General and Applied Plant Physiology – 2009, Volume 35 (3–4), pp. 153–161
©2009 ISSN 1312-8183 Published by the Institute of Plant Physiology – Bulgarian Academy of Sciences
Available online at http://www.bio21.bas.bg/ipp/

Special Issue (Part I) – Proceedings of the XI National Conference on Plant Physiology
18–19 November 2009, Sofia, Bulgaria

BIOTIC STRESS RESPONSE OF *SCENEDESMUS INCRASSATULUS* INFECTED BY *PHLYCTIDIUM SCENEDESMI*. CHANGES OF POLYPEPTIDE AND ISOENZYME PROFILES OF SUREROXIDE DISMUTASE AND CATALASE

Puneva I. **[*]** ***and D. Nedeva***

Acad. M. Popov Institute of Plant Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Str, bl. 21, 1113, Sofia, Bulgaria

Received: *10 March 2010* Accepted: *26 March 2010*

Summary. The effects of three different initial degrees of infection (30, 45 and 70%) of the green alga *Scenedesmus incrassatulus* with the unicellular fungal parasite *Phlyctidium scenedesmi* on cell growth, protein, polypeptide and isoenzyme profiles of both SOD and CAT in the host cells were investigated. The effects of the strong oxidant sanosil with algicide and fungicide action and pathogen invasion were compared. Protein amount decreased significantly under the influence of parasite invasion. Sanosil diminished drastically the polypeptide content. Trace protein and polypeptide amounts were present in zoospores of *Phlyctidium*. Two intensive new polypeptides were detected in the profile of zoospores. *Phlyctidium* increased CAT activity in the host cells especially in the case of the highest parasite initial infection. Sanosil enhanced CAT activity similarly to parasite invasion. In contrast, SOD activity decreased drastically after treatment with the highest parasite concentration. A new highly active isoenzyme (Rm 0.26) appeared in the profile of invaded *Scenedesmus* cells originated from *Phlyctidium* genome. The data concerning the changes of protein, polypeptide and isoenzyme profiles of the antioxidant enzymes CAT and SOD due to the parasite infection and oxidant treatment were discussed as a proof that parasite invasion acted at the molecular level causing oxidative stress.

***Key words**:* green alga, chytridial parasite, protein profiles, SOD, CAT, oxidative stress.

Abbreviations: BSA – bovine serum albumin, CAT – catalase, EDTA – diamino-ethane-tetra acetic acid, NBT – nitrotethrazolium blue, PAGE – polyacrylamide gel electrophoresis, PO – peroxidase, Rm – relative mobility, ROS – reactive oxygen species, SDS – sodium dodecyl sulphate, SOD – superoxide dismutase, TCA – trichloracetic acid.

[*]Corresponding author: ipuneva@bio.bas.bg

INTRODUCTION

Parasitic fungi which belong to the Chytridiomycetes have strong effects on their host populations although they are often neglected mainly owing to their insignificant biomass. Chytrids frequently cause significant algal mortality since the prevalence of infection during epidemics may exceed 90% (Polis and Strong, 1996; Kagami et al., 2007). It was established that disease resistance is associated with the activation of wide range of defence responses that serve to prevent pathogen infection. The ability of algae to cope parasite development with the excess of reactive oxygen species (ROS) is presumably one of the main factors that influence its survival (Mallick and Mohn, 2000). The production of ROS is an important defence mechanism in plants against pathogens (Sahoo et al., 2007) and one of the earliest responses of plants to attempted infection by pathogens (Grant and Loake, 2000). Algae as well as higher plants developed a defence system involving SOD, CAT, PO and glutathione reductase enable to neutralize the toxic effects of ROS (Baranenko, 2001; Roginsky and Barsukova, 2001; Wu et al., 2007). The overall results in literature have revealed significant effects of the infection on the induction of antioxidant enzymes and isozyme patterns in resistant and susceptible genotypes. Detailed information concerning activation of antioxidant system in algae invaded by pathogens is scarce although algae are recommended as biological monitors for biotic and environmental stresses. It was observed that the activities of protective enzymes (SOD, CAT and PO) involved in H_2O_2 metabolism have been activated to high levels in fungal elicited cell cultures due to oxidative burst. H_2O_2 treatment alone could mimic the oxidative burst (Zhao et al., 2007). There is little information on the role of ROS inducing common responses in plants by different stressors (Babu et al., 2003). Our study aimed to examine if two stressors (pathogen and sanosil) associated with ROS production could be involved in the elicitation of similar responses. In the present investigation we report the effect of different initial degrees of pathogen infection on cell growth, native protein, polypeptide, SOD and CAT profiles in *Scenedesmus incrassulatus* cells.

MATERIALS AND METHODS

Material

The investigation was carried out on the host-parasite system unicellular green alga *Scenedesmus incrassulatus Bohl (Chlorophyta)* and the obligatory unicellular fungal parasite *Phlyctidium scenedesmi Fott (Chytridiales*). The development of host and parasite was maintained in the optimal nutrient medium at conditions of intensive cultivation (Benderliev et al., 1993). The role of three initial degrees of infection (30, 45 and 70%) with *P. scenedesmi* on the algal cell growth was investigated gravimetricaly.

After 48 h treatment of both non-infected cells of *S. incrassatulus* and zoospores of *P. scenedesmi* (the unique unattached phase during the parasite development) with 0.0025% sanosil polypeptide and isoenzyme profiles of SOD and CAT were examined.

Viability testing

The effect of 24-h, 48-h and 72-h treatments with 0.025% sanosil on the pathogen viability (zoospores and cysts) was evaluated using the triphenyl tetrazolium test (Jones, 1987).

Protein extraction

Frozen algal cells were disintegrated in 0.1 M Tris-HCl buffer, pH 7.1. The homogenate was centrifuged at 12 000 x *g* for 30 min at 4°C. The supernatant was used as a crude enzyme extract. Aliquots of the enzyme extracts were stored at -20°C until enzyme analysis. Soluble protein and polypeptide profiles, SOD and CAT activities were analyzed electrophoretically.

Total protein content determination

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.

Native and SDS polyacrylamide gel electrophoresis

Native PAGE in 7.5% gel was carried out by the method of Davis (1964). SDS-PAGE under 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. The fraction of thermostable proteins was obtained by high temperature treatment (96°C for 10 min) of the crude extracts.

Enzyme visualization (after native PAGE)

The isoforms of CAT and SOD were detected by activity staining of crude samples separated on the native gels. Superoxide dismutase isoenzymes were detected on the gels by the method of Greneche et al. (1991). The gels were incubated for 30 min in the dark in a mixture containing 10 mg NBT, 75 mg Na_2-EDTA and 3 mg riboflavin dissolved in 100 ml tris-HCl buffer, pH 8.2. After that gels were illuminated for 15 min. Illumination was discontinued when maximum contrast between the achromatic zones and the background had been achieved**.** Activity staining of CAT was done following the method of Woodbury et al. (1971). The gel was treated with 10mM hydrogen peroxide for 20 min and after a brief rinse with distilled water, the gels were transferred to freshly prepared 1% solution of ferric chloride and potassium ferricyanide.

RESULTS

After the initial 30% and 45% infection *Scenedesmus incrassatulus* synthesized biomass up to 48h of its intensive cultivation but in significantly lower degree compared with non infected controls. In the same time after high initial infection (70%) the host growth was strongly repressed and nearly was absent till 24h and after that the absolute dry weight declined with 65% of dry mass in the beginning of the infection. This effect is the result of the appearance of the polyinfection, when the greater number of infected cells were completely destroyed (Fig. 1.)

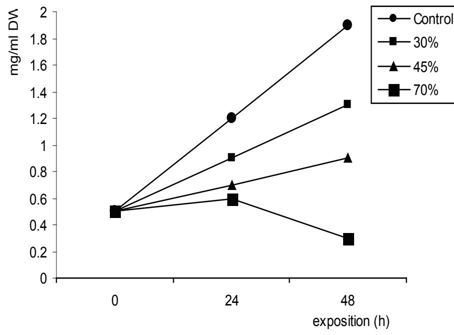

Fig. 1. Effect of initial infection with *Phlyctidium* on *S. incrassatulus* synthesized biomass.

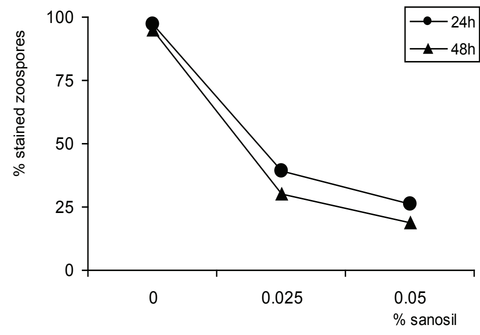

Fig. 2. Effect of sanosil on the *Phlyctidium* zoospore viability.

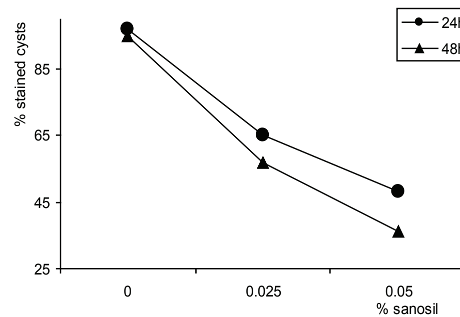

Fig. 3. Effect of sanosil on the *Phlyctidium* cyst viability.

Figures 2 and 3 illustrate the decrease of zoospore and parasite cyst viability after treatment with sanosil. During sanosil treatment (0.05%) they lost their viability by 76% and 59%, respectively. It is obvious that zoospores were more sensitive to the oxidant sanosil. The amounts of native proteins and especially that of Rubisco declined. The degree of the observd decrease depended on the concentration of the parasite, the highest degree of infection being the most effective.

The protein profiles of *Scenedesmus* changed under the influence of *Phlyctidium* invasion. It is noteworthy the very low protein amount of zoospores and poor profile, only three protein bands revealed. Lower thermostable protein amount was manifested under the influence of parasite invasion. Low thermostable protein amount was presented in zoospores. Polypeptide profiles confirm the data concerning the effect of parasite invasion on native protein profiles (Fig. 4). The intensity of polypeptide bands decreased in invaded variant in comparison with control cells. Only few faint polypeptide bands were revealed in zoospores among them two bands were specific for zoospore profile. Only traces of thermostable polypeptides were registered in control, invaded and zoospore cells (Fig. 5). Different SOD isoenzyme profiles were observed in control, invaded cells and in zoospores (Fig. 6). It is evident that the profile of invaded cells was enriched with SOD isoenzymes

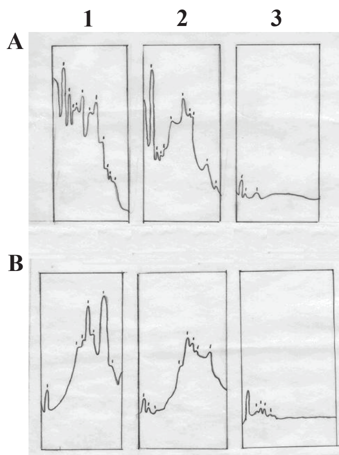

Fig. 4. Protein profiles (native – A and thermostable B) of *Scenedesmus* cells infected by *Phlyctidium* zoospores. 1 – *Scenedesmus* cells - control; 2 – *Scenedesmus* + *Phlyctidium*; 3 – *Phlyctidium* zoospores.

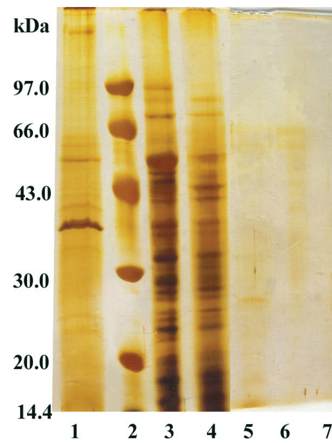

Fig. 5. Polypeptide profiles of Scenedesmus cells infected by *Phlyctidium*. 1 – *Phlyctidim* zoospores; 2 – molecular markers; 3 – *Scenedesmus* cells-control; 4 – *Scenedesmus* +*Phlyctidium*; 5–7 thermostable polypeptides.

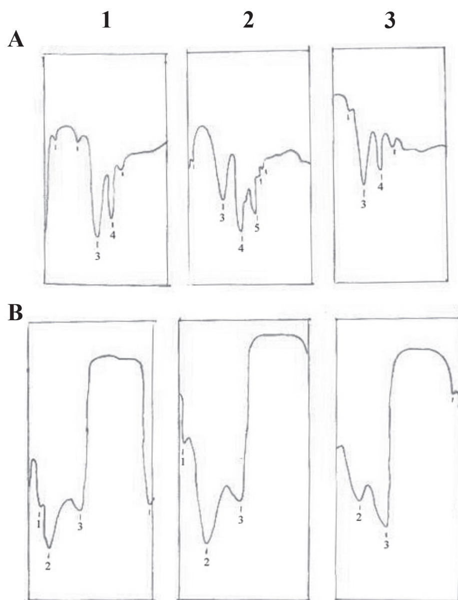

Fig. 6. Isoenzyme profiles of SOD (A) and catalase (B). 1 – *Scenedesmus* cells - control; 2 – *Scenedesmus* + *Phlyctidium*; 3 – *Phlyctidium* zoospores. A – SOD - densitometric scans; A_1 – SOD – cylindrical gels. B – Catalase – densitometric scans.

originating from *Phlyctidium* genome. Catalase isoenzyme profiles differ by the enzyme activity distribution among the individual enzymes. Isoenzyme N 2 was the most active in invaded cells and isoenzyme N 3 being the most active in the zoospores (fig. 6-B). Parasite invasion led to decrease of Rubisco amount in all variants investgated (Fig. 7). No visible effect of sanosil on polypeptide profile was observed. In the isoenzyme profile of SOD from invaded *Scenedesmus* cells new isoenzymes appeared (Fig. 8). These isoenzymes originated from zoospores. Sanosil treatment caused the appearance of the same isoenzymes as in the case of parasite invasion. It is interesting that sanosil treatment of zoospores increased the activity of all existing SOD isoenzymes. That means presence of oxidative stress in zoospores, too. *Phlyctidium* invasion affected also CAT activity by increasing the activity of the existing enzymes (Fig. 9). Unlike SOD, sanosil treatment did not affect CAT activity. It is noteworthy that sanosil increased CAT activity of zoospores.

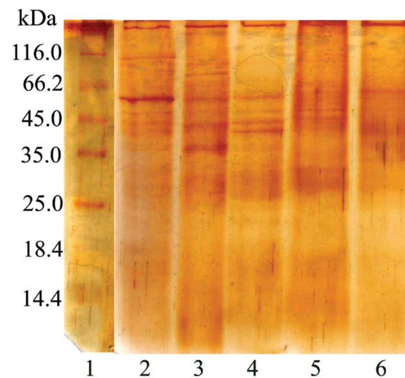

Fig. 7. Polypeptide profiles of *Scenedesmus* cells infected by *Phlyctidium* and treated by sanosil. 1 – molecular markers; 2 – *Scenedesmus* cells-control; 3 – *Scenedesmus* + *Phlyctidium*; 4 – *Scenedesmus* cells treated with sanosil; 5 – *Phlyctidium* zoospores; 6 – *Phlyctidium* zoospores treated by sanosil.

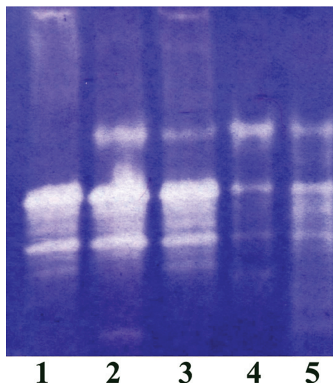

Fig. 8. Isoenzyme profiles of SOD. 1 – *Scenedesmus* cells - control; 2 – *Scenedesmus* + *Phlyctidium*; 3 – *Scenedesmus* cells treated by sanosil, 4 – *Phlyctidium* zoospores; 5 – *Phlyctidium* zoospores treated by sanosil.

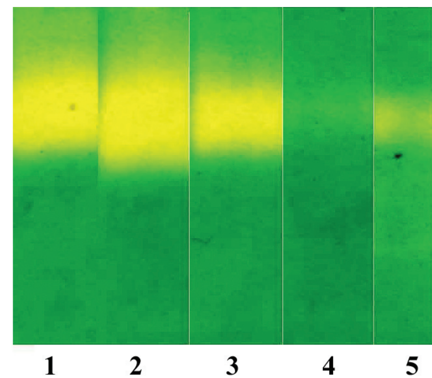

Fig. 9. Isoenzyme profiles of catalase. 1 – *Scenedesmus* cells - control; 2 – *Scenedesmus* + *Phlyctidium*; 3 – *Scenedesmus* cells treated by sanosil; 4 – *Phlyctidium* zoospores; 5 – *Phlyctidium* zoospores treated by sanosil.

DISCUSSION

Our results concerning the impact of the initial infection degree on the growth of the host alga are in concert with the findings of Kagami et al. (2007). They established that an important factor and parameter of fungal infection is the percent of the infected cells.

The amounts of native proteins and especially that of Rubisco declined in the invaded algal cells. The degree of the decrease depended on the concentration of the parasite, the highest degree of infection of 70% being the most effective. The thermostable protein profiles were also affected. These results were in close correlation with the data concerning growth retardation of host cells in terms of biomass production (dry weight). Five SOD isoenzymes were detected in control and invaded cells of *Scenedesmus* and 3 isoenzymes in zoospores. Pokora et al. (2003) reported for 4 SOD isoenzymes in S. obliquus, one MnSOD, two FeSOD and one Cu/ZnSOD. Three new isoenzymes appeared when *Scenedesmus* cells were 30 and 45 % infected by *Phlyctidium* zoospores. The highest degree of infection caused a decrease of SOD activity and three of the isoenzymes disappeared from the profile. These results were in support of the proposition that cells response to stress depending on its duration and degree. This indicates that the change of SOD activity was very sensitive to the environment (Wu et al., 2007; Li et al., 2005). Different SOD isoenzyme profiles established in control, invaded and zoospore cells confirmed the finding of Amanatidou et al. (2001) that the cellular SOD profile is variable between organisms and can change with ambient environmental conditions. Since Kong and Sang (1999) have shown that SOD was one of the key enzymes to eliminate active oxygen in algal cells once the pathogen invasion increases, the cellular detoxification system was stimulated and the synthesis of SOD was started. As these changes took place at the molecular level in the cells, they happened much earlier than growth or reproduction. We also detected three CAT isoenzymes in agreement with the statement that the presence of three isoforms of catalase enzyme is more common in higher plants (Rao et al., 1996). Catalase activity has been shown to be associated with all cyanobacterial species (Obingeret al. 1998). The alteration in protein profiles and in the activity of antioxidant enzymes are part of the process of survival and a proof that these biomolecules play a significant role in pathogen tolerance. The interpretation of plant responses to stress is exceedingly complex because the mechanisms involved differ depending on the species, tissues, the stage of development, physiological status of the plant, and the degree and duration of the stress (Baek and Skiner, 2003). The new SOD isoenzyme which appeared in infected *Scenedesmus* cells probably originates from the parasite zoospores. On the basis of the similarity of the isoenzyme profiles of SOD and CAT in the non-infected algal cells subjected to oxidative stress and the invaded algal cells a conclusion could be drawn that the biotic stress of the host alga was connected with oxidative stress.

Acknowlegments: This investigation was supported by research grant DOO2-317 of the Bulgarian National Science Fund.

REFERENCES

Amanatidou A, MH Bennik, LG Goris, EJ Smid, 2001. Superoxide dismutase plays an important role in the survival of Lactobacillus sake upon exposure to elevated oxygen level. Arch Microbiol, 176: 79-88.

Babu TS, TA Akhtar, MA Lampi, S Tripuranthakam, DG Dixon, BMGreenberg, 2003. Similar stress responses are elicited by copper and ultraviolet radiation in the aquatic plant Lemna gibba: implication of reactive oxygen species as common signals. Plant Cell Physiol, 44: 1320-1329.

Baek K-H, DJ Skinner, 2003. Alteration of antioxidant enzyme gene expression during cold acclimation of near-isogenic wheat lines. Plant Sci, 165: 1221-1227.

Baranenko VV, 2001. Pea chloroplasts under clinorotation: lipid peroxidation and superoxide dismutase activity. Adv Space Res, 27: 973-976.

Benderliev KM, ID Pouneva, NI Ivanova, 1993. Fungicide effects of triton-N on *Phlyctidium*. Biotechnol Techn, 7: 335–338.

Grant JJ, GJ Loake, 2000. Role of reactive oxygen intermediates and cognate redox signalling in disease resistance. Plant Physiol, 124: 21-29.

Greneche M, J Lallemand, O Michaud, 1991. Comparison of different enzyme loci as a means of distinguishing ryegrass varieties by electrophoresis. Seed Sci. Technol., 19, 147-158.

Jones RP, 1987. Measures of yeast death and deactivation and their meaning. Process Biochem, 22: 117-128.

Kagami M, A Bruin, BW Ibelings, E Van Donk, 2007. Parasitic chytrids: their effects on phytoplankton communities and food-web dynamics. Hydrobiol, 578: 113-129.

Kong FX, WL Sang, 1999. Physiological and biochemical response of *Scenedesmus obliquus* to combined effect of Al, Ca, and low pH Bull. Environ Contam Toxicol, 62: 179-186.

Laemmli UK, 1970. Cleavage of the structural proteins during the assembly of the head of the bacteriophage T4. Nature, 277: 680-683.

Li X, X Ping, Sh Xiumei, W Zhenbin, X Liqiang, 2005. Toxicity of cypermethrin on growth, pigments, and superoxide dismutase of Scenedesmus obliquus. Ecotoxicol Env Safety, 60: 188-192.

Lowry OH, NJ, Rosebrough, AL Farr, RL Randal, 1951. Protein measurement with the Folin phenol reagent. J Biol Chem, 193: 265-275.

Mallick N, FH Mohn, 2000. Reactive oxygen species: response of algal cells. J Plant Physiol, 157: 83-93.

Nesterenko MV, M Tilley, J Upton, 1994. A simple modification of Blum's silver stain method allows for 30 minute detection of proteins in polyacrylamide gels. J Biochem Biophys Meth, 28: 239-242.

Obinger C, G Regelsberger, A Pircher, G Strasser, A Peschek, 1998. Scavenging of superoxide and hydrogen peroxide in blue-green algae (cyanobacteria). Physiol Plant, 104: 693-698.

Pokora W, J Reszka, Z Tukaj, 2003. Activities of superoxide dismutase (SOD) isoforms during growth of Scenedesmus (Chlorophyta) species and strains grown in bacth-cultures. Acta Physiol Plant, 25: 375-384.

Polis GA, DR Strong, 1996. Food web complexity and community dynamics. American Naturalist, 147: 813-846.

Rao MV, G Paliyath, DP Ormrod, 1996. Ultraviolet-B and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. Plant Physiol, 110:125-136.

Roginsky V, T Barsukova, 2001. Superoxide dismutase inhibits lipid peroxidation in micelles. Chem Phys Lip, 111: 87-91.

Sahoo MR, M Das Gupta, PC Kole, JS Bhat, A Mukherjee, 2007. Antioxideative enzymes and isozymes analysis of taro genotypes and their implications in *Phytophthora* blight disease resistance. Mycopathol, 163: 241-248.

Woodbury W, AK Spenser, MA Stahmann, 1971. An improved procedure using ferricyanide for detecting catalase isoenzymes. Anal Biochem, 44: 301-305.

Wu Z, P Deng, X Wu, S Luo, Y Gao, 2007. Allelopathic effects of the submerged macrophyte *Potamogeton malaianus* on *Scenedesmus obliquus.* Hydrobiol, 592: 465-474.

Zhao W, D I Diz, M E Robins, 2007. Oxidative damage pathways in relation to normal tissue injury. British J Radiol, 80: 23-31.