations (10%) of fungal filtrate (Fig. 5, see arrow). It can also be observed that some treatments induce an overexpression of some isoforms, for example the isoperoxidase from the first position on the gel in case of E2 variant (Fig. 5, see arrow) and the isoperoxidase from the first position on the gel in case of E4 variant (Fig. 5, see arrow).

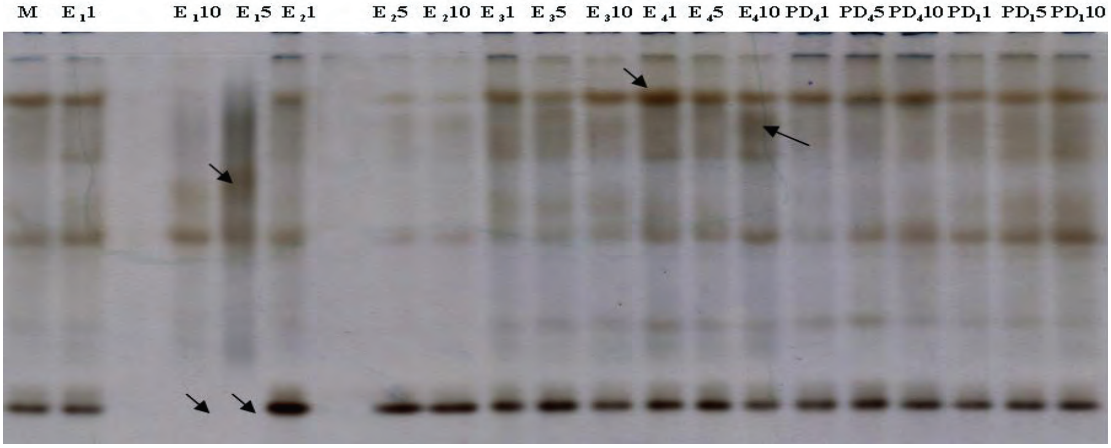

Figure 5. POX electrophoretic spectra of untreated soybean cell suspension (M) and in treated cell suspensions:
E1, E2, E3, E4, PD1-E4 and PD2-E1. The second digit of each sample represents the concentration of the fungal filtrate 1%, 5% or 10%.

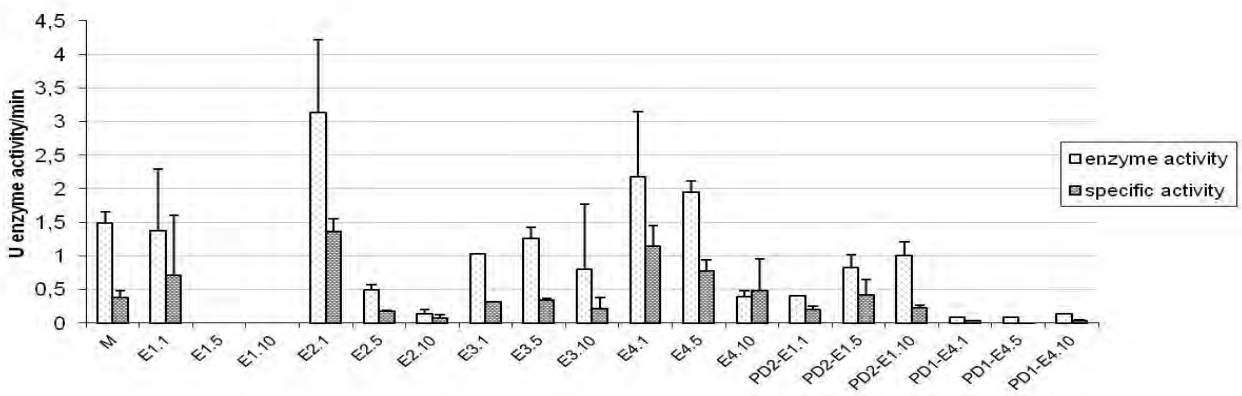

Figure 6. CAT activity in untreated cell suspensions (M) and in treated cell suspensions: E1, E2, E3, E4, PD1-E4 and PD2-E1.
The second digit of each sample represents the concentration of the fungal filtrate 1%, 5% or 10%.

CAT activity is generally decreasing comparing with the control, except for several experimental variants which present an increase: E2 for a 1% fungal elicitor concentration and E4 at 1% and 5% (Fig. 6). In all these variants POX activity was also higher. Electrophoresis of CAT in soybean cell suspensions showed a single isoenzyme (Fig. 7) that is absent in experimental variant E1, in higher concentrations, results which are correlated with the quantitative assay of CAT. Also, in 5% E2 and 10% E3 variants the CAT isoform is not expressed.

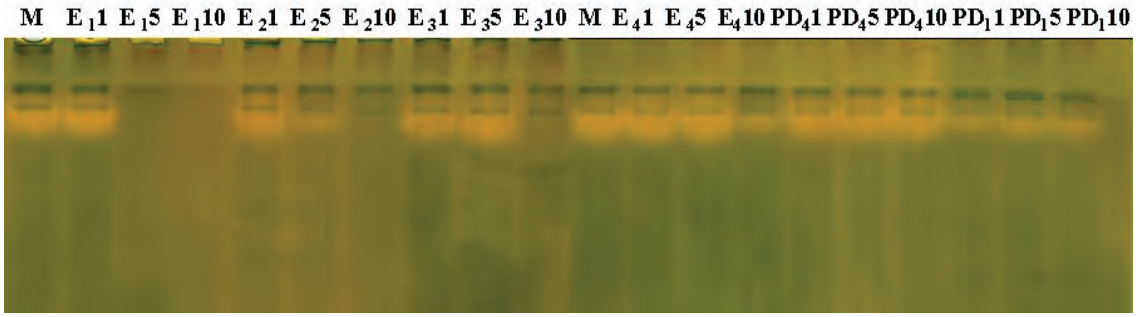

Figure 7. CAT electrophoretic spectra of untreated soybean cell suspension (M) and in treated cell suspensions:
E1, E2, E3, E4, PD1-E4 and PD2-E1. The second digit of each sample represents the concentration of the fungal filtrate 1%, 5% or 10%.

Peroxidase extracellular enzymatic activity in soybean cell suspensions was higher than at cellular level, which indicates an intense excretion of peroxidases in extracellular medium. As in case of intracellular peroxidases, the enzymatic activity in culture medium was generally higher than the control, except for E1 and PD-E1 variants (Fig. 8).

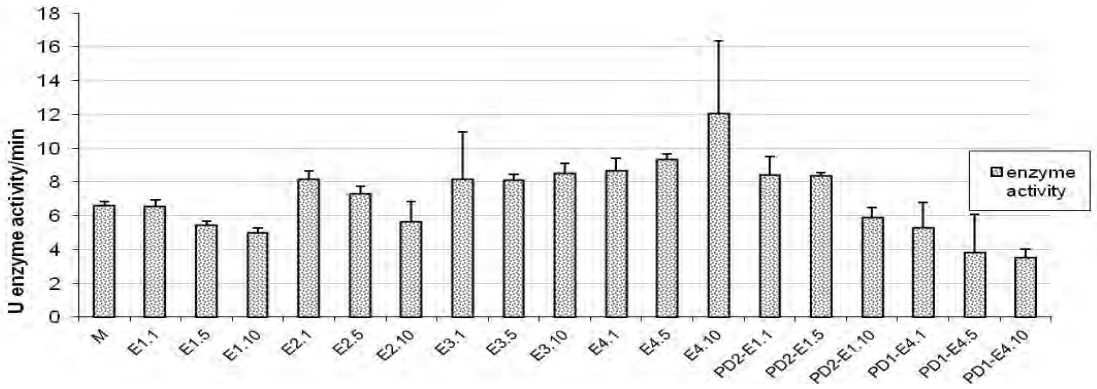

Figure 8. POX extracellular activity in untreated cell suspensions (M) and in treated cell suspensions:
E1, E2, E3, E4, PD1-E4 and PD2-E1. The second digit of each sample represents the concentration of the fungal filtrate 1%, 5% or 10%.

Although our previous results showed an induction of catalase-detoxifying H_2O_2 excess pathway (COGĂLNICEANU et al., 2010, HELEPCIUC et al., 2014 in press), the current results indicate an involvement of peroxidase rather than catalase in oxidative stress response of the cell to biotic elicitors. Similar results were mentioned previously in scientific literature, when single elicitors or elicitors from just one species where used. For example, the results obtained by SAISAVOEY et al. (2014) in *Pueraria mirifica* cell suspension treated with a plant endogenous elicitor, methyl jasmonate, showed that catalase also decreased in elicited variants comparing with the control. Also, a study reveals that in *Euphorbia pekinensis* cell culture treated with fungal elicitors from *Fusarium* sp., at 48 hrs catalase activity is slightly higher in elicited variants, but reaches a maximum six days after the elicitor administration (GAO et al., 2011).

However, in our study, the E4 culture filtrate variant succeeded to induce an increase in POX, CAT and SOD enzymatic activities, at 1% and 5% concentration, after 24 hrs treatment. As mentioned before, the E4 variant was obtained by filtration of fungal cultures of four different species, with a total of eight fungal strains, so it contains the highest variety of elicitors comparing with the other variants. Among them, there is a phytopathogenic species *Botritys cinerea*, and three antagonistic species, *Trichoderma viride*, *Trichoderma harzianum* and *Penicllium chrysogenum*. Previous studies reported the activation of various defense responses when mixtures of fungal elicitors were applied (NAVAZIO et al., 2007). It is known that interference of signalling pathways provides a flexibility of plant defense response, conferring improved disease resistance in plants (PIETERSE et al., 2006). Probably our mixtures of fungal filtrates used contained molecules that elicit different signalling pathways which induced antioxidant defense response.

CONCLUSIONS

Fungal filtrates activated the antioxidant defense response in our system, inducing an increase in SOD, CAT and POX antioxidant activity. The best defense response in soybean cell suspensions was obtained by treatment with E4 variant. All three antioxidant enzymes registered higher enzymatic activities comparing with the control. For the rest of the experimental variants, SOD and POX had generally higher enzymatic activities than the control, while CAT showed a lower activity. The mixture of the eight fungal strains from E4 induced the most efficient defense response, activating the antioxidant response reflected by increased enzymatic activity in the experimental system used.

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Gina Cogălniceanu, Monica Mitoi, Florenţa-Elena Helepciuc, Florentina Aldea
Institute of Biology Bucharest, Romanian Academy,
296 Splaiul Independenţei, 060031 Bucharest, Romania.
E-mail: gina.cogalniceanu@ibiol.ro;
monica.carasan@ibiol.ro

Sorin Matei, Gabi-Mirela Matei
Research Institute for Soil Science and Agrochemistry,
Bdul Mărăşti 61, 011464 Bucharest, Romania.

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