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Research article

# DEVDase activity is induced in potato leaves during *Phytophthora infestans* infection

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ABSTRACT

Programmed cell death (PCD) occurs in plants, animals and several branches of unicellular eukaryotes as a part of developmental and/or defense processes. Caspase proteases are universal mediators of animal apoptosis, a type of PCD. In plants, there are not animal caspase homologs; therefore, the characterization of caspase-like activities is of considerable importance to our understanding of PCD in plants. Here we report for the first time the involvement of caspase-3-like activity in the resistance mechanism of potato to *Phytophthora infestans* infection. We showed that disease development in infected potato leaves is dependent of caspase-3-like activity. Unlike plant DEVDases previously reported, this DEVDase activity was sensitive to the serine protease inhibitor PMSF. As reported for other subtilisin- like proteases with caspase activity, potato DEVDase activity was mainly localized in the apoplast. We demonstrated that in total protein extract DEVDase activity accounts for a 60% of serine proteases; however, this percentage increases to 100% in the apoplast. Additionally, this caspase-3-like activity is constitutively expressed in the apoplast of potato leaves. Total DEVDase activity is induced only in potato cultivars with high field resistance to *P. infestans*. These results show that potato caspase-3-like protease could constitute a tool in the potato defense mechanisms resulting in partial resistance, although further assays would be necessary in order to elucidate its role.



## 1. Introduction

Programmed cell death (PCD) is a fundamental, genetically controlled process that is responsible for removal of unwanted and potentially dangerous cells; thus, it contributes significantly to both, the development and defense of multicellular organisms [1–3]. In animal cells, a central core execution switch for apoptosis, which is a defined type of PCD in the animal kingdom, is the activation of caspases (Cys-containing Asp-specific proteases). These proteolytic enzymes become activated in the course of apoptosis and introduce specific breaks after aspartate (D) residues in a limited number of cellular proteins, thus mediating PCD [4,5]. In mammalian PCD, caspases play a role as initiators and effectors [6]. Caspases-2, -8, -9 and -10 have been classified as initiator caspases, and they cleave inactive pro-forms of effector caspases, thereby activating them. On the other hand, caspases -3, -6 and -7, have been classified as effector caspases and they cleave other protein substrates within the cell, to trigger the apoptotic process and ultimately leading to cell demise [7,8].

Given the role of caspases in animal PCD, in the last decade scientific effort has been directed to investigate the possibility of existence of these proteins in plants [9]. However, there is no evidence in plant genomes for genes orthologous to caspases [10]. Only distant homologs of caspases, metacaspases, that may be involved in plant PCD have been found [11–13]. However, accumulating evidence in recent years suggests the existence of caspase-like activities in plants and its functional involvement in various types of plant PCD, although many types do not depend upon caspase-like proteases and do not share aspects of apoptosis [9,14–16]. Several caspase activities have been detected in plants using synthetic caspase substrates [10]. Furthermore, protein inhibitors of caspases (specific inhibitor of animal caspases-1 and-3 and the baculovirus

*Abbreviations:* Ac-DEVD-AFC, *N*-Acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin; Ac-DEVD-CHO, *N*-acetyl-Asp-Glu-Val-Asp-CHO (aldehyde); DTT, dithiothreitol; E-64, trans-epoxysuccinyl-L-leucylamido(4-guanidino) butane; IAP, inhibitor of apoptosis; PARP, Poly ADP-ribose polymerase; PCD, programmed cell death; *P. infestans*, *Phytophthora infestans*; PMSF, phenyl methyl sulfonyl fluoride; PVP, polyvinylpyrrolidone; *S. tuberosum*, *Solanum tuberosum*; Z-VAD-FMK, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone.

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anti-apoptotic proteins p35 and IAP) were efficient in preventing plant PCD induced by bacterial, fungal and viral infections [17,18], indicating that enzymes structurally distinct from classical caspases may operate as caspase-like proteases in plant PCD [2].

In many animal cell apoptosis pathways, activation of the effector caspases is considered to be the final step. Among the spectrum of various caspases, caspase-3 is believed to be the major executioner to induce the cleavage of the PARP (Poly ADP-ribose polymerase), DNA fragmentation, chromatin condensation, and final programmed death in animal cells [4,19,20]. For this reason, in plants, like in animals, caspase-3-like activities have also attracted attention [2,14,21,22].

In this study, we have described and characterized the presence of caspase-3-like activity in potato leaves. Additionally, we have determined the induction of DEVDase activity in potato leaf apoplast during *Phytophthora infestans* infection.

## 2. Results and discussion

### 2.1. Caspase-3-like activity is related to potato leaves disease development and cell death during infection

During *P. infestans*–*Solanum tuberosum* interaction, hypersensitive response (HR)-like necrotic reactions occurs in all known forms of genetic resistance to *Phytophthora* (race-specific resistance, nonhost resistance and partial resistance) in order to restrict the invasion of the pathogen [23,24]. Contributing to the general knowledge of the defense mechanism of plants, several reports show that, like in animals, caspase-like proteases are universal mediators of PCD that induce HR [17,18,25]. In order to elucidate if caspase-3-like activities are necessary for completion of HR-like necrotic reactions in potato leaves during potato–*P. infestans* interaction, we examined the effect of specific peptide inhibitors of caspases on disease development and on cell death induced in potato leaves. Results obtained show that Ac-DEVD-CHO and Z-VAD-FMK pretreated leaves have a major disease development compared to controls (Fig. 1A). The disease development in leaves (Fig. 1B) was twice as much, approximately, in leaves pretreated with Ac-DEVD-CHO compared with its respective control. When leaves were pretreated with Z-VAD-FMK, disease development was 20% higher than in control ones (Fig. 1B).

To evaluate the effect of caspase inhibitors on plant cell death during *P. infestans* infection, we quantified by spectrophotometric analysis the Evans blue uptake by cells (Fig. 1C). Results obtained shown that, an increase in Evans blue uptake was observed in control cells leaf 3 and 4 days post-*P. infestans* inoculation. *P. infestans* is a hemibiotrophic oomycete with a biotrophic phase, that occurs during 2–3 days post-inoculation (dpi) and a necrotrophic phase occurs after 4–5 dpi [26]. The results obtained are consistent with that potato–*P. infestans* interactions displaying partial resistance, during the biotrophic phase, have been associated with hypersensitive response (HR)- like necrotic reactions that help to restrict pathogen spread [24]. On the other hand, leaves pretreated with caspase inhibitors before *P. infestans* infection produced a significant reduction in Evans blue uptake compared with untreated and infected leaves. In Ac-DEVD-CHO infiltrated leaves and infected with *P. infestans* during 72 and 96 h, a decrease in Evans blue uptake by cells of 50% and 55% respectively, was observed. Z-VAD-FMK pretreatment showed a reduction in Evans blue uptake of 40 and 30% after 72 and 96 h of *P. infestans* infection, respectively. These results are consistent with several reports that, using synthetic caspase inhibitors, proof that caspase-like activities, specifically caspase-3-like activity, are required for completion of cell death in plants [4,10]. Therefore, the effect of caspase inhibitors decreases HR-like necrotic reactions and produces an increase of the disease development in infected leaves (Fig. 1A and B). Finally, results here showed suggest a possible role of caspase-3-like activity in the completion of hypersensitive response (HR)- like necrotic reactions that restrict the pathogen spread in potato leaves.

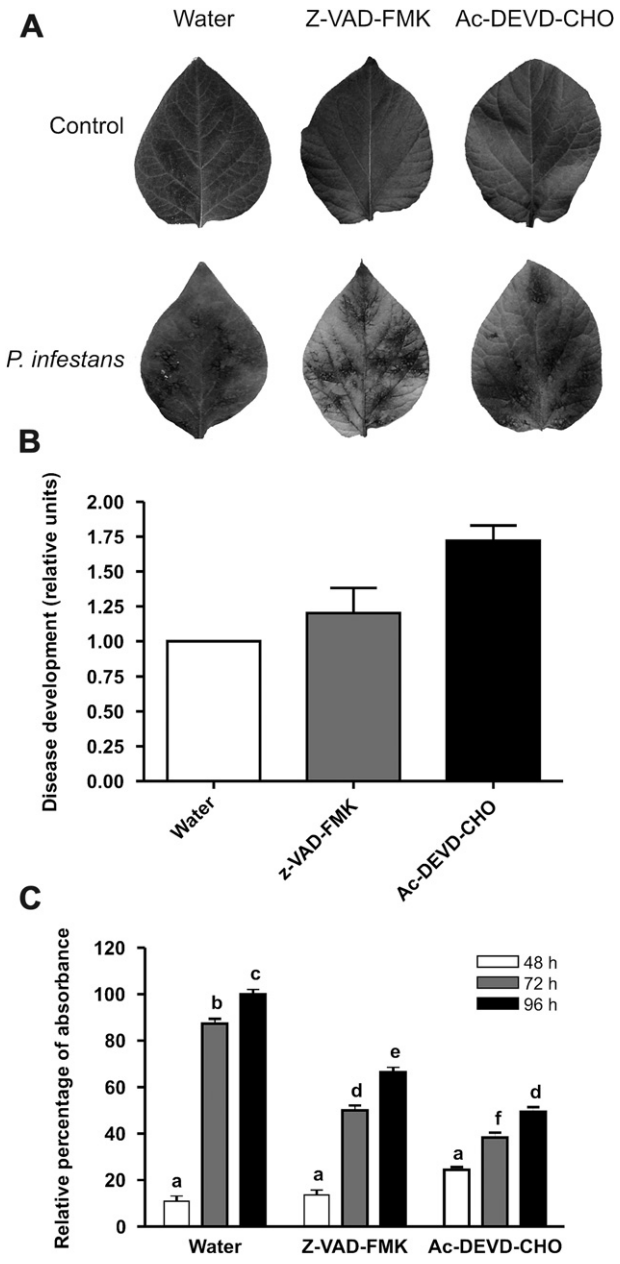


**Fig. 1.** Effect of caspase inhibitors on disease development and cell death in potato leaves infected with *P. infestans*. **A**: Detached leaves of *S. tuberosum* cv. Pampeana were submitted to absorption by petiole of water or a water solution containing 50 μM of the caspase-3 inhibitor Ac-DEVD-CHO or the pan-caspase inhibitor Z-VAD-FMK. After total absorption, leaves were inoculated by spray with water or *P. infestans* (infected) and incubated 5 days. **B**: Disease development was quantified after 5 days of *P. infestans* infection using the ImageJ software. Results are expressed as relative units of infected area, taking as 1 the surface area affected by *P. infestans* in leaves pretreated with water (control) and subsequently inoculated with *P. infestans*. Disease development was quantified in control leaves (white bars), leaves pretreated with Z-VAD-FMK (gray bars) and leaves pretreated with Ac-DEVD-CHO (black bars). The data represent the mean ± SEM of three replicates. **C**: Cell death was estimated by monitoring Evans blue uptake by spectrophotometry at 620 nm. Values were normalized as a percentage of DEVDase activity detected in extracts of leaves treated with water and then infected with *P. infestans* during 96 h. The data represent the mean ± SEM of three replicates. Different letters indicate significant differences with $p < 0.001$ and $p < 0.01$ for one-way ANOVA and Tukey tests respectively.

### 2.2. DEVDase activity level correlates to the degree of potato field resistance to P. infestans

Like in animals, plant caspase-3-like DEVDase belongs to the most- studied ones [27], along with the caspase-1-like YVADase [28]. Here we performed proteolytic assays in order to elucidate if caspase-3-like activity is present and/or induced in potato leaves after *P. infestans* infection. Additionally, we evaluated the possible correlation between caspase-3-like activity and the degree of field resistance of *S. tuberosum* to *P. infestans*. This type of resistance (partial resistance) is durable and thus commercially more attractive than race-specific resistance and it is assumed to be multiple genes based [29,30]. To perform these assays, caspase-3-like activity was measured in extracts of potato leaves from two potato cultivars with different degree of field resistance to *P. infestans*. Whereas cv. Bintje is susceptible to *P. infestans* infection, cv. Pampeana is moderately resistant to this pathogen. Protein extracts of leaves, inoculated with water or *P. infestans* during 0, 1, 6, 12, 24 and 48 h were analyzed. Data obtained indicate the presence of caspase-3-like activity in healthy leaves of both cultivars. Fig. 2 shows that this activity was increased faster and higher in cv. Pampeana than in cv. Bintje during *P. infestans* infection. In cv. Pampeana (Fig. 2A) caspase-3-like activity was increased in a 50 and 90% after 24 and 48 h of *P. infestans* infection, respectively. In contrast, no significant differences in caspase-3-like activities were observed in cv. Bintje leaves during *P. infestans* infection (Fig. 2B). These results show the existence of caspase-3-like activity in leaves and the presence of a caspase-3-like activating pathway during potato–*P. infestans* interaction. These results are consistent with reports that, using synthetic fluorogenic caspase substrates show that caspase-like activities are induced during plant–pathogen interactions [33]. Several proteases have been associated with the potato degree resistance to *P. infestans*. We have previously reported the positive correlation between potato degree resistance to *P. infestans* and the level of expression of two aspartic proteases, named *St*AP1 and *St*AP3. On the other hand, a papain-like cysteine protease (C14) has been associated with the immunity of plants towards *P. infestans* infection [31,32]. In addition, results obtained here suggest for the first time a possible correlation between the level of caspase-3-like activity and the degree of potato field resistance to *P. infestans*. However, this suggestion must be supported by the analysis of caspase-3-like activity in other potato cultivars with different degree of field resistance to *P. infestans*.

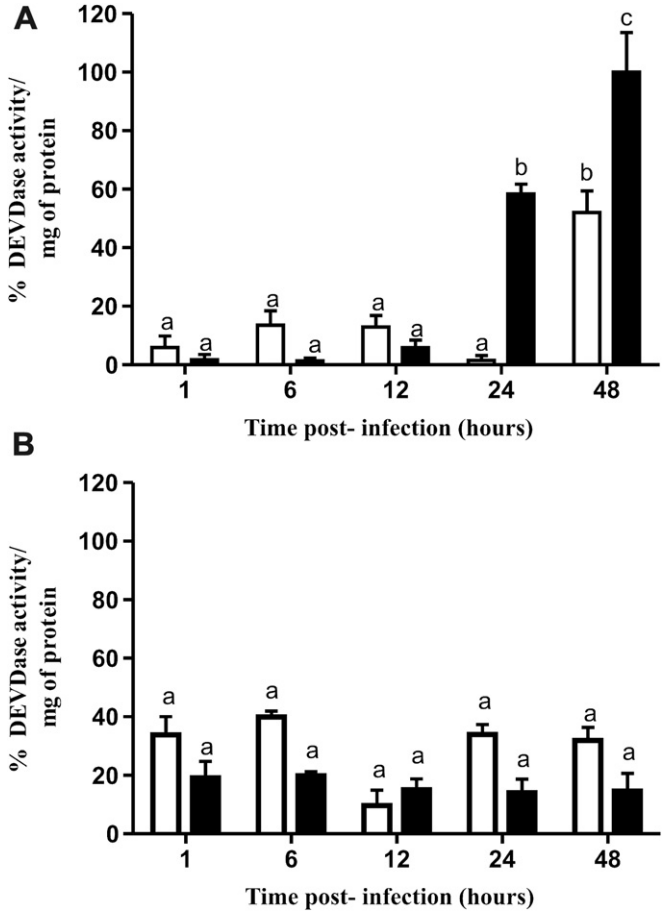


**Fig. 2.** Percentage of DEVDase activity in potato leaves infected with *P. infestans*. **A**: cv. Pampeana; **B**: cv. Bintje. Percentage of total DEVDase activity detected in extracts (corresponding to 1 mg of proteins) of detached leaves inoculated with water (white bars) or *P. infestans* (black bars) and incubated during 1, 6, 12, 24 and 48 h. DEVDase activity determined in extracts of healthy leaves was subtracted from other values to each cultivar tested. Values were normalized as a percentage of the mean of DEVDase activity detected in extracts (corresponding to 1 mg of protein) of 48 h cv. Pampeana infected leaves. The data represent the mean ± SEM of three replicates. Different letters indicate significant differences with $p < 0.001$ and $p < 0.01$ for one-way ANOVA and Tukey tests respectively.

### 2.3. Partial biochemical characterization of total DEVDase activity

Caspase activity in presence of protease inhibitors was quantified in order to characterize possible enzymes structurally distinct from classical caspases but that operate as caspase-like proteases. To perform these assays, caspase-like activity of protein extracts from *P. infestans* infected leaves during 24 h was determined. Results obtained show that DEVDase activity decreases a 60% in presence of the serine protease inhibitor PMSF (1 mM) (Fig. 3). However, no significant changes in the values of caspase activity were observed when the proteolytic assays contained cysteine protease inhibitor E-64 (30 μM), aspartic protease inhibitor pepstatin A (150 μM) or the pan-caspase inhibitor Z-VAD-FMK (15 μM). As expected, a decrease of a 95% was observed in presence of caspase-3 substrate mimetic inhibitor Ac-DEVD-CHO (15 μM). The sensitivity of the DEVDase activity to both, caspase-3 inhibitor and PMSF, suggests that the cleavage of Ac-DEVD-AFC is carried out by

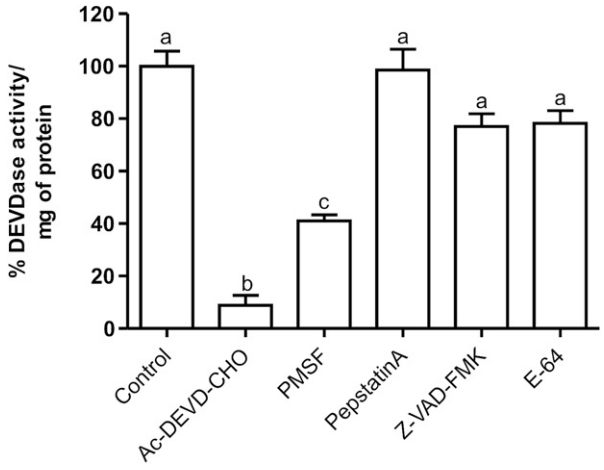


**Fig. 3.** Effect of general protease and caspase inhibitors on *P. infestans* induced DEVDase activity *in vitro*. Total extract of cv. Pampeana leaves infected with *P. infestans* during 24 h were incubated with buffer (control), PMSF (1 mM), E-64 (30 μM), pepstatin A (150 μM), Ac-DEVD-CHO (15 μM) or Z-VAD-FMK (15 μM) for 1 h at 37 °C and pH 5.2. Mixtures were then incubated with 15 μM Ac-DEVD-AFC substrate at 37 °C during a 3.5 h reaction. Values were normalized as a percentage of the mean of DEVDase activity determined in control extracts. The data represent the mean ± SEM of three replicates. Means with different letters differ at $p < 0.001$ and $p < 0.01$ according to one-way ANOVA and Tukey tests respectively.

two types of proteases (caspase-3-like protease and serine proteases) or by a single group of proteases sensitive to both types of inhibitor. These results demonstrate the existence/activation of serine proteases with caspase-3-like activity in potato leaves after infection with *P. infestans*. This is in accordance with the fact that some caspase-like activities in plants are attributable to plant subtilisin- like proteases, named as saspases and phytaspases [34]. By contrast, neither phytaspase nor saspase could hydrolyze a DEVD based caspase substrate [34,35]. The results obtained here suggest a new/s candidate/s (serine protease with DEVDase activity) to complete the family of plant 'caspase-like' proteases previously named as "incomplete" [34].

Animal caspases exhibit certain sensitivity to ions and pH [36]. To further characterize DEVDase activity in this patho-system, the effect of ions and pH were determined. As Table 1 shows, no significant effect was observed in DEVDase activity in presence of different amounts of NaCl (1–300 mM) and $MgCl_2$ (1–150 mM). Results obtained are in disagreement with data reported for the cytosolic caspase-3-like activity found in barley [21] and with the results reported to sapases 1 and 2 from oat. For these proteases, a concentration of 100 mM NaCl or $MgCl_2$ reduces these caspase activities to a 50% approximately. Various cellular functions are influenced by essential trace elements such as the divalent cations zinc (Zn2+) and manganese (Mn2+) [37,38]. Zn2+ directly inhibits the *in vitro* activity of various recombinant caspases, including caspase-3, -6 and -8 [36,39]. In plant cells, Zn2+ produce changes in the VADase activity of saspases 1 and 2 from oat [40]. Here we determined that, when Mn2+ was added in the enzymatic reaction, an increase of 40–60% in DEVDase activity was observed (Table 1). The presence of Zn2+ at 3 μM concentration produced a decrease in potato leaf caspase-3-like activity of 50% approximately. Contrary, the presence of Zn2+ at 30 mM concentration produce an increase of potato leaves DEVDase activity of 40% and 190% for 3 mM and 30 mM Zn2+, respectively. Similar results were observed for caspase-3 activity of human Burkitt lymphoma B cells [41]. These results indicate that, depending on Zn2+ concentrations, zinc can exert opposite effects on caspase-3 activation and apoptosis in cells: concentrations below 50 μM inhibit caspase-3 activation whereas higher concentrations of zinc activate a death pathway associated with apoptotic-like features and caspase-3 activation.

pH optima for plant-DEVDase activities are acidic. For example, in *Arabidopsis thaliana* seedlings [42] and in germinating white spruce seeds [43], DEVDase activity exhibited maximal activity at pH 5 and 5.5 respectively [10]. Unlike this, and in concordance with animal caspases, whose optimum pH is 7 [36], caspase-3-like activity in *S. tuberosum* leaves infected with *P. infestans* showed maximal pH activity at neutral pH, and no activity could be detected at basic pH (Fig. 4).

**Table 1**
Effect of different salts on DEVDase activity of leaf extracts infected for 24 h.

| Salt | Activity (% of control) |
|---|---|
| None | 100.00 ± 3.54 |
| NaCl | |
| 3 mM | 92.00 ± 8.89 |
| 30 mM | 65.08 ± 7.26 |
| 300 mM | 85.41 ± 13.98 |
| $MgCl_2$ | |
| 3 mM | 97.67 ± 5.79 |
| 30 mM | 89.46 ± 8.74 |
| 150 mM | 97.88 ± 11.75 |
| $MnCl_2$ | |
| 3 mM | 160.31 ± 2.26 |
| 30 mM | 140.32 ± 8.49 |
| 150 mM | 124.18 ± 13.07 |
| ZnAc | |
| 3 μM | 57.09 ± 5.59 |
| 0, 3 mM | 92.51 ± 15.01 |
| 3 mM | 143.73 ± 24.17 |
| 30 mM | 287.96 ± 18.77 |

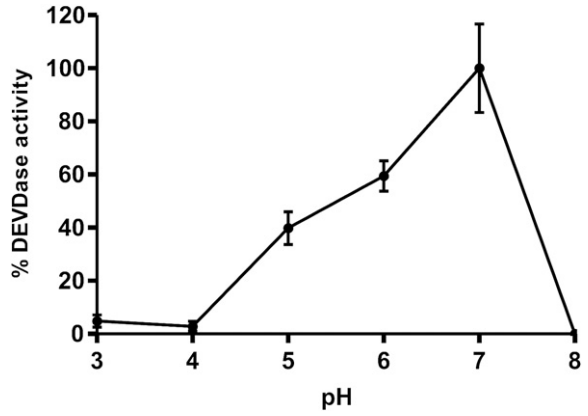


**Fig. 4.** Effect of pH on DEVDase activity in potato leaves after *P. infestans* infection. Total extracts of leaves infected with *P. infestans* during 24 h were assayed for hydrolytic activity at different pHs in citrate–phosphate buffer with Ac-DEVD-AFC substrate at 37 °C as described in Materials and methods. Values were normalized as a percentage of the mean of DEVDase activity determined at pH 7 (maximum activity). The data represent the mean ± SEM of three replicates.

### 2.4. Part of the DEVDase activity is localized in the apoplast

Like caspases, phytaspases and saspases are synthesized as proenzymes, which are autocatalytically processed to generate a mature enzyme. Both types of enzymes are implicated in plant PCD during both abiotic and biotic stresses. However, unlike caspases, phytaspases and saspases appear to be constitutively processed and secreted from healthy plant cells into the intercellular space [34,35,40]. In order to determine if caspase-3-like activity is present in the potato apoplast, we determined DEVDase activity in IWFs of healthy and infected potato leaves. Fig. 5 shows that there is DEVDase activity in intercellular space of healthy and *P. infestans*

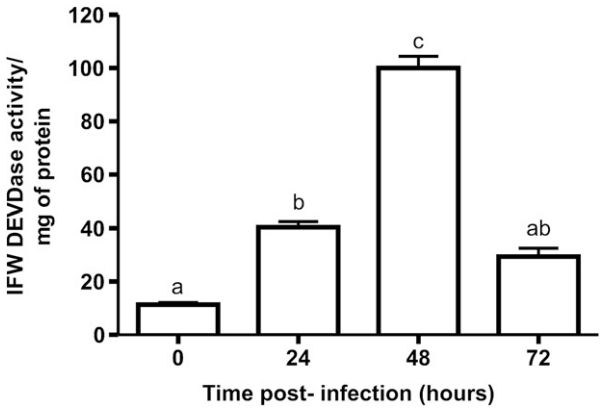


**Fig. 5.** Percentage of DEVDase activity in potato leaves intercellular washing fluid (IWF) during *P. infestans* infection. Intercellular washing fluid of healthy and infected leaves during 0, 24, 48 and 72 h with *P. infestans* were obtained as described in Materials and methods. Aliquots of these IWF containing 1 mg of protein were incubated in the presence of 15 μM Ac-DEVD-AFC substrate at 37 °C during 2.5 h. DEVDase activity determined in IWF of healthy leaves was subtracted from other values. Values are normalized as a percentage of the mean of DEVDase activity detected in IWF of leaves after 48 h of *P. infestans* infection. The data represent the mean ± SEM of three replicates. Different letters indicate significant differences with $p < 0.001$ for one-way ANOVA and Tukey tests.

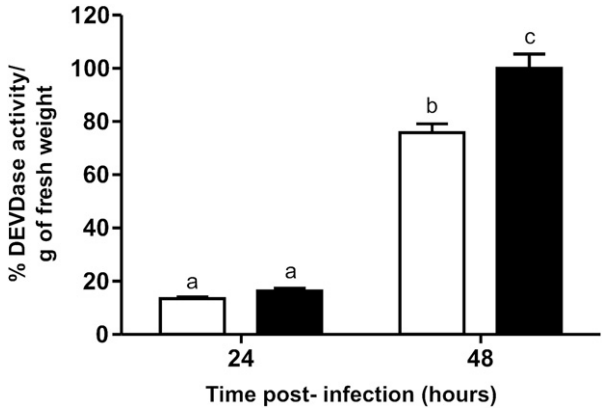


**Fig. 6.** DEVDase activity percentage in IWF and soluble extracts of potato leaves after infection with *P. infestans*. Total extracts (black bars) and IWF (white bars) from leaves inoculated with *P. infestans* during 24 and 48 h were obtained as described in Materials and methods. Aliquots of these IWF corresponding to 1 g of fresh weight were incubated in presence of 15 μM Ac-DEVD-AFC substrate at 37 °C during 2.5 h. DEVDase activity determined in IWF and extracts of healthy leaves was subtracted from other values. Values were normalized as a percentage of the mean of DEVDase activity detected in extracts of leaves of cv. Pampeana after 48 h of *P. infestans* infection (maximum activity). The data represent the mean ± SEM of three replicates. Different letters indicate significant differences with $p < 0.001$ for one-way ANOVA and Tukey tests.

infected potato leaves. Results obtained here show that, as phytaspase, caspase-3-like protease activity in potato leaves apoplast is constitutive in healthy leaves. These results are in disagreement with those reported for saspases that showed that saspase activity in oat extracellular fluids is only detected after induction of PCD [40]. In infected leaves, apoplastic DEVDase activity was 80% of the DEVDase of total extracts (Fig. 6). Like saspases, that are induced after pathogen attack [34], DEVDase activity in intercellular space of potato leaves infected with *P. infestans* (during 24 and 48 h) was higher (3 and 9 times, respectively) than the values determined for DEVDase activity in healthy leaves (Fig. 5). However, unlike phytaspases that, in response to death-inducing stimuli are re-localized to the cell interior, potato caspase-3-like activity, like saspases, is induced in the potato leaf apoplast upon pathogen infection (Fig. 5). Additionally, in Fig. 7, we demonstrate that accumulated caspase-3-like activity during *P. infestans* infection is attributable to potato serine proteases, since the presence of the serine protease inhibitor PMSF inhibits in a 100% the DEVDase activity detected in the apoplast of healthy and infected potato leaves.

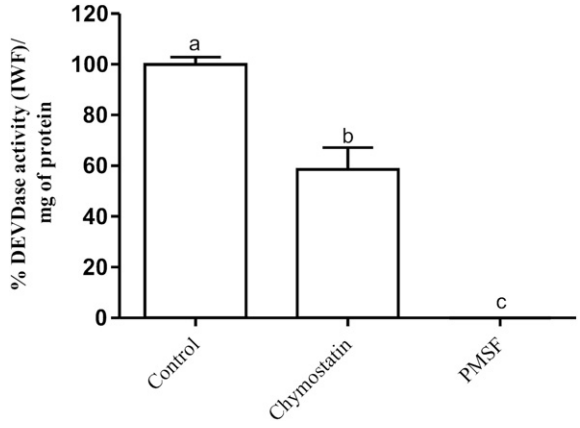


**Fig. 7.** Effect of general protease inhibitors on DEVDase activity of IWF of potato leaves infected with *P. infestans*. IWF from leaves infected with *P. infestans* during 48 h were pre-incubated with the serine and cysteine protease inhibitors PMSF (1 mM) and chymostatin (200 μM) for 1 h at 37 °C and pH 5.2. Then, mixtures were incubated with 15 μM Ac-DEVD-AFC substrate at 37 °C during 1.5 h. Values are normalized as a percentage of DEVDase activity detected in IWF of leaves of cv. Pampeana after 48 h of *P. infestans* infection without inhibitors. The data represent the mean ± SEM of three replicates. Means with different letters differ at $p < 0.001$ and $p < 0.01$ according to one-way ANOVA and Tukey tests respectively.

## 3. Conclusion

As a conclusion, here we demonstrate the presence of caspase-3-like activity in IWF from healthy and *P. infestans* infected potato leaves. The importance of this activity to restrict pathogen spread and its possible correlation with field potato resistance to *P. infestans* remains elusive. Further assays would be necessary in order to identify potato apoplastic proteins with caspase-3-like activity and to elucidate the role of caspase-3-like activity in potato resistance to *P. infestans*.

## 4. Material and methods

### 4.1. Plant and fungal material: growth conditions

Potato plants of *S. tuberosum* L. *cv*. Pampeana INTA and *cv* Bintje were grown in pots containing a 1:1 (v/v) mixture of soil/vermiculite and maintained at 25 °C for 15 days with a 12 h photoperiod. Plants were then transferred to 18 °C with the same photoperiod for six weeks. *P. infestans* race R2 R3 R6 R7 R9, mating type A2, was obtained from the INTA Collection, Balcarce (Argentina). *P. infestans* was grown on potato tuber slices incubated in closed plastic boxes and maintained in darkness at 18 °C. Seven days post- inoculation, mycelia was harvested in sterile water and stimulated to release zoospores by incubation for 2–3 h at 4 °C. After filtration through muslin, the resulting suspension was observed with a light microscope to quantify spores and sporangia. Concentration was adjusted to $10^5$ sporangia $ml^{-1}$.

Detached leaves were placed on Petri dish capsules with wet filter paper and infected by spraying with a suspension containing $10^5$ sporangia of *P. infestans* while control leaves were sprayed with sterile distilled water. Petri dishes were placed at 18 °C and 100% humidity until leaves were harvested at different times post-inoculation.

### 4.2. In vivo treatment of leaves with caspase and protease inhibitors

Detached leaves from *S. tuberosum cv*. Pampeana were weighted and submitted to absorption through petiole of 50 μl (per approximately 0.4 g of fresh weight) of distilled sterile water or a solution containing the cell permeable broad spectrum Z-VAD-FMK (50 μM) or specific Ac-DEVD-CHO (50 μM) caspase inhibitors. After all the volume was absorbed (approximately 2 h), leaves were placed in Petri dishes and sprayed with water (control) or with a suspension containing $10^5$ sporangia $ml^{-1}$ of *P. infestans*. Leaves were incubated in Petri dishes at 18 °C, 100% humidity and with a 12 h photoperiod. Disease development was recorded 5 days after inoculation and infected area was quantified using the ImageJ software (http://rsb.info.nih.gov/ij/). In addition, after 0, 48, 72 and 96 h of infection leaves were collected and incubated in 1% aqueous Evans blue with shaking for 15 min. Stained leaves were rinsed well with deionized water until no more blue stain was eluted and dried by filter paper. Dry leaves were grinded with a mortar and a pestle in liquid nitrogen. Powder was resuspended at 0.3 g $ml^{-1}$ with 50 mM buffer HEPES pH 7.5 and 2 mM DTT, prior to centrifugation at 9600 × *g* for 5 min. The supernatant was collected and optical density was determined spectrophotometrically at 620 nm (Hitachi spectrophotometer).

### 4.3. Total soluble extracts

After 0, 1, 6, 12, 24 and 48 h of *P. infestans* infection, leaves of *S. tuberosum* cv. Pampeana and Bintje, were frozen in liquid

nitrogen and grounded to a powder with a cold mortar and pestle. The powder was collected and resuspended in 5 volumes of ice cold caspase extraction buffer (50 mM Hepes pH 7.5, 20% glycerol, 1% PVP and 2 mM DTT). Samples were maintained at 4 °C, vortexed for 20 min and centrifuged (9.6 × *g*, 20 min, 4 °C). Supernatants were collected, its volume measured and used for protease assays.

### 4.4. Isolation of intercellular washing fluid (IWF)

Intercellular washing fluids of potato infected leaves cv. Pampeana for 0, 24 and 48 h were obtained as previously described [44]. Tissue was immersed in infiltration buffer (50 mM HCl-Tris, pH 7.5, 0.6 M NaCl, 0.1% (v/v) 2-mercaptoethanol and 20 μl/15 ml of Tween20) and subjected to three vacuum pulses of 10 s separated by 30 s intervals. Infiltrated leaves were recovered, dried on filter paper, placed in fritted glass filters, inserted in centrifuge tubes and centrifuged for 20 min at 400*g* (3000 rpm) at 4 °C. The recovered extract was used immediately or conserved at −20 °C. As a marker enzyme for the cytosolic and vacuolar fraction Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and α-Mannosidase (EC 3.2.1.24) activity were measured [45].

### 4.5. Protein concentration

Protein concentration was measured by the bicinchoninic acid method [46] using bovine serum albumin (BSA) as standard.

### 4.6. In vitro caspase activity assay

Total extracts and IWF were dialyzed overnight against 50 mM sodium acetate pH 5.2, using a cellulose membrane (Sigma D9652-100). Proteolytic activity was measured in reaction buffer composed of 50 mM sodium acetate pH 5.2, 2 mM DTT and 15 μM mammalian caspase-3 substrate *N*-Acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin (Ac-DEVD-AFC) dissolved in Dimethyl sulfoxide. Release of fluorophore by cleavage was measured (excitation wavelength of 405 nm and emission wavelength of 525 nm) using a microplate fluorimeter (Fluoroskan ascent, Thermo Electron Corporation) during a 2.5 h reaction time at 37 °C. Results were corrected against a blank containing only buffer and substrate. Relative activity was standardized to the quantity of total proteins present in the sample. All assays were performed in triplicate and DMSO concentration was less than 1.5%.

To analyze the effect of protease and caspase inhibitors on caspase substrate cleavage, soluble extracts and IWF from leaves of *S. tuberosum* cv. Pampeana control and infected for 24 and 48 h respectively with *P. infestans* were incubated in caspase reaction buffer containing one of different caspase and protease inhibitors: 15 μM Ac-DEVD-CHO, 30 μM E-64, 1 mM PMSF, 150 μM pepstatin A, 15 μM Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone) or 200 μM chymostatin for 1 h at 37 °C. Subsequently, extracts were incubated with 15 μM of the Ac-DEVD-AFC substrate.

For determination of DEVDase optimal pH 50 mM citrate/phosphate buffer was used in the pH range of 3–8. To test the effect of cations on DEVDase activity, ZnCl2, ZnAc, MnCl2 or NaCl were added at different concentrations to the caspase reaction buffer (50 mM sodium acetate pH 5.2 and 15 μM Ac-DEVD-AFC). In the determination of the effect of divalent cations, a concentration of 2 mM β- mercaptoethanol was used instead of DTT because it does not chelate these ions with the same strength.

### 4.7. Statistical analysis

Results are reported as the means ± SEM of the combined experiments. Statistical analysis was performed by one-way ANOVA followed by Tukey test for comparisons among multiple groups. Analysis was done using SigmaStat 3.5 (http://www.systat.com/).

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