PROFILES OF ANTIOXIDANT ISOENZYMES IN THE PATHOSYSTEM *SCENEDESMUS–PHLYCTIDIUM* AFTER TREATMENT WITH PLANT GROWTH REGULATORS

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Summary. The present study was undertaken to investigate the possibilities of some plant growth regulators – abscisic acid, fluridone, methyl jasmonate, salicylic acid and the cytokinin 4-PU-30 to participate in the disease tolerance induction in the unicellular green alga *Scenedesmus incrassatulus* invaded with the unicellular fungal parasite *Phlyctidium scenedesmi*. We established that protein profiles in host cells changed drastically after the parasite invasion. Pretreatment of algal cells with PGR (4-PU-30 and MeJA) depressed the depletion of soluble proteins in *Scenedesmus* cells caused by *Phlyctidium* invasion. The presence of SA and MeJA in the cultivation medium caused a significant enhancement of the amount of the fastest moving thermostable protein band. In ABA-, fluridone- and 4-PU-30-pretreated invaded algal cells significant activation of peroxidase which plays an important role in resistance induction was observed. SA stopped catalase enhancement in the invaded cells. Parasite invasion was connected with both qualitative and quantitative changes in the spectrum of SOD. The data obtained support the suggestion that ABA, MeJA, 4-PU-30 and SA are involved in plant-pathogen interactions and acquisition of pathogen tolerance.

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Key words: green alga,chytridial parasite, abscisic acid, methyl jasmonate, salicylic acid, cytokinin, antioxidant enzymes, resistance.

Abbreviations: PGRs–plant growth regulators; ABA–abscisic acid; JA–jasmonic acid; MeJA–methyl jasmonate; SA–salicylic acid; ROS–reactive oxygen species; PR–pathogenesis related; SOD–superoxide dismutase; TCA–trichloracetic acid; BSA–bovine serum albumin.

INTRODUCTION

It is well known that the production of reactive oxygen species (ROS) is stimulated by various environmental and biotic stresses such as invasion by pathogens (Foyer and Noctor, 2005; Suzuki and Mittler. 2006). ROS could potentially affect many cellular processes involved in plant/pathogen interactions (Baker and Orlandi, 1995). The production of ROS is an important defense mechanism in plants against pathogens (Sahoo et al., 2007). However, excessive production of ROS may cause the disruption of cellular functions, finally leading to cell death. The equilibrium between ROS production and scavenging is extremely important. Particular attention is paid to the ROS scavenging antioxidants including SOD and CAT (Edreva, 2005).

Plant cells have evolved efficient defense antioxidant enzymatic and non enzymatic mechanisms to cope the danger posed by the presence of ROS (Mallick and Mohn, 2000). A lot of data concerning higher plants prove that ABA acts as a hormone responsible for the adaptation to different kinds of stress (Palva et al., 2002). According to Jiang and Zhang (2001) exogenous application of ABA enhances superoxide radical and H_2O_2 levels followed by an increase in the activity of the antioxidant enzymes SOD, CAT and ascorbate peroxidase.

The role of plant hormones in the complicated host-pathogen relationship is not sufficiently elucidated (Czerpak et al., 2006). Contradictory data exist in the literature. A special attention has been given to jasmonic acid

(JA) and its methyl ester as key molecules of the lipoxygenase signaling pathway mediating the defense response to infection (Sembdner and Partier, 1993; Czerpak et al., 2006). The experiments concerning the role of plant growth regulators in the disease response are mainly carried out with higher plants, but the results which clarify how these substances operate at the biochemical level in green algae are still limited.

In the present study, we investigated the influence of ABA, fluridone, MeJA, SA and the cytokinin - 4-PU-30 on the infection process and the capacity of these plant hormones to induce resistance in the host - parasite system unicellular alga - unicellular fungal parasite.

MATERIALS AND METHODS

Plant material

The investigation was carried out on the host-parasite system unicellular green alga *Scenedesmus incrassulatus* Bohl and the obligatory unicellular fungal parasite *Phlyctidium scenedesmi Fott (Chytridiales).* The development of host and parasite was maintained in an optimal nutrient medium at conditions of intensive cultivation (Benderliev et al., 1993). The roles of ABA (10^{-5}M), fluridone (10^{-7} M), MeJA (10^{-5} M), SA (1.8. 10^{-3} M) and 4-PU-30 (10^{-5} M) in the resistance induction were elucidated in two series of experiments. In the first one the influence of direct exogenous treatment on uninfected algal cells with MeJA, SA and 4-PU-30 for 7 days was investigated. In the second series the preliminary treated with these substances algal cells were infected for 48 h with *Ph. scenedesmi*. After the removal of PGR algal cells were resuspended in a cultural medium optimal for the parasite development and infected with *P. scenedesmi*.

Protein extraction

Frozen algal cells were disintegrated in 0.1 M Tris-HCL buffer, pH 7.1. All steps of extract preparation were performed at 4 ^{o}C The homogenate was centrifuged at 12 000 x *g* for 30 min at 4 ^{o}C. The supernatant was used as a crude enzyme extract. All samples were stored at -20 ^{o}C until enzyme

analysis.

The fraction of thermostable proteins was obtained by high temperature treatment (96 °C for 10 min) of the crude extracts. Native polyacrilamide gel electrophoresis (native PAGE in 7,5 % gel) was carried out by the method of Davis (1964). SDS-PAGE under the denaturing (SDS) and reducing (β-ME) conditions in 12 % PAG was performed by the method of Laemmli (1971). Silver staining method by Nesterenko et al. (1994) was used for polypeptide profile visualization.

Protein content in the crude extracts was determined after TCA precipitation according to the method of Lowry et al. (1951) using BSA as a standard.

Enzyme visualization

Peroxidase isoenzymes were detected on the gels by the method of Ornstein (1964) with benzidine as an H-donor.

Superoxide dismutase isoenzymes were detected on the gels by the method of Greneche et al. (1991).

Catalase isoenzymes were stained as described by Woodbury et al. (1971).

RESULTS

The bulk of protein amount in control *Scenedesmus* cells was localized in one slow migrating band (Rubisco) and in some of the moderate moving bands (Fig.1). In the presence of ABA and fluridone, the intensity of moderate moving bands decreased. Protein profiles changed most drastically after parasite invasion. In the presence of the parasite the Rubisco band disappeared and the quantity of the moderate moving protein bands significantly declined after ABA and fluridone treatment. In the case of MeJA, SA and 4-PU-30 pretreatment Rubisco degradation was delayed.

Rubisco band was missing in the spectrum of thermostable proteins (Fig.2). Most thermostable proteins were localized in the fastest moving bands. The presence of MeJA and SA in the cultivation media caused a significant enhancement of the fastest moving band. No protective effect of

PGRs applied on the thermostable protein spectrum after pretreatment of algal cells was established.

In the control *Scenedesmus* cells faint polypeptide bands were detected (Fig. 3). Direct treatment of algal cells with MeJA, SA and 4-PU-30 induced the synthesis of both available and new polypeptides, the effect of 4-PU-30 being the most significant. The protecting effect of MeJA, SA and 4-PU-30 pretreatment on protein degradation under the influence of the parasite invasion was observed. MeJA was a better protector compared to SA and 4-PU-30.

The activity of peroxidase isoenzymes in control and PGR-treated

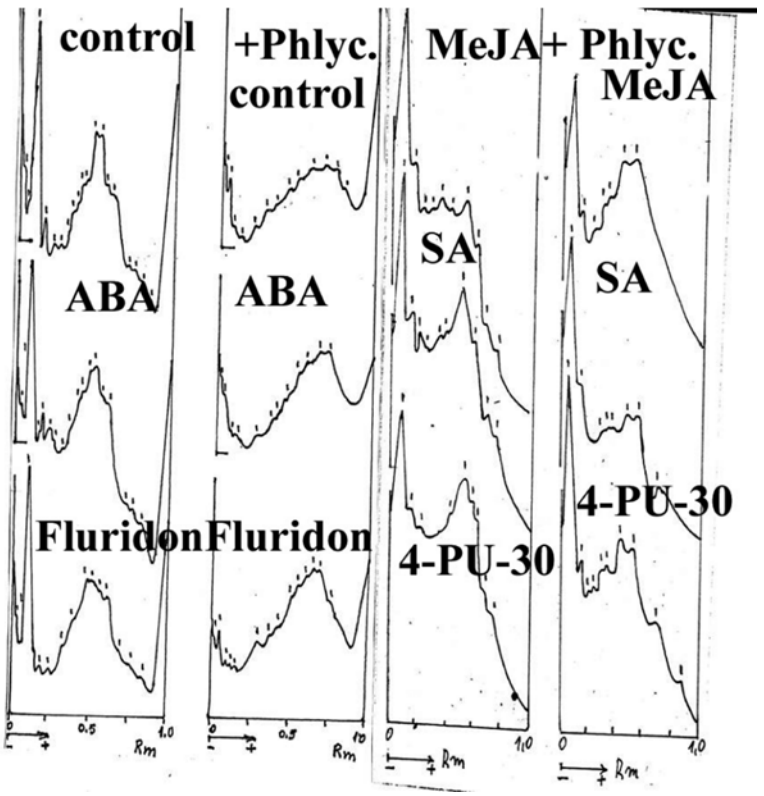

Fig. 1. Densitometric scans of soluble protein spectra (native PAGE) of unicellular green algae *S. incrassatulus*. A hundred μg protein was loaded per each tube. The protein profile was visualized on gels by staining with Coomassie Blue.

Scenedesmus cells was negligible. In the case of Phlyctidium invasion a significant activation of the fast moving peroxidase isoenzyme was

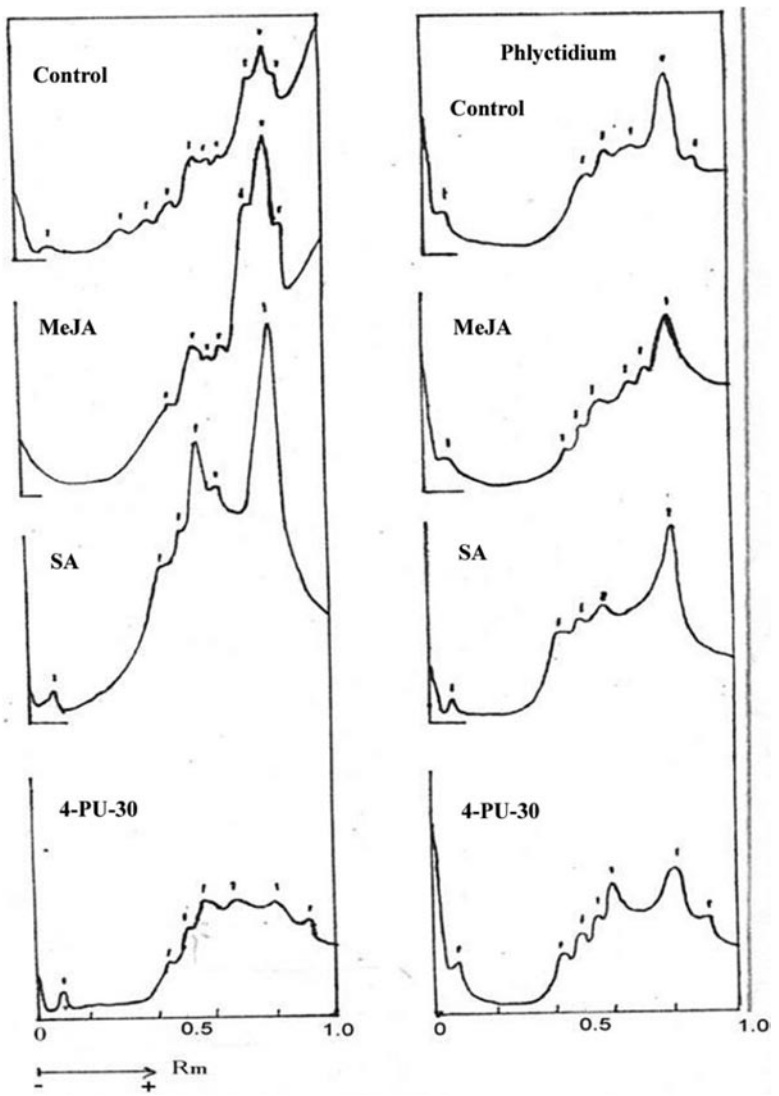

Fig. 2. Densitometric scans of thermostable soluble protein spectra (native PAGE) of unicellular green algae *S. incrassatulus*. A hundred μg protein was loaded per each tube. The protein profile was visualized on the gels by staining with Coomassie Blue.

observed under the influence of all PGRs applied. The activity of peroxidase isoenzymes increased strongly in ABA, fluridone and 4-PU-30-pretreated parasite invaded host cells (Fig. 4).

In contrast to peroxidase, the catalase isoenzyme profiles showed high activity of catalase detected in the algal cells (Fig. 5). The activity of catalase in control *Scenedesmus* cells was presented by two high active isoenzymes. Their activity in the uninfected algal cells increased under the influence of the PGRs applied. Parasite invasion activated catalase, a new faster moving isoenzyme appeared in invaded algal cells. The activation of catalase isoenzymes was more pronounced in the *Phlyctidium* infected cells pretreated by ABA, MeJA and 4-PU-30. SA stopped the enhancement of catalase activity by parasite invasion.

The isoenzyme profiles of SOD (Fig. 6) showed high activity in control *Scenedesmus* cells, localized in the moderate moving izoenzymes. Parasite invasion influenced both the activity of the present SOD isoenzymes and

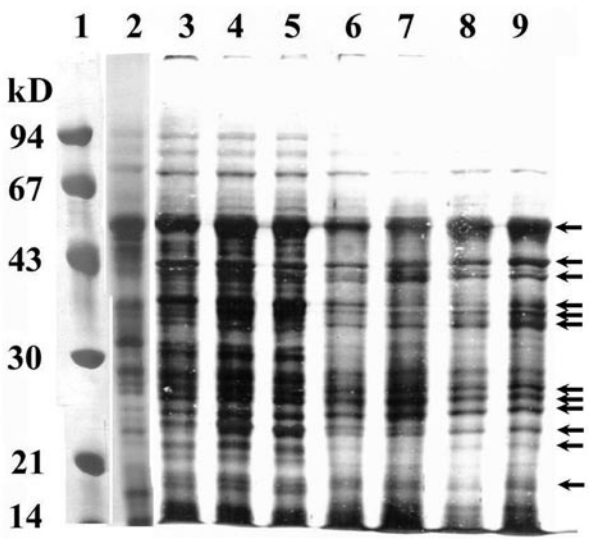

Fig. 3. Polypeptide spectra of total proteins of unicellular green algae *S. incrassatulus*. 1- protein molecular weight markers; (2-5) – total polypeptides from control cells treated by MeJA, SA and 4-PU-30; (6-9) – total polypeptides from invaded cells pretreated by MeJA, SA and 4-PU-30. Ten μg proteins were loaded on each lane.

the number of isoenzymes visualized. Some of the newly appeared bands could be inherent to the parasite or synthesized de novo in *Scenedesmus* cells in response to the parasite invasion (Fig. 7). No remarkable direct effect of PGRs was observed. In the case of PGR pretreatment of invaded cells, a very strong synergestic effect of the parasite invasion and all PGRs applied was found. A special effect of SA on the activity of slow migrating isoenzyme N3 coud be emphasized.

DISCUSSION

The present results showed that protein profiles changed most drastically after the parasite invasion. In parasite invaded algal cultures the Rubisco

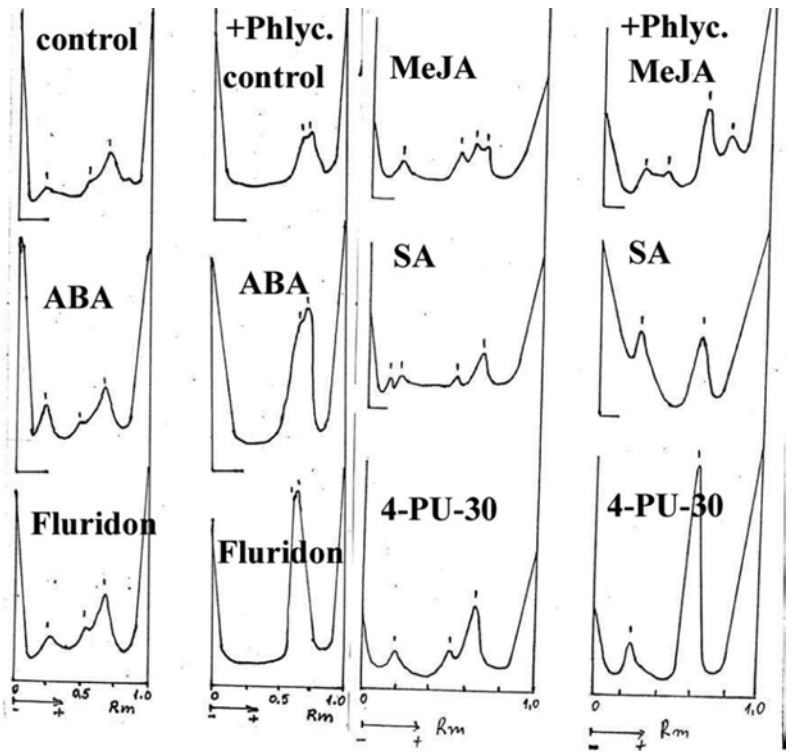

Fig. 4. Densitometric scans of anionic peroxidase isoenzymes of unicellular green algae *S. incrassatulus*. Two hundred μg protein was loaded per each tube.

band disappeared and the quantity of the moderate moving protein bands significantly declined. Mehta et al. (1992) reported that oxidative stress caused a rapid *in vivo* degradation of Rubisco in two species of higher plants and two algal species. There was no stimulating effect of the direct PGR treatment on the content and composition of soluble proteins of *S. incrassatulus* unlike the data of Czespak et al. (2006). Pretreatment of algal cells with MeJA, SA and 4-PU-30 delayed significantly Rubisco degradation under the influence of the parasite.

In the control cells of *Scenedesmus* 23 faint polypeptide bands were detected. Wu et al. (2007) revealed 11-12 polypeptides in *Scenedesmus obliquus* cells, but Czerpak et al. (2006) found only 8 polypeptides in the *Chlorella* cell. Direct treatment of algal cells with SA, 4-PU-30 and MeJA

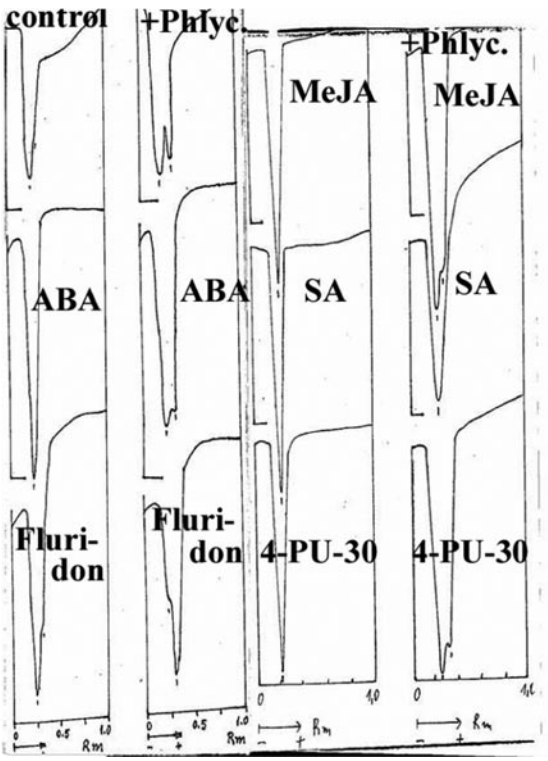

Fig. 5. Densitometric scans of catalase isoenzymes of unicellular green algae *S. incrassatulus*. Twenty five µg protein was loaded per each tube.

induced the synthesis of both available and new polypeptides, the effect of 4-PU-30 being the strongest. SA, JA, H_2O_2, GA_3 and ABA, are known to induce the expression of defense or stress-related genes in plants, each employing a signal transduction pathway (Dong, 1998). It was suggested that the production /accumulation of PR proteins in plants in response to invading pathogens and/or related abiotic stress situations was one of the crucial components in the inducible repertoire of the plant's self-defense mechanism (Jwa et al., 2001)

We established that the parasite invasion caused also the enhancement of polypeptide amounts. Quantitative changes in the polypeptide pattern of total cellular proteins after treatment of *Chlorella* cells with JA were also observed by Czerpak et al. (2006).

Phlyctidium invasion itself as well as the direct PGR treatment did not change peroxidase activity in the host alga cells. This result is contradictory

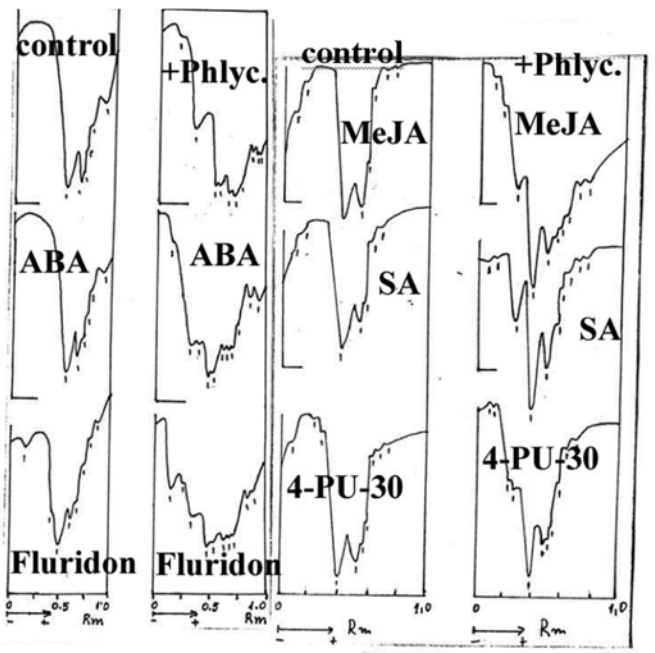

Fig. 6. Densitometric scans of superoxide dismutase isoenzymes of unicellular green algae *S. incrassatulus*. A hundred µg protein was loaded per each tube.

to the data of Wu et al. (2007) showing that *Potamogeton malaianus* stimulated peroxidase, SOD and catalase activities in *Scenedesmus obliquus* at the low initial cell density. In the case of *P. scenedesmi* invaded algal cells a significant activation of the fast moving peroxidase isoenzyme was obtained. The activity of peroxidase isoenzymes increased strongly in ABA-, fluridone- and 4-PU-30-pretreated invaded host cells. It was found that MeJA reduced *Scenedesmus* viability and enhanced peroxidase and glutamate dehydrogenase activities and regulated α-esterase activity in algal cultures (Pouneva et al., 1994). It is well known that peroxidases are implicated to play a major role in plant pathogen interaction (Castillo, 1992).

The catalase activity in the uninfected algal cells increased under the

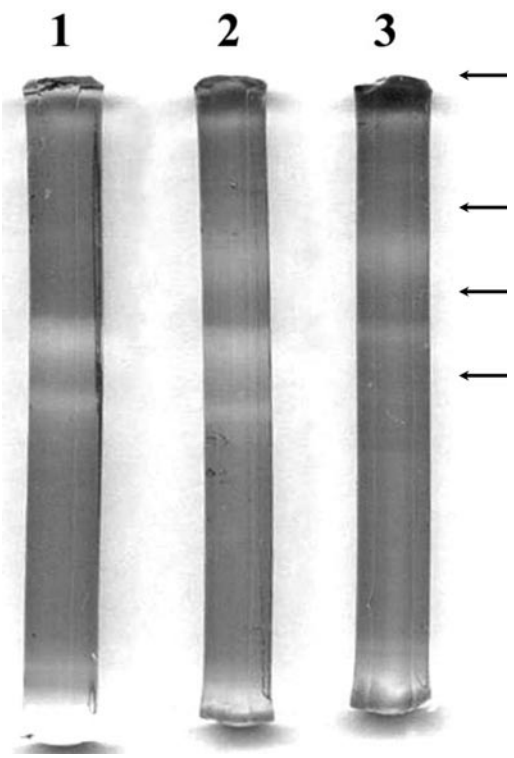

Fig. 7. SOD isoenzyme profiles: 1. Control cells of *S. incrassatulus*; 2. *S. incrassatulus* cells invaded with *P. scenedesmi*; 3. Zoospores of *P. scenedesmi*.

influence of the PGRs applied. Parasite invasion activated catalase, a new faster moving isoenzyme appeared in the invaded algal cells. The activation of catalase isoenzymes was more pronounced in the *Phlyctidium* infected cells pretreated with ABA, MeJA and 4-PU-30. SA inhibited this activation. This effect of SA confirms the well known result obtained for higher plant (Janda et al., 1999).

The high SOD activity found in control *Scenedesmus* cells supports the suggestion about the key role of this enzyme in eliminating ROS in algal cells (Kong and Sang, 1999). Moreover, Li et al. (2005) established that the change of SOD activity under the influence of cypermethrin started earlier than the change of growth. This indicated that the change of SOD activity was very sensitive to the environment. As these changes took place at the molecular level in the cells, they happened much earlier than growth or reproduction (Rabinovich and Fridovich, 1985). They consider that SOD serves as a very sensitive biomarker and could be used for early warning of unfavorable environmental changes. SOD isoenzyme profile was significantly changed after the parasite invasion. Parasite invasion influenced both the activity of the present isoenzymes and the number of isoenzymes visualized. The role of SOD in plant defense response against systemic virus infection has been well documented (Riedle-Bauer M. 2000; Sahoo et al., 2007). SOD increased under stress to protect plants from oxidative damage (Mckersie et al., 1996; Lesser, 2008) and an apparent correlation of higher SOD activity with disease resistance was established by Sahoo et al. (2007). Many authors have proved that pathogen infection of plants results in increased ROS generation which in turn causes the induction of antioxidant enzymes, including SOD, in the resistant genotypes.

The data obtained support the suggestion that ABA, MeJA, 4-PU-30 and SA are involved in plant-pathogen interactions and acquisition of pathogen tolerance.

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