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Short communication

Induction of different chitinase and β-1,3-glucanase isoenzymes in sunflower (*Helianthus annuus* L.) seedlings in response to infection by *Plasmopara halstedii*

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Abstract

Chitinase and β-1,3-glucanase activities were assayed in roots, hypocotyls and cotyledons of downy mildew-susceptible and -resistant sunflower (*Helianthus annuus* L.) cultivars. While the highest β-1,3-glucanase activity was in roots, that of chitinase activity was in hypocotyls. Inoculation of both sunflower cultivars with *Plasmopara halstedii* resulted in a marked increase of chitinase and β-1,3-glucanase activities. The increase was observed earlier in incompatible than in compatible reactions. Both enzymes occurred in root tissue as a complex mixture of isoenzymes. At least three different peaks with chitinase activity and three with glucanase activity could be resolved by gel filtration chromatography on Sephacryl S-100 and chromatofocusing on PBE 94 (pH 7–4). Following ammonium sulfate precipitation and ion-exchange on CM- and DEAE-Trisacryl, three glucanase and chitinase fractions, referred to as basic, neutral and acidic, were separated on the basis of their chromatographic behaviour. A different pattern of distribution of chitinase and β-1,3-glucanase fractions was observed between inoculated and non-inoculated plants in both resistant (cv. RS-105) and susceptible (cv. Peredovik) cultivars. In healthy plants β-1,3-glucanase was mainly found in the basic (cv. Peredovik) and neutral (cv. RS-105) fractions, whereas chitinase was in the basic fraction for both cultivars. The neutral and acidic fractions of chitinases were induced in the compatible and incompatible reactions. Inoculation of the plants induced the neutral β-1,3-glucanase fraction in resistant and susceptible cultivars and the acidic only in the susceptible one. Induction of the basic fraction of both activities was not observed in any case.

The cultivated sunflower, *Helianthus annuus* L., is one of the most important annual crops in the world grown for edible oil. It is host of a number of bacteria and fungus species that cause diseases, extensive damages and, thus, important crop losses. One of the most studied diseases of sunflower is the downy mildew, caused by the biotrophic fungus *Plasmopara halstedii* (Farlow) Berlese et de Toni [Gulya et al., 1991]. Despite of the ample phytopathological knowledge of the disease [Gray and Sackston, 1985], the existence of numerous resistant cultivars and the knowledge of the bases of their inheritance [Miller and Gulya, 1991], little is known about the biochemical bases of such resistance. This situation is not specific for downy mildew but quite general since studies on plant defense responses, specially antifungal enzyme activities, in sunflower are limited [Jung et al., 1993; Siefert et al., 1994; Tena and López-Valbuena, 1983].

Chitinases and β-1,3-glucanases seem to play an important role in the plant defense against phytopathogenic microorganisms [Boller, 1993; Collinge et al., 1993; Joosten and de Wit, 1988]. They are coordinately and differentially induced in a number of plant tissues by pathogen attack, elicitors, or ethylene

[Vögeli et al., 1988]. Both enzymes often occur in a complicated pattern of isoforms, differing in physical properties, enzyme activity, antigenicity, cellular compartmentation, tissue localization, and antifungal activity [Boller, 1993; Collinge et al., 1993]. This multiplicity of isoenzymes implies that plants may respond in a tissue-specific or stimulus-specific manner [Margis-Pinheiro et al., 1993]. The isoenzymes are classified in two main groups, the acidic and basic enzymes, although at least three different groups have been established for each of the two enzymes based on the structural analysis of their genes [Linthorst, 1991].

We are studying the possible role of chitinases and β-1,3-glucanases as a defense mechanism in sunflower against *Plasmopara halstedii*, causal agent of the downy mildew. Here, we present data showing differential induction of specific chitinase and β-1,3-glucanase isoenzymes in response to fungal infection, in both resistant and susceptible cultivars.

For inoculation of resistant and susceptible sunflower cultivars, germinated seeds were suspended for 4 h in a suspension of $5 \cdot 10^6$ conidia/ml and then planted. Chitinase and β-1,3-glucanase enzymes were extracted, and their activities determined, as described in Cabello et al. [1994]. Both hydrolytic activities were detected in different parts of the inoculated and non-inoculated plants of downy mildew-resistant (RS-105) and susceptible (Peredovik) sunflower cultivars (Figure 1). In non-inoculated plants, both enzyme activities increased with aging in 5- to 15-day-old seedlings (Figure 1). While β-1,3-glucanase activity was higher in roots than in hypocotyls and cotyledons (Figure 1), the highest chitinase activity was present in the aerial part of the plant (Figure 1). In all the tissues studied, it was found that, during the course of infection (Figure 1), chitinase and β-1,3-glucanase were increased, reaching maximum values earlier in the incompatible reaction (RS-105 *P. halstedii*) than in the compatible one (Peredovik *P. halstedii*) (Figure 1). Increased levels of chitinase and β-1,3-glucanase activities were observed as early as 5 days after inoculation in both incompatible and compatible reactions, although at this time no distinct differences in induced chitinase and β-1,3-glucanase levels were seen between the two cultivars (Figure 1). The kinetics of the increases in both activities were similar in all cases but, while maximum values of chitinase and β-1,3-glucanase activities were detected 10 day after inoculation in the incompatible reaction (Figure 1), maximum values of chitinase and β-1,3-glucanase activities in the compatible reaction were reached 15 days after inoculation (Figure 1). Chitinase and β-1,3-glucanase induction was always larger in root tissue, both activities being higher in the incompatible reaction than in the compatible one.

Gel filtration chromatography (Figure 2A) and chromatofocuing (Figure 2B) techniques revealed the existence of multiple isoenzymes of chitinases and β-1,3-glucanases in sunflower roots. The complexity of chickpea chitinases and β-1,3-glucanases prompted us to adopt a simplified protocol of enzyme fractionation in the studies on the specific distribution and inducibility of different isoforms. Similarly basic, neutral and acidic fractions of chitinases and β-1,3-glucanases were separated by sequentially ion-exchange chromatography on CM- and DEAE-Trisacryl (Pharmacia) as described in Cabello et al. [1994]. There was a comparatively low recovery of both activities and because of that the existence of other minor isoenzymes could have been missed; low levels of recovery have in general been obtained when purifying activities in different plant systems. In non-inoculated 10-day-old plants, chitinase activity was predominantly found in the neutral fraction (80% of the total recovered activity) in both cultivars, whereas β-1,3-glucanase activity was predominantly in the neutral (74%) and basic (73%) fractions in the resistant and susceptible cultivars, respectively (Table 1). Inoculation of the susceptible cultivar with *P. halstedii* resulted in an increase of chitinase and β-1,3-glucanase activities in the neutral (69% and 41%, respectively) and acidic (31% and 47%, respectively) fractions. The acidic isoforms increased the most (9.6- and 19.5-times the basal level for chitinase and β-1,3-glucanase, respectively) (Table 1). In the incompatible reaction, an increase of chitinase in the neutral (69% and 4.4-times the basal level) and acidic (30% and 15.7-times the basal level) fractions and of β-1,3-glucanases in the neutral fraction (with a 96% of the activity recovered unbound to either column) were found (Table 1). While similar chitinase isoforms (neutral and acidic) were induced in both compatible and incompatible reactions (Table 1), neutral β-1,3-glucanase was the only isoform increased in the incompatible reaction (Table 1). This fraction besides with the acidic β-1,3-glucanase were induced in the compatible reaction.

Results presented here confirm previous observations made in the other plant systems, first as referred to the asymmetric distribution of chitinases and β-1,3-glucanases in different tissues [Cabello et al., 1994] and second to their induction in response to fungal inoculation, which is earlier and higher in the resis-

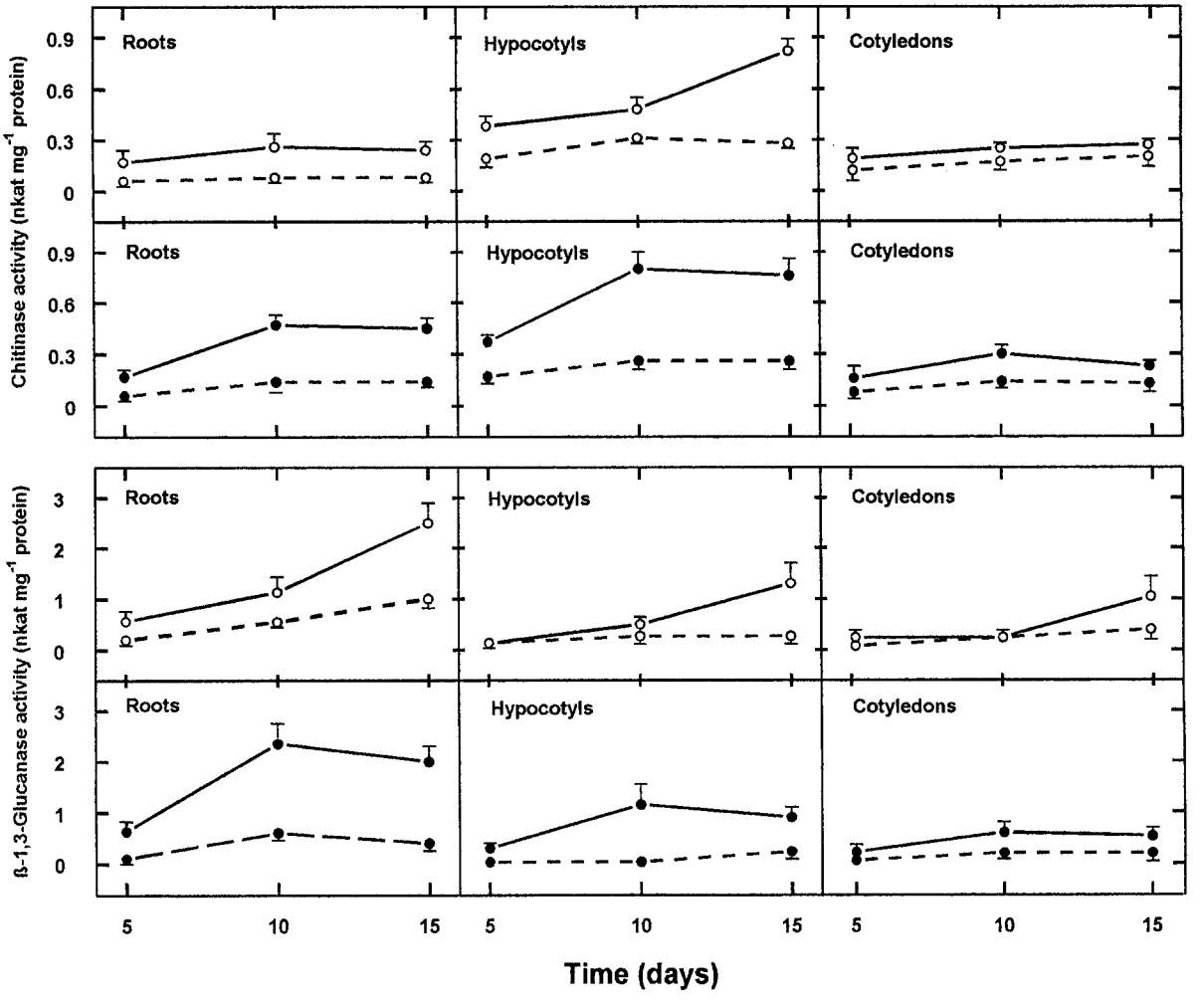

Figure 1. Time course increase of chitinase and β-1,3-glucanase activities in roots, hypocotyls and cotyledons from inoculated sunflower cultivars, susceptible (○——○) and resistant (●——●) to *Plasmopara halstedii*, and from unionculated controls (○- - -○, ●- - -●).

tant cultivar [Joosten and de Wit, 1988]. The earlier induction of chitinase and β-1,3-glucanase activities in the resistant sunflower plants may explain, at least in part, the restriction of the fungus in tissue near the infection site, as has been published by Gray and Sackston [1985]. In sunflower, similarly to other systems [Cabello et al., 1994; Joosten and de Wit, 1988], chitinases and β-1,3-glucanases from root tissue were present as multiple isoenzymes (Figure 2). Specific induction of chitinase and β-1,3-glucanase isoforms have been described in a number of plants systems in response to pathogens or with aging [Boller, 1993; Collinge et al., 1993; Joosten and de Wit, 1988; Linthorst, 1991]. Infection of sunflower plants resulted in an alteration of the distribution of both hydrolases, with specific fractions being induced. In the incompatible and compatible reactions an induction of the chitinase and β-1,3-glucanase neutral fractions and chitinase acidic fraction was observed, which was higher in the former one (Table 2). These results suggest coordinated induction of similar sets of chitinase and β-1,3-glucanase isoforms in response to the pathogen attack. In addition acidic isoforms of β-1,3-glucanase also increased in susceptible plants. In summary, our data suggest that extracellular chitinases and β-1,3-glucanases in sunflower can be part of the plant-defense battery against *P. halstedii*. Both activities may act in inhibiting fungal growth and limiting the colonization of the plant.

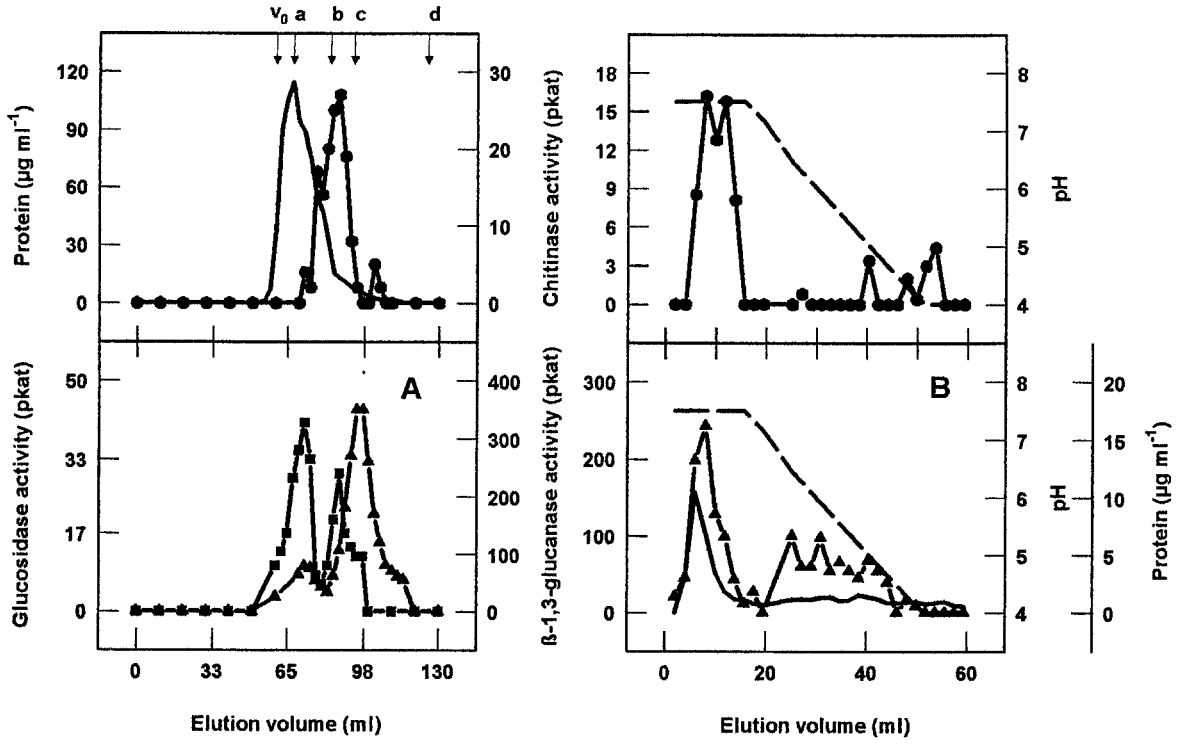

Figure 2. Gel filtration (A) and chromatofocusing (B) of chitinase and β-1,3-glucanase isolated from intact roots of 10-day-old sunflower plants. The cell extract obtained after ammonium sulfate precipitation was subjected to gel filtration on Sephacryl S-100 and chromatofocusing on PBE 94 in the pH range 7–4, as described by Cabello et al. (1994). Chitinase activity (pkat) (–●–), β-1,3-glucanase activity (pkat) (–▲–), β-glucosidase activity (pkat) (–■–), pH (- - -) and proteins (μg ml^{-1}) (——). Arrows indicate elution volume of standards molecular mass protein markers (Vo: void volume; a: 200 kDa; b: 150 kDa; c: 66 kDa; d: 29 kDa).

Table 1. Chitinase and β-1,3-glucanase activities in different protein fractions obtained after CM- and DEAE-Trisacryl separation steps. Indicated values correspond to mg of protein and nkat of enzyme activities. Root extracts obtained after ammonium sulfate precipitation (total) corresponding to healthy (H) and inoculated (I) sunflower 10-day-old plants (cvs. Peredovik and RS-105) were separated in three different groups, termed as basic, neutral and acidic, by ion exchange chromatography on CM- and DEAE-Trisacryl as described by Cabello et al., 1994. The experiment was repeated three times; values of a typical experiment are presented

Enzyme fraction	Chitinase		β-1,3-glucanase		Protein	
	H	I	H	I	H	I
Cv. Peredovik						
Unfractionated	0.205	0.580	1.23	3.70	3.00	3.00
Basic	0.006	0.002	0.22	0.10	0.30	0.26
Neutral	0.050	0.150	0.06	0.34	0.33	0.36
Acid	0.007	0.067	0.02	0.39	0.27	0.30
Cv. RS-105						
Unfractionated	0.350	1.030	1.85	8.55	3.00	3.00
Basic	0.007	0.004	0.08	0.04	0.17	0.18
Neutral	0.056	0.250	0.46	2.40	0.38	0.40
Acid	0.007	0.110	0.08	0.05	0.26	0.35

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