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# Effect of β-aminobutyric acid (BABA) on protection against *Phytophthora infestans* throughout the potato crop cycle

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**Abstract.** The protective effect of β-aminobutyric acid (BABA) on two potato cultivars (Bintje and Pampeana) with different levels of horizontal resistance against *Phytophthora infestans* was investigated during the crop cycle. Plants were treated with BABA 16, 23, 30, 38, 44, 51, 61 or 75 days after emergence. After each application, the percentage of protection and the content of glucanases, chitinases and phenolic compounds were determined in detached leaves. The foliar pretreatment with BABA up to 30 days after emergence showed a 60% protection percentage against *P. infestans* in cv. Pampeana, while cv. Bintje almost reached 20%. The results indicated that the time of application of BABA and the susceptibility of the cultivars affect both the protection against late blight and the expression of defence molecules like glucanases, chitinases and phenolic compounds.

**Additional keywords:** plant inducers, *Solanum tuberosum*.

## Introduction

Potato late blight, caused by *Phytophthora infestans* (Mont.) de Bary, is a devastating disease that is controlled by frequent fungicide applications. This results in important consequences for the environment, the farmer's health and the cost of production. An effective and low-cost strategy to minimise late blight incidence is the utilisation of cultivars with horizontal resistance, partial resistance, multigenic or field resistance, which are controlled by several so- called 'minor' genes, found in *Solanum tuberosum* (Guzman 1964). These confer incomplete protection in the aerial part of potato plants, slowing the dissemination of late blight in the field (Wilson and Coffey 1980). In areas where the disease is most common and severe, the use of varieties with horizontal resistance reduces the risk of early occurrence and quick disease development in the field. Cultivars Bintje and Pampeana, utilised in this work, represent a model of horizontal resistance in breeding programs in Argentina (Huarte 2001). As it has been reviewed by Tuzun (2001), plants with multigenic resistance display defences to a variety of pathogens and the same is true for plants in which systemic resistance has been induced. Tuzun (2001) suggested that the genes involved in multigenic resistance are also involved in induced systemic resistance. As a consequence, the advances in the use of induced resistance and the multigenic resistance cultivars in breeding programs could combine to contribute to integrated disease management strategies.

Induced resistance is defined as the stimulation of natural defence mechanisms in plants by biological agents or chemical compounds (inducers), biocompatible with the environment. Different inducers can produce similar but not identical defence responses (Gozzo 2003). Also, for each inducer, the response mechanism varies according to the plant–pathogen system. One of the described inducers is the plant activator β-aminobutyric acid (BABA).

The broad spectrum protective effect of BABA against numerous plant diseases has been well documented (Jakab *et al*. 2001; Cohen 2002). In several cases, treatment with BABA induced the accumulation of pathogenesis-related proteins (PRs) in treated tissue before and after challenge with the pathogen (Cohen *et al*. 1994). The β-1,3-glucanases and chitinases are two important groups of PRs, which are induced either by pathogens or by chemical inducers. The induction of these PRs is an important parameter for the evaluation of the plant–pathogen interaction in cultivars with multigenic resistance (Vleeshouwers *et al*. 2000). Both enzymatic activities are involved in the degradation of the cell wall of fungi and bacteria (Kombrink *et al*. 1988). In previous work, we have observed a substantial reduction in foliar infection by *P. infestans* in moderately susceptible and moderately resistant commercial potato cultivars treated with BABA (Andreu *et al*. 2006). However, there are relatively few reports about the biochemical responses of potato leaves, although leaves are the primary infection site in the field. Together with PRs, phytoalexins and phenolic compounds have been postulated to be involved in defence responses against fungal infection. Phytoalexins and phenolics are important features in the resistance of moderately resistant cultivars (Andreu *et al*. 2001). In addition to glucanases, phytoalexins have been reported to be induced in potato tubers by BABA treatment in foliage (Olivieri *et al*. 2005; Andreu *et al*. 2006).

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Studies have been published about the changes in resistance against *P. infestans* with increasing plant age in potato (Visker *et al*. 2003). However, the effect of plant activators in different stages of plant growth is not known. Assuming that BABA applications could be a part of an integrated disease management program, it is of interest to know if the time of application in the cropping cycle is important and how it affects the defence response in cultivars with different levels of multigenic resistance. Thus, the aim of the present work was to investigate the effect of BABA on protection against *P. infestans* and on the induction of defence molecules, throughout the potato crop cycle.

## Methods

### *Plant material*

Experiments were performed under greenhouse conditions with seed tubers of cultivars Pampeana INTA (MPI 59.789/ 12 × Huinkul MAG) (moderately resistant to *P. infestans*) and Bintje (susceptible to *P. infestans*). They were used due to their different foliar and tuber late blight polygenic resistance. Seed tubers were planted at 10-cm depth in steam-pasteurised substrate greenhouse potting mix in 7-L plastic pots. During plant growth, the temperature ranged between 15 and 24°C and natural daylight was supplemented by high-pressure sodium lamps (400 W) in a 14–10-h day/night cycle. Plants were irrigated with a sprinkler system. Fifty plants were used per treatment and cultivar and the experiments were repeated three times.

### *Phytophthora cultures*

All experiments were performed with the isolate of *P. infestans* race R2R3R6R7R9 mating type A2. This was maintained on potato tuber slices in closed plastic boxes in the dark at 18°C and 90% relative humidity, for 6–7 days until new sporulation occurred. The mycelium was harvested in sterile water. The release of zoospores was stimulated by incubation at 4°C for 2 h. The mycelium was filtered through a 15-μm nylon filter cloth and the zoospore suspension was observed under light microscope for quantification before using it as inoculum. The concentration of zoospores was adjusted to 40 000 zoospores/mL using a haemacytometer.

### *BABA treatments*

The inducer BABA was dissolved in water (40 mM) and was used for plant foliar spray on potato plants. Applications were made with a fine glass atomiser (~5 mL per plant). Water was used as a control. BABA was applied to different groups of three plants, each one corresponding to different times of treatment. In other words, each group corresponds to only one time of application. The application to the different groups was made at 16, 23, 30, 38, 44, 51, 61 or 75 days after emergence (dae).

### *Evaluation of late blight severity and foliage protection*

Disease severity and foliage protection were assessed by the detached-leaf method (Goth and Keane 1997). Three days after each foliar application of BABA or water, five leaflets were detached per plant from the upper portion of the foliage (3rd and 4th leaf from the top) from each cultivar. At the laboratory, detached leaves were placed abaxial side up in 20-cm-diameter plastic Petri dishes sealed with 2% agar water. The leaflets were inoculated by placing a 50-μL droplet of a zoospore suspension (40 000 zoospores/mL) in the centre part of each leaflet. Immediately after inoculation, the leaflets were incubated in a growth chamber at 15°C in the dark for 24 h. After this period, incubation continued in the growth chamber at 18°C and 16-h light period (Philips fluorescence tubes type 33, intensity of 12 W/m$^2$).

Disease development in treated and untreated leaves was recorded 7 days after inoculation by visual estimation of the leaf area with late blight lesions. The disease severity index was estimated using a scale from 1 to 10, where: 1 = no lesion; 2 = a few circles; 3 = up to 5% of leaves affected; 4 = 5–10% affected; 5 = 10–25% affected; 6 = 25–50% affected; 7 = 50–75% affected; 8 = 75–85% affected; 9 = 85–95% affected, and 10 = 95–100% of the leaf area showing late blight symptoms (Andreu *et al*. 2006). Foliage protection percentage (FPP) was calculated as follows: FPP (%) = 100 (1−$x/y$), where $x$ and $y$ are disease severity values for treated and control plants, respectively, as described by Cohen *et al*. (1994).

### *Preparation of total leaf soluble extract for biochemical assays*

Three days after each BABA or water treatment, leaflets were cut and inoculated by spray with zoospore suspension (40 000 zoospores/mL) of *P. infestans*. Five days after inoculation, potato leaflets (4–5 g) were homogenised in two volumes of buffer solution containing 100 mM sodium acetate, pH 5.2, 5 g/L sodium metabisulfite. Homogenates were filtered through cheesecloth and centrifuged at 12 000*g* for 20 min. The resulting supernatant represented the total leaf soluble extract. The extracts were conserved at −20°C.

### *Enzymatic activities*

For β-1,3-glucanases activity measurements, 200 μL of total soluble extracts were dialysed against sodium acetate buffer 50 mM, pH 5.2, overnight and then centrifuged at 12 000*g* for 5 min. The β-1,3-glucanase activity was determined by the colourimetric method of Ashwell (Ashwell 1957) with laminarin (Sigma) as substrate. The reaction mixture consisted of 50 μL of 50 mM sodium acetate buffer, pH 5.2, containing 10 g/L laminarin, 0.01 mL of enzyme extract, and 0.05 mL of sodium acetate buffer, pH 5.2. After 30 min of incubation at 37°C, the enzyme reaction was heated in boiling water for 3 min and the reducing sugar released was measured using glucose as standard. The activity was expressed as one unit of activity equivalent to 1 nmol glucose/ h.g fresh weight.

Chitinase activity was determined by the method of Reissig by measuring the released GlcNAc residues from partially hydrolysed chitin (Reissig *et al*. 1955). The reaction mixture consisted of 200 μL sodium acetate buffer 50 mM, pH 5.2, 100 μL of chitin, and 200 μL enzymatic extract. After 3 h of incubation at 37°C, the samples were centrifuged at 12 000*g* for 15 min and 100 μL of each supernatant were mixed with 20 μL of borate buffer 0.8 M, pH 9. The reaction was stopped by boiling for 3 min. GlcNAc residues were determined by the Reissig

method. The absorbance was measured at 585 nm. One unit of activity was defined as the amount of enzyme that produces a change of 1.0 in the absorbance for 1 h and 1 g of fresh weight. All measurements were conducted twice.

### *Gel electrophoresis and immunoblot analysis*

For inmunoblot analysis, the leaf soluble extracts prepared for biochemical assays were subjected to electrophoresis in 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes in a semi-dry electrophoretic transfer cell (Trans-Blot, Bio Rad, Hercules, CA, USA). Western blot assays were performed as described previously (Andreu *et al*. 2006). Polyclonal antibodies, either anti-β-1,3-glucanases of potato or anti-basic chitinases of potato (both kindly provided by Kombrink) were used at 1 : 3000 v/v or 1 : 5000 v/v dilution, respectively (Kombrink *et al*. 1988).

### *Extraction and determination of phenolic compounds*

Three days after each BABA or water treatment, leaflets were cut and inoculated by spray with a zoospore suspension (40 000 zoospores/mL) of *P. infestans.* Five days after inoculation, phenolic compounds were extracted according to Andreu *et al*. (2006). Briefly, leaves (1 g frozen tissue) were homogenised with methanol/water (80 : 20 v/v, 10 mL), allowed to stand with continuous stirring for 30 min and then centrifuged at 12 000*g* for 20 min. Supernatants (100 µL) were taken for phenol quantification with Folin–Ciocalteu reagent (Bray and Thorpe 1954). The absorbance was measured at 650 nm. Chlorogenic acid (200 µg/mL) in methanol/water (80 : 20 v/v) was used as standard.

### *Statistical procedures*

Data were analysed for significance by a one-way ANOVA and means were compared at the $P < 0.05$ level of significance by multiple range comparisons (Tukey, SigmaStat).

## Results

### *Effect of BABA on foliage protection against late blight*

Results showed that the effect of BABA on foliage protection to late blight was only effective in cv. Pampeana (moderately resistant). A high level of protection against *P. infestans* was observed in the foliage during early growth stage, when BABA was applied 30 dae, as shown in Fig. 1. At 38 dae, at the beginning of the tuberisation stage, a decrease of 40% in the foliage protection level was observed. No significant foliage protection by BABA treatment was observed in cv. Bintje (susceptible) at any stage of the potato life cycle (Fig. 1).

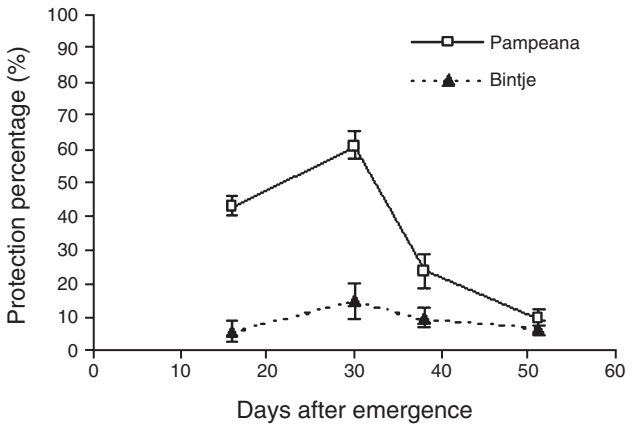


**Fig. 1.** Effect of β-aminobutyric acid (BABA) in foliage protection percentage to late blight in Bintje and Pampeana cultivars during the potato crop cycle. Foliage protection was evaluated by the detached-leaf method. BABA or water (control) were applied at indicated times. Three days after treatment, leaflets were inoculated with a drop of zoospore suspension of *Phytophthora infestans.* Foliage protection was evaluated 7 days after inoculation.

### *Effect of BABA on expression of PRs in foliage*

#### *Induction of glucanases*

Foliage was treated with BABA or water at the indicated times (Fig. 2). Three days after treatment, leaves were detached and inoculated or were not inoculated with *P. infestans.* The leaflets were processed for enzymatic activity 5 days after inoculation.

In both cultivars, the plant activator BABA induced high expression level of glucanases in inoculated leaves with respect to the control. The major value was obtained in Pampeana-inoculated leaflets coming from BABA-treated plants at 30 dae. At the beginning of the tuberisation stage (~38 dae), a significant reduction of activity was observed for inoculated leaves from BABA-treated and control plants, in both cultivars. In cv. Pampeana, the effect of BABA was not evident at this stage (Fig. 2*a*). However, in cv. Bintje, a strong increase in the levels of glucanases was observed after BABA treatment at 51 dae (the end of the tuberisation stage). Towards the end of the crop cycle, the glucanase increase by BABA was not detected. In order to know which isoforms were responsible for the glucanase activity, the expression of β-1,3-glucanases in foliage was analysed by western blot 23 dae, in response to BABA or infection with *P. infestans*. A protein between 30 and 35 KDa was recognised by the antiserum anti-basic β-1,3-glucanase. The increase of this isoform after infection in untreated leaflets was higher in Bintje than in Pampeana. However, in BABA-treated leaflets, a notable induction was observed in the two cultivars, both in inoculated and non-inoculated leaves.

#### *Induction of chitinases*

Leaf soluble extracts made for glucanase measurement were utilised for the chitinase analysis. In both cultivars, an increase in chitinase activity was observed in correlation with foliage maturity for all treatments. The highest expression of chitinases was obtained in cv. Pampeana by BABA treatment at intermediate developmental stages (until 38 dae) with respect to the control (Fig. 3*a*). However, in cv. Bintje, a higher induction by BABA was observed along almost the whole cycle, except for 23 dae, when a strong decrease in this activity was observed (Fig. 3*b*). In order to identify the isoforms responsible for the chitinase activity, the expression of chitinases in foliage was analysed by western blot 23 dae, in response to BABA and infection with *P. infestans*. Two

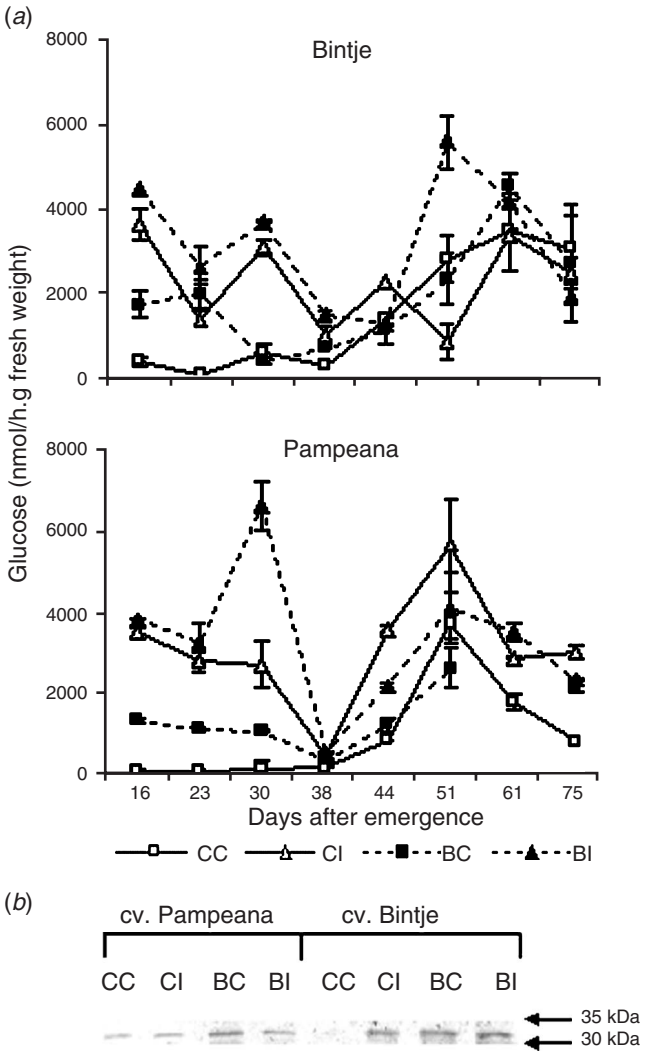


**Fig. 2.** Effect of β-aminobutyric acid (BABA) on glucanase expression in potato leaves. BABA or water (control) were applied at indicated times. Three days after treatment, leaflets were inoculated by spraying with a zoospore suspension of *Phytophthora infestans*. Five days after inoculation, the leaflets were processed for the total enzymatic activity determination (*a*) or for determination of specific protein accumulation by Western blot analysis (*b*). Enzymatic activity was determined at different times along plant growth and the analysis of expression by Western blot was made at 23 days after emergence. Ref: CC, untreated and non-inoculated leaflets; CI, untreated and inoculated leaflets; BC, leaflets treated with BABA and non-inoculated; BI, leaflets treated with BABA and inoculated.

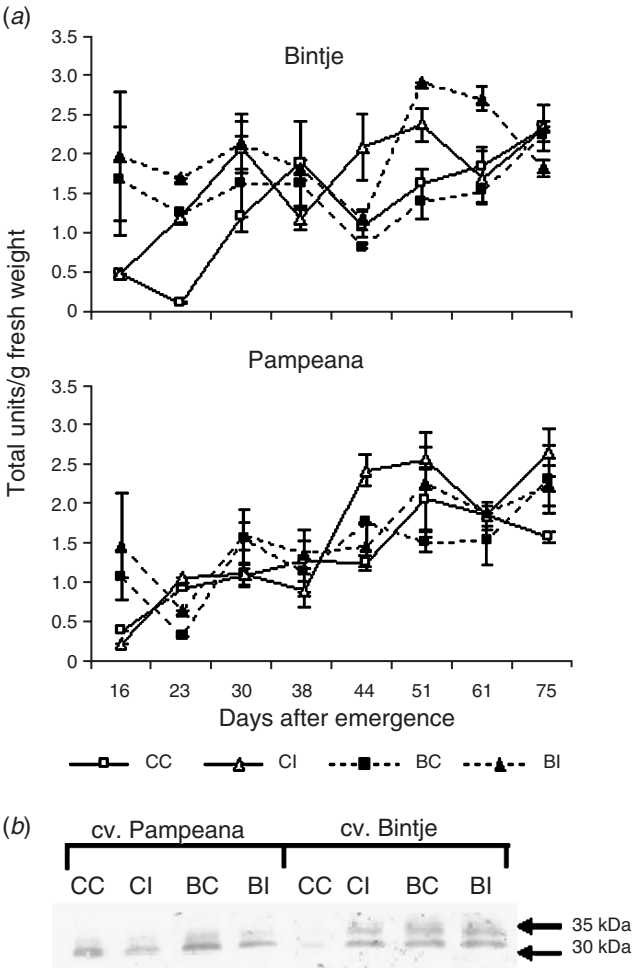


**Fig. 3.** Effect of β-aminobutyric acid (BABA) on chitinase expression in potato leaves. BABA or water (control) were applied at indicated times. Three days after treatment, leaflets were inoculated by spraying with a zoospore suspension of *Phytophthora infestans*. Five days after inoculation, the leaflets were processed for total enzymatic activity determination (*a*) or for determination of protein accumulation by Western blot (*b*). Enzymatic activity was determined at different times along plant development and the analysis of expression by Western blot was made at 23 days after emergence. Ref: CC, untreated and non-inoculated leaflets; CI, untreated and inoculated leaflets; BC, leaflets treated with BABA and non-inoculated leaflets; BI, leaflets treated with BABA and inoculated.

isoforms were recognised by the antiserum anti-basic chitinases. One of them was induced by BABA in cv. Pampeana and by BABA or infection in cv. Bintje.

### *Effect of BABA on the accumulation of phenolics*

The accumulation of phenolics was determined 5 days after inoculation with *P. infestans* in detached leaves coming from BABA-treated or untreated plants at indicated times (Fig. 4). In general, a lower increment in the levels of phenolics was detected in correlation with foliage maturity in both cultivars. The foliar application of BABA increased the levels of phenolics in infected leaflets at 30 and 60 dae, in both cultivars (Fig. 4*a*, *b*).

## Discussion

In the present work, the action of BABA in the defence response of potato plants to *P. infestans* throughout the potato crop cycle was studied, using cultivars with different degrees of horizontal resistance to late blight.

The results obtained indicated that BABA induced protection in foliage differentially, depending on the potato cultivar. The major protection was observed in the moderately resistant cv. Pampeana, during the whole crop cycle (Fig. 1). These results were consistent with previous studies in potato, which indicated

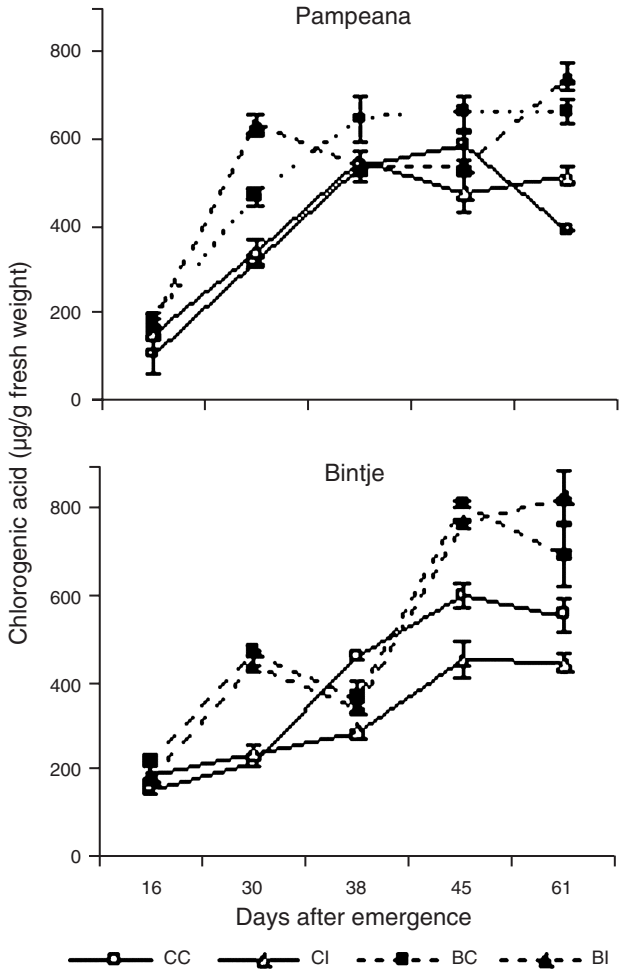


**Fig. 4.** Effect of β-aminobutyric acid (BABA) on accumulation of phenolic compounds in potato leaves. BABA or water (control) were applied at indicated times. Three days after treatment, leaflets were inoculated by spraying with a zoospore suspension of *Phytophthora infestans*. Phenolics were extracted and measured 5 days after inoculation. Ref: CC, untreated and non-inoculated leaflets; CI, untreated and inoculated leaflets; BC, leaflets treated with BABA and non-inoculated; BI, leaflets treated with BABA and inoculated.

that in two cultivars with similar characteristics, Kennebec (moderately susceptible) and Russet Burbank (moderately resistant), protection against late blight in foliage was observed at intermediate developmental stages (35 dae). Only this stage was analysed for the present study (Andreu *et al*. 2006). In addition, previous works have reported a significant difference in the development of late blight lesions in tomato plants, untreated and treated with BABA 5 weeks after planting (Cohen 2002). It can be concluded from our data that BABA reduces foliar disease in potato cultivars with moderate resistance to *P. infestans* and, alternatively, that the highest effectiveness in disease reduction corresponds to intermediate developmental stages, previous to the tuberisation stage, at least in greenhouse conditions. Similar effects for non-induced systems have been reported, in which young potato plants were found to be susceptible to late blight, potato plants of intermediate age were resistant and old potato plants became susceptible again (Mooi 1965; Stewart *et al*. 1983). Therefore, the effect of plant activator BABA would reinforce the existing pattern between resistance level and plant age, against the infection by *P. infestans.* There were no other reports where the effect of BABA applications was related to plant age. Contrary to the potato–*P. infestans* pathosystem, Shailasree *et al*. (2001) have shown that resistance induced in seeds with BABA remained operative through vegetative and reproductive growth of pearl millet plants. This result refers to the durability of protection by BABA, when the activator was applied to seeds. However, that is not comparable with foliar applications at different ages. Although they have also shown that the protection offered by BABA was independent of the constitutive resistance of the cultivars utilised.

Different mechanisms were reported to be involved in BABA-induced resistance depending on the plant–pathogen interaction (Jakab *et al*. 2001; Cohen 2002). In many cases, plant resistance was correlated with the accumulation of PR proteins (Cohen *et al*. 1994). Our results showed a correlation between the BABA induced resistance and the accumulation of β-1,3-glucanases and chitinases until the beginning of the tuberisation stage, in cv. Pampeana (Figs 2*a* and 3*a*). The beginning of the tuberisation stage seems to be a crucial moment in plant defence response when the redistribution of metabolites could modify the expression pattern of different PRs. Like glucanases and chitinases, other PR were measured in different cultivars and similar behaviours were observed (data not shown). A strong induction of chitinases in cv. Bintje was observed after BABA treatment along the potato crop cycle; however, its protection percentage was low (Fig. 3*b*). BABA also induced β-1,3-glucanases in both cultivars. However, the levels of induction in cv. Bintje were not correlated with protection observed (Fig. 2*b*). Therefore, we cannot establish a correlation between the resistance and the induction of PR proteins in the susceptible cv. Bintje. Our results show that there was also a cumulative effect of BABA and *P. infestans* in the moderately resistant cultivar with respect to PRs induction. The increase in the induction of β-1,3-glucanases and chitinases produced by BABA in potato leaves in response to the inoculation by *P. infestans* was consistent with previous studies in tomato plants under the same conditions (Cohen *et al*. 1994). In addition, there is evidence that β-1,3-glucanases are induced in parallel with chitinases in different situations (Mauch *et al*. 1984; Shinshi *et al*. 1987). A strong induction of genes related to the defence in susceptible cultivars such as Bintje has been reported by Ros *et al*. (2005). In their work, three susceptible cultivars were analysed, Bintje, Cara and Indira. A higher induction was observed in these cultivars 72 h after the challenge inoculation with respect to resistant cultivars. In particular, the induction of the gene that express for β-1,3-glucanases was 2–3-fold higher in Bintje than in the resistant cultivars.

However, we did not establish a correlation between the expression level of PRs detected by western blot and the protection induced in the susceptible cultivar. Further studies must be conducted to know which PR isoforms are involved in the activity detected and which isoforms could participate in the protection process.

Phenolic compounds were involved in defence response of potato cultivars with different levels of multigenic resistance (Andreu *et al*. 2001), although several reports showed the induction of phenols, or related compounds, after BABA treatments. Phenylalanine ammonia-lyase, considered to be the main enzyme of the phenylpropanoid pathway, which is the first intermediate in the biosynthesis of phenolics and flavonoids, has been reported to be induced by BABA in tomato leaves (Baysal *et al*. 2005). In addition, in previous work, we have also detected that foliar BABA treatments produced an increase in tuber phenols content after *P. infestans* inoculation (Andreu *et al*. 2006). In the present work, BABA induced accumulation of phenolic compounds at different times during the growth cycle, in both cultivars (Fig. 4). This could be correlated with protection observed in cv. Pampeana at the intermediate developmental stages. However, no correlation between the phenolic induction and the level of protection was observed for the susceptible cultivar.

The results obtained in the present work allowed a partial characterisation of some biochemical markers and certain phytopathological features involved in the response induced by BABA, in two cultivars with different degrees of multigenic resistance to *P. infestans*, throughout the potato crop cycle. Even though defence-related molecules were differentially expressed after treatment with BABA in both cultivars, these compounds are insufficient to generate a defence response in the susceptible cultivar. However, in the moderately resistant cultivar, PRs and phenolics could be part of a defence mechanism induced by BABA. Finally, it can be concluded that the time of BABA application and the susceptibility of cultivar are critical parameters in the determination of the protection in foliage against *P. infestans* in potato.

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