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BRIEF COMMUNICATION

Chitinase and peroxidase activities in sunflower hypocotyls: effects of BTH and inoculation with *Plasmopara halstedii*

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Abstract

Systemic acquired resistance (SAR) can be induced in plants by incompatible pathogens, pathogen derived extracts, or certain chemicals as benzothiadiazole (BTH). The aim of this work was to compare changes in peroxidase and chitinase activities, enzymes considered as PR-proteins, caused by BTH and the pathogen *Plasmopara halstedii*. Hypocotyls from susceptible and resistant BTH-treated sunflower seedlings showed increased peroxidase and chitinase activities. Inoculation with *P. halstedii* increased chitinase and peroxidase activities in inoculated hypocotyls from susceptible but not from resistant sunflower seedlings.

Additional key words: *Helianthus annuus*, plant defense, mildew.

Acquired resistance (AR) phenomena occur when plants became resistant to a broad range of pathogens. Ross (1961) demonstrated that pre-inoculation of *Xanthi-nc* tobacco plants with tobacco mosaic virus (TMV) conferred protection against some viral pathogens. After induction, protection against a subsequent infection can be restricted to the tissue treated with the inducer (local acquired resistance, LAR) or can affect plant tissues that have not been treated (systemic acquired resistance, SAR).

SAR shows some basic characteristics: *1*) it is triggered by necrotic lesions caused by pathogens in both incompatible and compatible interactions; *2*) it takes some time between induction and expression of protection; *3*) it results in a systemic protection of the entire plant; *4*) it is long lasting (several weeks or even months) and provides the plant with protection against a broad spectrum of pathogens, and *5*) it is correlated with the induction of a well-characterized set of genes that comprise those encoding the so-called pathogenesis-related proteins (PR-proteins).

PR-proteins represent major quantitative changes in soluble proteins during plant defense response. They were first detected in tobacco leaves reacting hypersensitively to tobacco mosaic virus (Van Loon *et al.* 1970). PR-proteins have been classified in five major groups with chitinase, lysozyme, or 1,3-β-glucanase activities. Other PR-proteins isolated from tobacco cannot be classified in these five groups and they are α-amylases (Heitz *et al.* 1991), peroxidases (Lagrimini *et al.* 1987) or inhibitors of microbial proteases (Freidrich *et al.* 1990). Chitinases, peroxidases and 1,3-β-glucanases are considered by Siegrist *et al.* (1997), as biochemical markers of SAR.

Besides pathogens, pathogen-derived elicitors (Wurms *et al*. 1999) and some chemical compounds can induce SAR. Some well characterized SAR inducers are:

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Abbreviations: AR - acquired resistance; BTH - benzo[1,2,3]thiadiazole-7-carbothioic acid S-methyl ester; DS - diffusion solution; INA - 2,6-dichloroisonicotinic acid; LAR - local acquired resistance; PR-proteins - pathogenesis related proteins; SAR - systemic acquired resistance; TE - total extract.
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benzo[1,2,3]thiadiazole-7-carbothioic acid S-methyl ester (BTH or CGA 2457704) (Friedrich *et al.* 1996, Tosi *et al.* 1999), salicylic acid (Jung *et al.* 1993, Jayaraj *et al.* 2004), 2,6-dichloroisonicotinic acid (INA) (Métraux *et al.* 1991), jasmonic acid (Jayaraj *et al.* 2004). BTH is a non-toxic synthetic chemical identified as a potent inducer of SAR in several crops (Gorlach *et al.* 1996, Katz *et al.* 1998, Godard *et al*. 1999, Stadnik and Buchenauer 1999). Previous results derived from experiments carried out in our laboratory (unpublished results) and Tosi *et al.* (1999) showed that BTH protects susceptible sunflower plants from *Plasmopara halstedii* infection. The aim of the work described in this paper was to study the effect of *P. halstedii* and BTH on peroxidase and chitinase activities in plants resistant and susceptible to this biotrofic fungus.

Two sunflower (*Helianthus annuus* L.) lines were used: HA89 (susceptible to all *Plasmopara halstedii* races) and X55 (resistant to *P. halstedii* races 1 to 10), while cv. Peredovick was used for the inoculum maintenance. The race 2 of *P. halstedii*, the causal agent of downy mildew, was used.

Seeds of cv. Peredovick were surfaced sterilized in 70 % ethanol for 4 min, rinsed with distilled water, then immersed in 0.5 % sodium hypochlorite for 20 min, rinsed twice with distilled water and finally rinsed a third time with sterile distilled water. For germination, seeds were rolled up in wet double filter paper, covered with aluminum foil and kept at 30 ºC for 48 h. Inoculation was made following the whole seedling immersion method (Cohen and Sackston 1973) with an inoculum density of 90 000 zoosporangia cm^{-3}. Seedlings were grown in wet *Perlite*, in a growth chamber at 20 ºC, and a 16-h photoperiod with irradiance of 300 μmol m^{-2} s^{-1} and 60 % RH. After 9 d, they were kept at 100 % RH for 48 h to induce sporulation of the fungus.

Three kinds of experiments were carried out. In the first one, seeds from susceptible and resistant sunflower lines were prepared for germination as previously described. After 36 h, seedlings were divided into two groups: one of them was immersed in 200 mg dm^{-3} of *Bion 50°*, commercial form of BTH, for 12 h at dark and 25 ºC. The other one was immersed in distilled water in the same conditions as BTH treated ones. Both BTH and BTH non-treated seedlings were washed with distilled water before planting in pots. In the second experiments, the seedlings were divided into two groups: one of them was inoculated and the other was immersed in distilled water and kept under the same conditions as the inoculated seedlings. In the third experiments, we proceeded as described for the first ones, but after BTH or water treatment, all seedlings were inoculated with *P. halstedii*. This kind of experiment was carried out for peroxidase but not for chitinase activity. In all the experiments, treated and control untreated seedlings were grown in three pots of 15 plants each. After 9 d, hypocotyls were cut and used for analyses.

Peroxidase was extracted from two different sources: total extract (TE) and diffusion solution (DS). TE was obtained following Svalheim and Robertsen, (1990). DS was obtained as follows: hypocotyls were submerged in bidistilled water [0.24 g(f.m.) cm^{-3}] and kept in darkness at 25 ºC for 30 min. The resultant solution was used as enzyme source. Peroxidase activity was measured using the spectrophotometric method of Ngo and Lenhoff (1980) and was expressed as change in absorbance at 590 nm, A_{590} g^{-1}(f.m.) min^{-1}.

For statistical analysis, a factorial design with two factors was used. The first factor was sunflower line, (susceptible or resistant) and the second factor was treatment (control/BTH; control/inoculated or control inoculated/BTH inoculated plants). Both were fixed effects factors. If interaction between factors was significant, pair wise comparisons among groups were carried out using Tukey´s penalization with balanced sample sizes or Bonferroni´s penalization when sample sizes were not equal. When interaction was not significant comparisons were obtained in a direct way using the principal effects of the factors. In some cases, data were transformed in order to get variance homogeneity as required for variance analysis models.

Chitinase activity was measured only in TE because it could not be detected in diffusion solution (DS). For enzyme extraction, the method of Boller *et al.* (1983) was followed. Chitin substrate was prepared following the method of Skuijins *et al*. (1965) and 0.1 cm^3 of extract, were incubated at 37 ºC for 6 h. Three replicates and a blank were prepared from each extract. In blank incubation mixtures, extract was added at the end of the incubation period. The reaction was stopped by cooling on ice, and then reaction mixtures were centrifuged at 10 000 *g* and 4 ºC for 30 min. The resulting *N*-acetyl-glucosamine was determined as described by Reissig *et al*. (1955). Standards of *N*-acetylglucosamine ranging from 0 to 200 μg cm^{-3} were measured by the same method and used as a reference. Protein content in extracts was determined by Bradford (1976). Chitinase activity was expressed as mg(*N*-acetylglucosamine liberated) g^{-1}(protein) min^{-1}.

Peroxidase activity measured in TE was always enhanced by BTH treatment (Table 1), in susceptible and resistant plants ($P < 0.001$), in those plants inoculated with the fungus and in non-inoculated plants ($P = 0.001$). BTH treatment also increased peroxidase activity, in DS (Table 1) in both resistant and susceptible plants ($P < 0.001$).

The inoculation with *P. halstedii* enhanced peroxidase (Table 1, $P < 0.001$) and chitinase (Table 1, $P < 0.00001$) activities in TE of susceptible plants but not in resistant ones. In DS (Table 1), we found no difference in peroxidase activity between inoculated and non-inoculated plants.

BTH treatment, raised chitinase activity (Table 1) in

both resistant and susceptible plants ($P < 0.01$). Susceptible plants responded to *Plasmopara* inoculation raising chitinase activity in TE but resistant plants did not (Table 1). This can mean that chitinase activity increase is not a defense response but a symptom of plant disease due to fungus infection.

Table 1. Peroxidase activity [ΔA_{590} g^{-1}(f.m.) min^{-1}] in diffusion solution (DS) and total extract (TE) and chitinase activity [mg(*N*-acetylglucosamine) g^{-1}(protein) min^{-1}] in total extract (TE) of hypocotyls from resistant (R) and susceptible (S) BTH-treated plants (BHT), *P. halstedii*-inoculated plants (I) or *P. halstedii*-inoculated plants that were previously treated with BTH (IBTH). C - control plants treated with water instead of BTH or immersed in water instead of inoculum solution. Each value represents the mean from three independent experiments with three replicates each. Every enzymatic extract was measured three times. Sunflower hypocotyls were collected 9 d after treatment. Values followed by different letters in a column are significantly different.

Treatment		Peroxidase		Chitinase
		DS	TE	TE
BTH	SC	0.94a	35.26a	0.50a
	SBTH	1.29b	47.68b	0.90b
	RC	1.28b	52.13b	0.48a
	RBTH	1.66c	67.54c	0.79b
P. halstedii	SC	1.41a	53.79a	0.32a
	SI	1.84a	83.50b	0.78b
	RC	1.56a	46.13a	0.33a
	RI	1.41a	54.83a	0.50a
BTH+*P. halstedii*	SIC	1.83a	71.80a	
	SIBTH	2.58a	115.76b	
	RIC	1.77a	68.36a	
	RIBTH	2.20a	99.01b	

In our experiments, BTH treatment protected plants against *P. halstedii*. We have only found a small amount of sporangia in the base of the hypocotyls of very little number of susceptible inoculated plants treated with BTH and never in cotyledons. On the contrary, susceptible BTH untreated-plants showed intense presence of sporangia after inoculation, especially in cotyledons and hypocotyls. Other authors (Tosi *et al*. 1999) have already described this protection. It is important to point out that, as above described, there was no direct contact between BTH and *P. halstedii*. We think that, perhaps, this chemical compound activated, in sunflower seedlings, some defense responses against pathogen.

Because BTH treatment protected plants against *P. halstedii* and BTH increased peroxidase and chitinase activities, it could be thought that this protection was due to these enzyme activities enhancements. Peroxidases could limit the spread of the pathogen because they act in cross-linking of some cell wall compounds (Katz *et al.* 1998). Svalheim and Robertsen (1990) described a strong induction on peroxidase activity in cucumber hypocotyls in response to a fungus infection and Reimers (1992) found increased activity of a cationic peroxidase in rice after incompatible interaction with *Xanthomonas oryzae* pv. *oryzae*. Furthermore, in tobacco plants, peroxidase activity measured in a resistant cultivar is higher than that detected in a susceptible one (Wyatt *et al.* 1991). Because in our experiments with BTH in non-inoculated seedlings, resistant control plants showed higher peroxidase activity, in DS and in TE, than control susceptible ones, it is possible that, part of the protection effect of BTH treatment could be due to the elevation of this enzyme activity. Nevertheless, this hypothesis must be proved.

Chitinases could play two different roles in defense mechanism against fungal pathogens. They have, by themselves or in combination with 1,3-β-glucanases, antifungal activity *in vivo* and *in vitro*. This is probably due to their capability for hydrolyzing structural components of cell walls. Nevertheless, as showed by Ludwig and Boller (1990), *in vitro* both enzymes can inhibit fungal growth only in a transitory way, because fungi can adapt even to high contents of these hydrolases. On the other hand, the hydrolysis of fungal cell walls by chitinase, releases small fragments, such as oligo-saccharides, that can act as elicitors (Ryan and Farmer 1991). Elicitors can activate plant defense response such as induction of PR-proteins.

Our results about BTH effect on chitinase activity (Table 1), agree with those described by other authors in several plant species (in sugar beet Burketová *et al.* 1999, 2003/4, in apple Brisset *et al.* 2000). All of them referred an accumulation of chitinase and 1,3-β-glucanase in response to BTH. In our experiments, the enhancement in enzymatic activities took place in both susceptible and resistant plants. As *P. halstedii* does not have chitin in its cells walls, we think that BTH put on some unspecific defense mechanisms. Unspecific effects are common in plant defense against pathogens. In tobacco plants, pathogen as virus, that does not have chitin, induce chitinases expression. This, could favor the idea that plant defense is, in part, the result of a coordinated and unspecific action in space and time of several reactions against different kinds of pathogens and even in response to abiotic stresses.

P. halstedii inoculation increased peroxidase and chitinase activities in hypocotyls of sunflower plantlets from susceptible line but not in those from resistant. This could indicate that the enhancement of these enzymatic activities is not a defense response but rather a consequence of an altered metabolism of plants after infection.

Briefly, BTH pre-treatment of sunflower seedlings put on a mechanism that protected them from further infection with *P. halstedii* and it can increase chitinase and peroxidase activities in hypocotyls. This enhancement was a part of the defense response but probably it was not the base of the protection effect.

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