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# PROTEINASE INHIBITOR ACCUMULATION IN APHID-INFESTED BARLEY LEAVES

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**Key Word Index**—*Hordeum vulgare*; Gramineae; *Schizaphis graminum*; *Rhopalosiphum padi*; aphids; defense proteins.

**Abstract**—Proteinase inhibitors (chymotrypsin and trypsin inhibitors) accumulation in barley leaves was studied when the plants were infested with the aphids *Schizaphis graminum* and *Rhopalosiphum padi*. Aphid infestation resulted mainly in the accumulation of chymotrypsin inhibitors (about two fold increase) and this response was higher with *S. graminum*. Frontera, a cultivar which was more resistant to aphids, accumulated more inhibitors than other cultivars. Compounds responsible for the inhibitory activities were separated by gel filtration chromatography. The separation revealed two peaks of chymotrypsin inhibitors and one peak of trypsin inhibitor. $M_r$ estimations for these proteins indicated sizes similar to those inhibitors found in barley seeds. These inhibitors had no effect on survival of *S. graminum* reared on artificial diets, but decreased survival of *R. padi*. 

## INTRODUCTION

Morphological and biochemical factors are involved in defense of barley against insects [1]. Among morphological barriers are waxes and pectins in the foliar surface. Chemicals include the alkaloid gramine, flavonoids and phenolic compounds. In addition, barley has polypeptides, such as thionins, with known antifungal activity [2] and proteinase inhibitors [3]. The accumulation of proteinase inhibitors (PIs) has been considered as a defensive response that protects plants from insects and microorganisms by inactivating hydrolytic enzymes of the aggressors [4].

The presence of PIs in barley has been described mainly in seeds, but they are also found in leaves and roots [5, 6]. These inhibitors act mainly against trypsin, chymotrypsin and some microbial proteases [7]. It is widely known that PIs can be induced by mechanical damage [8, 9] or by aggressors such as insects [10], fungi [8, 11], and viruses [12]. The inducibility of PIs and their protective role has been demonstrated mainly in dicots [4, 13], but in cereals is less documented (a review see [14]). A correlation was found between PI basal levels in barley leaves with the susceptibility of some cultivars to the grasshopper *Campanula pellucida* [15]. Cultivars with more chymotrypsin inhibitor activity were more resistant to the grasshopper. These authors did not demonstrate either a PI induction by the insect nor a deterrent effect of PIs toward that pest. The effects of PIs on sucking insects, such as aphids, are unknown. Since the damage caused by aphids in barley leaves may be severe, it is likely that aphid infestation will increase PI accumulation. In this paper, we study this hypothesis and measure the biological activity of barley PIs on aphids reared on artificial diets.

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## RESULTS AND DISCUSSION

### *Cultivar susceptibility*

Population growth rate of aphids and chlorophyll content were measured in order to evaluate the susceptibility of barley cultivars to the insects. Cultivar Aramir showed the highest aphid population growth rate, while cv. Frontera had the lowest aphid population growth rate (Table 1). Cultivar Frontera as expected, showed a higher chlorophyll content than the cultivar Aramir after six days of infestation. For these reasons, cv. Aramir was considered the most susceptible cultivar to aphid attack (Table 1). Since the different susceptibility observed

Table 1. Susceptibility of barley cultivars to the aphid *S. graminum*. Six day-old plants were infested with three aphids per plant. Measurements of chlorophyll content and aphid population growth rate were done after six days of infestation

| Cultivar | Aphids[a] | | Chlorophyll[b] |
|---|---|---|---|
| | final number per plant | population growth rate ($\ln[N_f/N_i]$ day$^{-1}$) | % of non infested control[c] |
| Frontera | 18.2 ± 1.2 | 0.30 ± 0.01 | 87.4 ± 1.8 |
| Libra | 24.6 ± 0.3 | 0.35 ± 0.00 | 94.7 ± 1.3 |
| Leo | 27.1 ± 1.3 | 0.37 ± 0.01 | 80.0 ± 1.8 |
| Aramir | 28.5 ± 0.9 | 0.38 ± 0.01 | 78.4 ± 2.3 |

[a]Mean of 3 measurements ± s.e.
[b]Mean of 3 pots (with 10 plants each) ± s.e.
[c]With respect to the non infested plants of the same cultivar ± s.e. Chlorophyll contents of controls were 16.6, 15.1, 16.5 and 17.2 μg chlorophyll g$^{-1}$ fr. wt for Frontera, Libra, Leo and Aramir, respectively.

in barley cultivars may be related to the capacity of plants to increase PIs after infestation, we selected cvs Frontera and Aramir for further experiments.

*PI activities*

Considering all cultivars, chymotrypsin inhibitor activity in healthy leaves fluctuated between 0.068 and 0.094 UI mg$^{-1}$ of protein during the first 14 d (Fig. 1). Trypsin inhibitor activity varied between 0.071 and 0.087 UI mg$^{-1}$ of protein. No significant differences among inhibitor activities were found during this period in different cultivars ($P < 0.05$, Fig. 1). Since basal activity showed a slight variation with time, the increments of activities were expressed as relative activity with respect to control plants for comparison purposes. In all infestation experiments, the maximum increment of inhibitory activity was reached after about 48 h of infestation for both types of inhibitors and depended on the number of aphids per plant (Fig. 2). Frontera accumulated more chymotrypsin inhibitor activity than Aramir, reaching a two fold increase when infested with 60 aphids per plant (Fig. 2(A) and (B)). In contrast, in cv. Aramir, this activity never exceeded 40% over control plants. No differences in trypsin inhibitor activity were found at different aphid densities in both cultivars (Fig. 2(C) and (D)). *S. graminum* always induced a greater accumulation of PIs than *R. padi* (Fig. 3). The basal and accumulated chymotrypsin and trypsin inhibitors levels were inversely correlated with population growth rate of aphids ($r > 0.93$ in all cases).

Increased PI levels in infested barley were discrete compared with those found in dicots [16]. Nonetheless, the damage caused by these insects was sufficiently severe to trigger a signal that drives PI induction. Infestation with the aphid *R. padi* led to smaller PI activities than infestation with *S. graminum*. This could be due to the different feeding behavior of these species. Whereas *S. graminum* ingests food in barley seedlings preferentially from phloem tissue, which does not have the indole alkaloid gramine, *R. padi* ingests from phloem and non-phloem tissues, such as the mesophyll parenchyma.

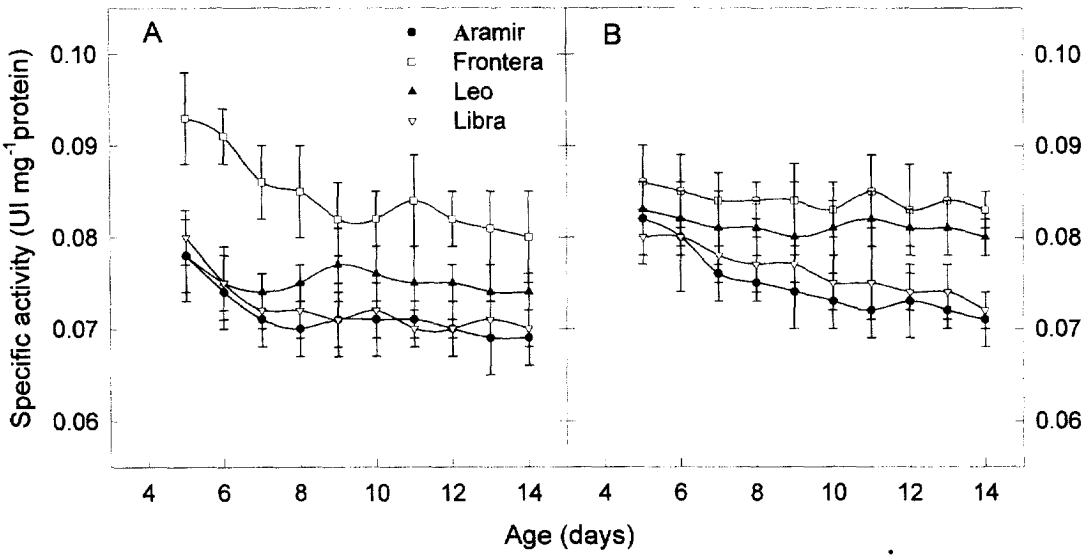


Fig. 1. Inhibitory activity toward chymotrypsin (A) and trypsin (B) in healthy barley leaves of different age. Each point is the mean of 3 measurements ± s.e.

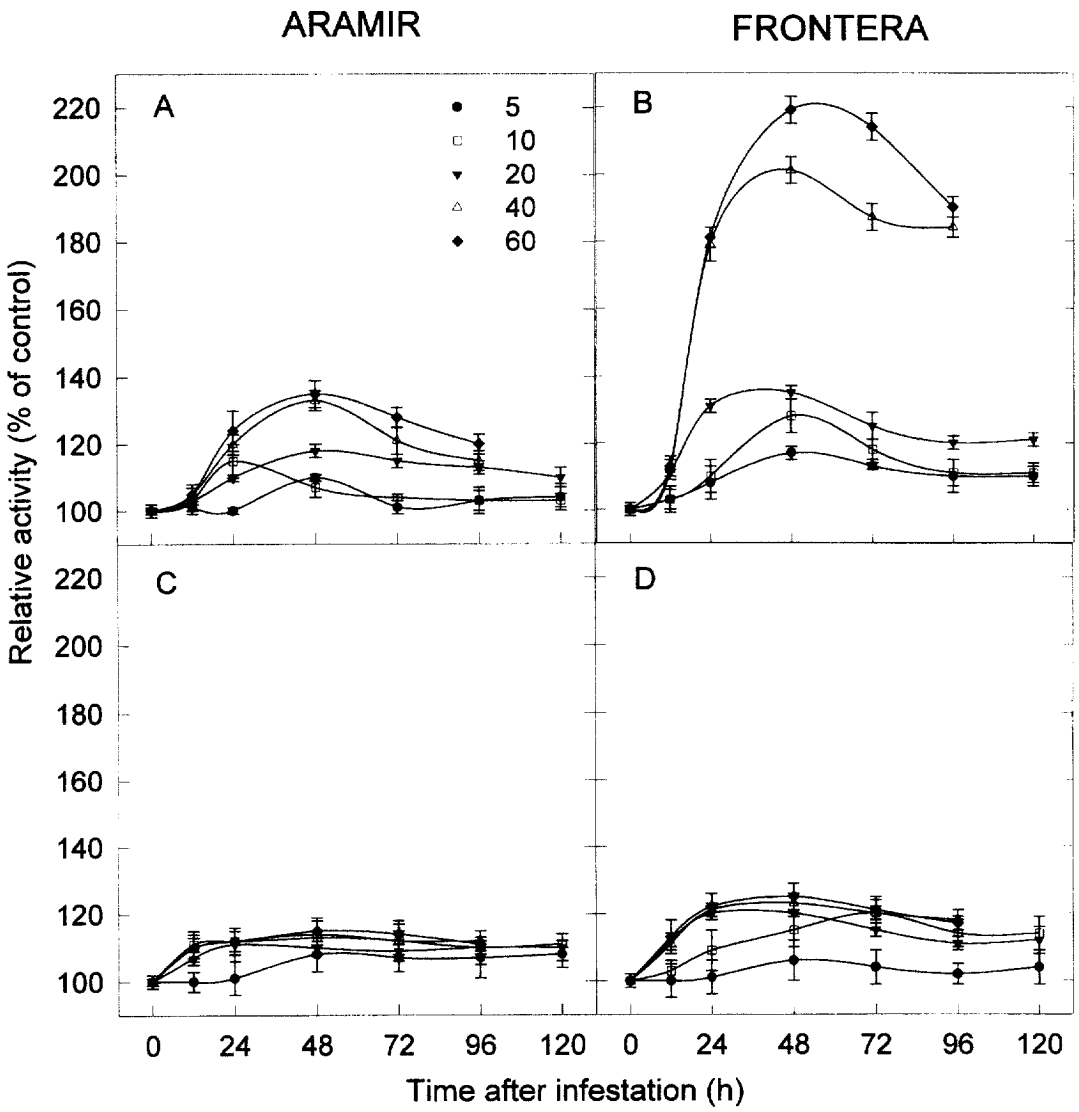


Fig. 2. Inhibitory activity toward chymotrypsin (A, B) and trypsin (C, D) in infested barley leaves. Seven-day-old barley plants cv. Aramir (A, C) and Frontera (B, D) were infested with *S. graminum* (5, 10, 20, 40 and 60 aphids per plant). Results are expressed as relative activity (activity compared to the non-infested controls with 3 measurements ± s.e.).

which have the compound [17]. In addition, *S. graminum* may cause more damage in the mesophyll cells because of the high activity of pectinases found in its saliva [18].

*Separation of PIs*

Gel filtration experiments with crude extracts showed that the activities of both inhibitors were recovered in the high $M_r$ fraction when separated in a Sephadex G-15 column. This procedure was carried out with extracts from control plants and infested with 40 aphids per plant for 48 h. High $M_r$ fractions were collected and concentrated, and then loaded into a Sephadex G-75 column in order to separate inhibitors by $M_r$. Extracts from non infested and infested plants of both cultivars gave the same elution profile (Fig. 4), differing only in the level of activity. Inhibitory activity against chymotrypsin was found in two peaks (mainly in fractions 11 and 14). The calibration done with standard proteins allowed us to estimate the $M_r$ of *ca.* $7 \times 10^3$ to $8 \times 10^3$ and $19 \times 10^3$ for these two chymotrypsin inhibitor peaks, respectively.

Barley chymotrypsin inhibitors CI-1 and CI-2 from seeds have been studied by Boisen *et al.* [7], who estimated a $M_r$ of $6.5 \times 10^3$ to $9 \times 10^3$ for monomeric forms and about $22 \times 10^3$ to $24 \times 10^3$ for iso-forms of dimers. The $M_r$ calculated by gel filtration ($7 \times 10^3$ to $8 \times 10^3$ and $19 \times 10^3$) for the compounds present in the two peaks of chymotryp-

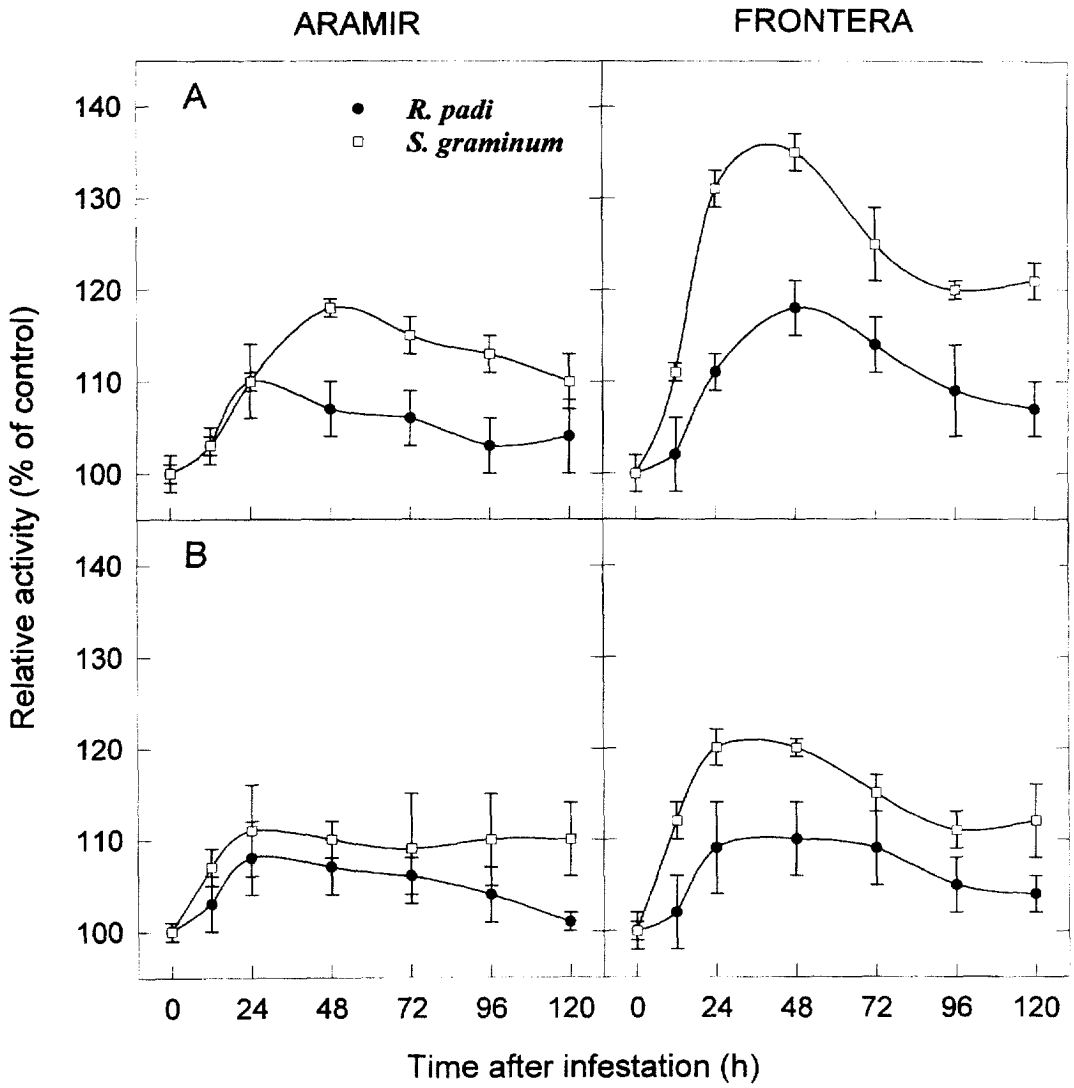


Fig. 3. Inhibitory activity toward chymotrypsin (A) and trypsin (B) in barley infested with two species of aphids. Seven-day-old barley plants cv. Aramir and Frontera were infested with 20 nymphs of the aphids *S. graminum* or *R. padi* per plant. Results are expressed as relative activity (activity compared to controls with 3 measurements ± s.e.).

sin inhibitor activity obtained from barley leaf extracts are close to the values reported by the above authors. These proteins could correspond to iso-inhibitors that may appear in young barley leaves .

The trypsin inhibitory activity eluted as a single major peak (mainly in fraction 12), with an estimated $M_r$ of *ca.* $13.8 \times 10^3$ (Fig. 4), which is also similar to that reported for embryo trypsin inhibitor. An estimated $M_r$ of $14.4 \times 10^3$ was reported by Mikola and Suolinna [19]. Boisen and Djurtoft [20] found that it has a $M_r$ of *ca.* $16 \times 10^3$; Odani *et al.* [21] sequenced the inhibitor and determined a $M_r$ of $13.3 \times 10^3$. Our results agree with the observation that the trypsin inhibitor found in the embryo would be the same found in young leaves [6].

*Effect of barley PIs on aphids reared on artificial diets*

Aphid food appears to be based mainly on sugars and aminoacids, although it has been demonstrated by aphid stylet collections that wheat sieve tubes possess abundant proteins [22]. BSA was used to observe the effect of a foreign protein on the survival of aphids. Survival of the aphid *S. graminum* fed with diets with BSA or soluble barley leaf proteins was similar, but survival of *R. padi* was better on diets with BSA than on diets with soluble barley proteins (Fig. 5). Diets with both, BSA and barley

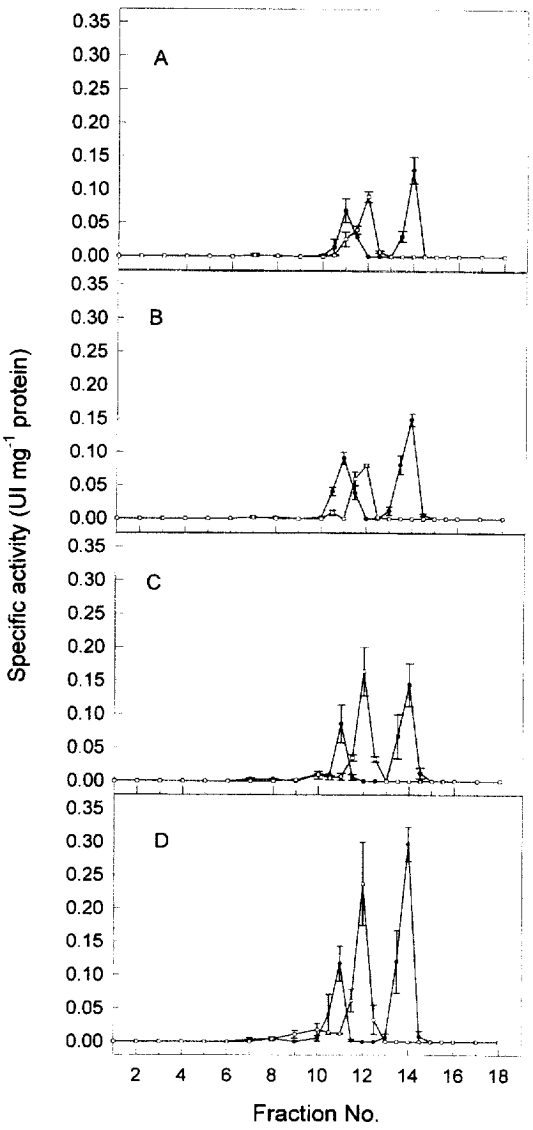


Fig. 4. Gel filtration chromatography of barley PIs by Sephadex G-75. Barley plants cv. Aramir and Frontera were infested for 48 h with *S. graminum* (40 aphids per plant). Extracts of cv. Aramir control (A), infested (B) and Frontera control (C) and infested (D), were filtered previously in a column with Sephadex G-15, concentrated and then loaded into a column with Sephadex G-75. The activity of chymotrypsin (●) and trypsin (○) inhibitor were measured for each fraction. Each point is the mean of 3 measurements ± s.e.

proteins, also decreased *R. padi* survival to levels similar to those found in diets with barley leaf proteins (data not shown). The activity of the three peaks with PI activity isolated from the Sephadex G-75 column was measured (Fig. 6). Again, no differences were found when *S. graminum* was fed with BSA or different fractions (*t*-test, $p < 0.05$). *R. padi* was more affected with PI fractions than with BSA when applied 0.1 to 0.25 mg ml$^{-1}$. It should be noted that, according to the PI activities in the fractions, they are found in leaves in levels corresponding to 0.05 to 0.1 mg ml$^{-1}$. Diets without aminoacids were also performed, but survival was low and no significant differences were found in diets with BSA or total soluble proteins (data not shown).

Not all insects appear to have digestive proteases. Some of them, including aphids, depend on sugars and free amino acids that can be absorbed from the phloem sap. In some aphids, however, some proteases and other peptidases, in addition to amylases and cell wall degrading pectinases, have been described [23]. Activity of a trypsin, cathepsin and acidic peptidases have been described in salival gland and stomach of *Myzus persicae*, *Eriosoma lanigerum*, *Pterocomma populae*, *R. padi* and *Schizolachnus* sp. [24]. Also, a protease activity was found in the intestine of the aphid *Viteus vitifoliae* [24]. Although some species of aphids present protease activity in their guts, it is believed that they do not require these enzymes since their food obtained from the phloem contains small amounts or no proteins [24]. Studies with severed stylets of *R. Padi* showed, however, the existence of active protein turnover and more than 100 proteins in sieve tubes of wheat [22]. The calculated protein concentration in sieve tube sap is about 0.1 μg μl$^{-1}$. Thus, protein ingestion by aphids may be higher than previously thought. The higher tolerance shown by *R. padi* to protein diets could be explained by the presence of a protease in the gut [24]. Proteins from barley extract and isolated PI fractions affected the survival of *R. padi* more than that of *S. graminum*. Whether these PIs from barley leaves are resistance factors against *R. padi*, should be a subject of further study. It is also known that aphids can avoid cell compartments rich in deterrent or toxic compounds when feeding [18]. Even though PIs may not be a main factor in the resistance to aphids, the accumulation of PIs induced by aphids may be of importance as a barrier to another pathogen or pest that attacks barley.

Resistance of barley cultivars to insects may depend on several factors. Among constitutive and inducible secondary compounds, gramine [25], phenolics [26] and flavonoids [1] have been shown to be deterrent to aphids. Cultivar Frontera has 1.4 mmol of gramine kg$^{-1}$ fresh wt. while cv. Aramir has an undetectable amount (unpublished data). Then, the presence of gramine in leaves affects the feeding behavior of aphids, probing frequently in resistant cultivars searching for food and avoiding deterrent compounds [17]. This damage could trigger defensive mechanisms, such as PI accumulation.

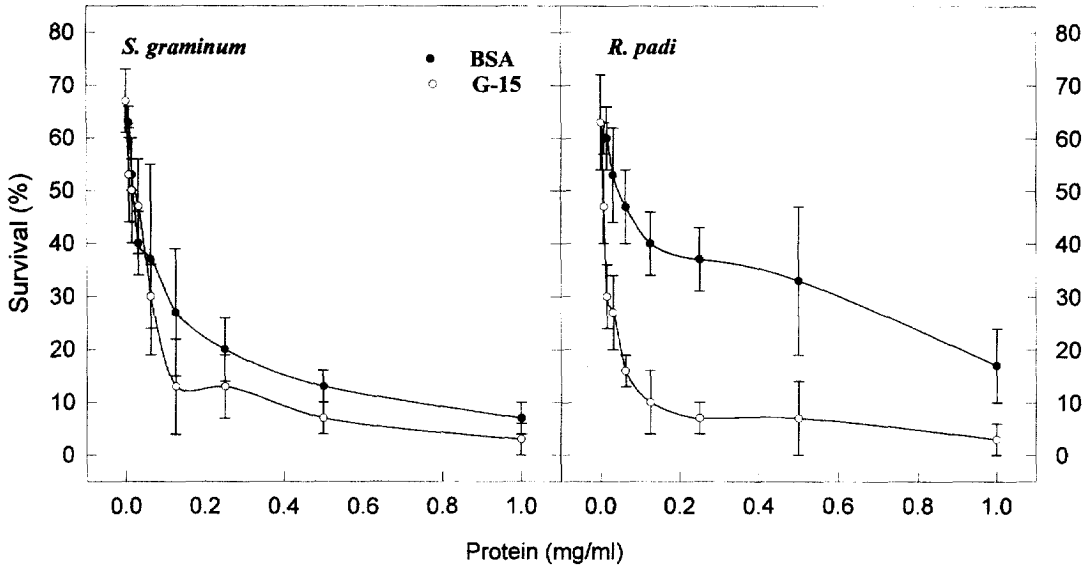


Fig. 5. Effect of barley extract proteins on the survival of aphids reared on artificial diets. Ten individuals of *S. graminum* and *R. padi* were reared for 48 h on diets containing BSA or barley soluble proteins separated in a Sephadex G-15 column. Each point is the mean of 3 measurements ± s.e.

## EXPERIMENTAL

### *Plant material*

Barley (*Hordeum vulgare* L.) seedlings cvs. Aramir, Frontera, Leo and Libra were grown in pots with vermiculite and irrigated with a nutritive solution (Phostrogen®) twice per week. The plants were cultivated in a growth chamber at 24 ± 2° and 14 h of light.

### *Aphid infestation*

To measure cultivar susceptibility, 7-day-old plants of each cultivar were infested with three nymphs of the aphid *Schizaphis graminum* Rondani biotype C per plant. After 6 days, aphids were removed and the population growth rate and chlorophyll content were calculated. For PI experiments, 7-day-old plants were infested with 5 to 60 aphids per plant for 12–120 h. The aphid *Rhopalosiphum padi* L. was also used in some infestation assays.

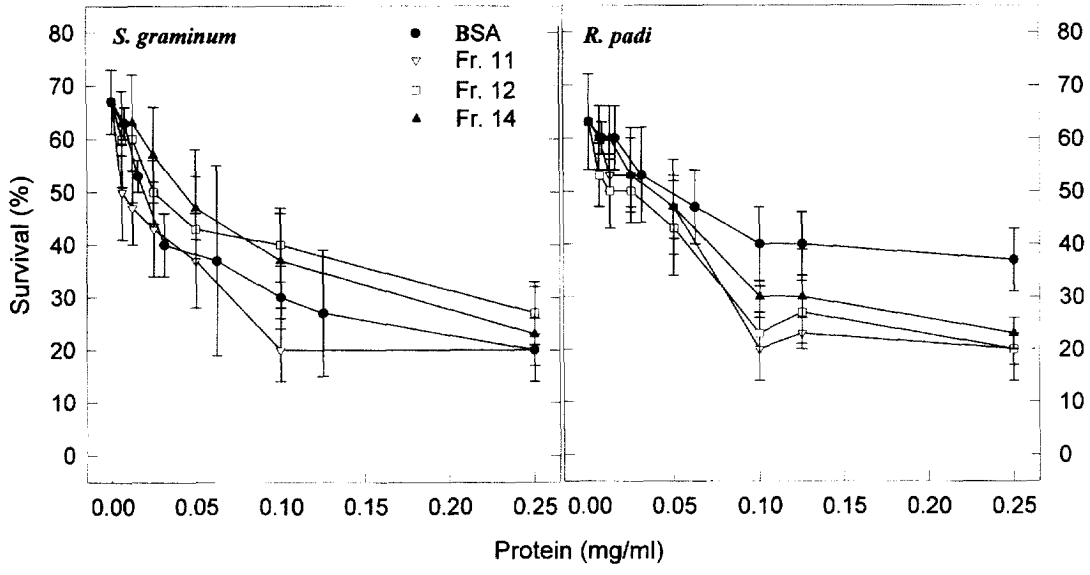


Fig. 6. Effect of leaf barley PIs on the survival of aphids. Aphids were reared on artificial diets as indicated in Fig. 5. Diets contained BSA or proteins from fractions with PI activity separated in a Sephadex G-75 column (see Fig. 4). Each point is the mean of 3 measurements ± s.e.

*Chlorophyll content*

Extracts were obtained grinding 0.5 g of tissue in a mortar with 10 ml of 96% EtOH and filtered. Chlorophyll content were calculated following the method described in Ref. [27].

*Population growth rate*

This growth rate ($r$) was estimated according to the formula: $r = \ln(N_f/N_i)\ \Delta t^{-1}$; where $N_f$ and $N_i$ are the final and initial number of individuals, respectively; and $\Delta t$, time in days.

*Proteinase inhibitor activity*

Barley leaf extracts were obtained from infested and non-infested plants. Leaves were ground in a cold mortar and pestle with 0.05 M Tris–HCl buffer, pH 8.0 (3 ml buffer $g^{-1}$ tissue) containing 10 mM 2-mercaptoethanol and 5% PVP-40 [3], then filtered with a cheesecloth and centrifuged at 20,000 $g$ for 30 min. The supernatant was used as a crude extract to measure inhibitory activity. PI activity was measured spectrophotometrically by means of inhibition of esterase activity of trypsin and chymotrypsin using the method described in Ref. [28]. Both enzymatic activities were measured at 25°. The assays were initiated with the incubation of 0.1 ml of the extract with 5 μg of trypsin (Sigma) or 20 μg of chymotrypsin (Sigma) in 1 mM HCl and 0.05 Tris–HCl buffer (pH 8.0 for trypsin or pH 7.0 for chymotrypsin) for 10 min at room temperature in a final vol. of 0.2 ml. For trypsin assay, this incubation mixture was added to 3 ml of 1 mM *N*-benzoyl-L-arginine ethyl ester (BAEE, Sigma) containing 0.02 M $CaCl_2$ in 0.05 M Tris–HCl buffer, pH 8.0. $A$ was measured at 256 nm. For chymotrypsin assay, the incubation mixture was added to 3 ml of 1 mM L-tyrosine ethyl ester (TEE, Sigma) containing 0.02 $CaCl_2$ in 0.05 Tris–HCl buffer, pH 7.0. $A$ was measured at 235 nm. Inhibitor activities were expressed as inhibitor units per mg of protein. An inhibitor unit (UI) was considered as the amount of inhibitor that reduces the hydrolysis of 1 μmol of substrate per min in standard conditions. Proteases incubated with the extraction buffer were used as controls. Protein content of extracts was determined using the method of Ref. [29] using BSA as standard.

*Gel filtration chromatography*

Two columns were assembled in order to separate the protein fractions with inhibitory activity. Extracts were obtained from control and infested barley plants of cvs Frontera and Aramir, with 40 aphids per plant for 48 h. One 1 × 15 cm column was packed with Sephadex G-15 (Sigma), in which 1 ml of crude extract was loaded and then 1 ml fractions collected. High $M_r$ fractions were concentrated using a Centricon® concentrator ($M_r$ cut off: $3 \times 10^3$), (Amicon) about 20 times. 0.5 ml of these resulting samples where loaded into a 1 × 30 cm column packed with Sephadex G-75 (Sigma). Fractions of 0.5 ml were collected and concentrated again about 5 times. These fractions were then used to determine inhibitory activity. This latter column was calibrated with a standard protein kit (Sigma) containing aprotinin ($M_r$ of $6.6 \times 10^3$), cytochrome c ($M_r$ of $12.4 \times 10^3$), carbonic anhydrase ($M_r$ of $29 \times 10^3$) and bovine serum albumin ($M_r$ of $66 \times 10^3$).

*Artificial diets*

Aphid feeding assays were performed with artificial diets containing BSA, barley proteins separated through a Sephadex G-15 column or different fractions with PI activity. Diets were placed between 2 layers of Parafilm M and consisted on aminoacids, sucrose, vitamins and salts as described in Ref. [30]. Survival of aphids was measured at various times.

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